Novel mechanisms in PPARγ-mediated adipogenesis

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Cover: Differentiated SBGS cells stained with Oil red O.

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Novel mechanisms in PPARγ-mediated adipogenesis

Nieuwe mechanismen in PPARγ gemedieerde adipogenese (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. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 10 april 2008 des middags te 12.45 uur

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“Life is infinitely stranger than anything which the mind of man could invent”
Arthur Conan Doyle, 1892

Aan Marielle

Commissie: Prof. dr. P.J. Coffer
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dr. S. Mandrup
Prof. dr. H.T.M. Timmers
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Victor Romijn

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Chapter 1

General Introduction

Type 2 diabetes and PPARγ

Obesity-induced type 2 diabetes mellitus (T2DM) has grown to epidemic proportions in the last decades. The number of adults with T2DM in the United States has increased rapidly by almost 50% between 1991 and 2000 according to the Center for Disease Control and Prevention (CDC) (1). Furthermore, the World Health Organisation (WHO) predicts that the number of T2DM patiens worldwide will grow to as much as 300 million in the year 2025 (1).
T2DM is not characeterised by the lack of insulin production found for the much less common type 1 diabetes mellitus, but manifests itself by decreased sensitivity towards insulin. In both types of diabetes similar complications arise as a result of impaired insulin signalling and lack of response to increased glucose levels. Serious complications, mostly resulting from vascular problems, like kidney failure and blindness are often found in untreated diabetic patients. Both types of diabetes can be treated and, in the case of T2DM, this often involves administration of insulin sensitizing drugs. A commonly used group of insulin sensitizers are the thiazolidinediones (TZDs). In 1995 it was found that TZDs function as ligands for the transcription factor PPARγ, a member of the nuclear receptor superfamily (2) and the TZDs rosiglitazone and pioglitazone are now well known compounds, both in clinical and experimental settings.

PPAR proteins

Like other nuclear receptors, PPARs 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). Ligand binding stabilises the active conformation of the PPARγ LBD, allowing a switch from a repressed to an active state and subsequent recruitment of different protein complexes. All PPAR proteins require heterodimerisation with the nuclear receptor RXR to allow binding to the PPAR response element, within the promoter regions of target genes.
The PPAR family contains three different members PPARα, PPARβ/δ and PPARγ. PPAR proteins are all involved in lipid and glucose homeostasis. PPARα was first identified as a receptor mediating peroxisome proliferation in rodent hepatocytes (3). PPARα is expressed in tissues with high fatty acid catabolism such as liver and heart controling lipid and glucose metabolism by targeting genes involved in β-oxidation and free fatty acid uptake. PPARα is also involved in fasting as was pointed out by the finding that PPARα knock-out mice can not sustain long term food deprivation (3;4). Furthermore, PPARα knock-out mice exhibit a delayed response to inflammatory stimuli, indicating a role in the regulation of inflammatory responses (5). The function of PPARβ/δ on the other hand seems to lay in regulation of fatty acid oxida-

tion in oxidative myofibres (6). Muscle specific overexpression of PPARβ/δ in mice ("marathon mice") results in a phenotype comparable to long term endurance excersise, which is characterised by an increment in oxidative myofiber number, increased muscle fatty acid oxidation and resistance to muscle fatigue and deleterious effects of a high fat diet (6-8).

The third family member, PPARγ, is expressed in two different isoforms, namely PPARγ1 and PPARγ2 of which the latter contains an 30 residue extension at the N-terminus (9). PPARγ1 is mainly expressed in adipose tissue but is also found in other tissues including the insulin responsive tissues skeletal muscle and liver. PPARγ2, however, is exclusively expressed in adipose tissue. Activation of PPARγ by synthetic ligands causes full adipogenic differentiation in fibroblasts or preadipocytes, inhibition of growth and, most imporantly, increased insulin sensitivity. Homozygous PPARγ knock-out mice die at embryogenesis but heterozygous PPARγ knock-out mice are viable. Although not a consistent finding, mice heterozygous for PPARγ show a reduction in adipose tissue, are protected against diet- induced diabetes and display increased insulin sensitivity (10-12). Selective disruption of PPARγ2 in adipose tissue also led to a reduction in fat mass and a decrease in adipogenic gene expression similar to heterozygous PPARγ knock-out mice (13). However, selective disruption of PPARγ2 in adipose tissue has been associated with impaired insulin sensitivity. Interestingly, PPARγ2-specific knock-out mice are not affected in the anti-diabetic action of TZDs (14) similar to mouse models with a specific disruption of PPARγ in either muscle or liver (15;16).

PPARs can be viewed as lipid sensors and several substances have been suggested to function as natural ligands of PPARγ. These include eicosanoids and fatty acids, like nitro-linoleic acid (17). Many of these substances however show low specificity between the different PPAR members and low binding affinity. Interestingly, Tzameli and coworkers reported the excistense of a transiently produced but unidentified ligand which is produced during adipogenesis of 3T3L1 cells (18). For some of the compounds described as natural ligands, the existence of sufficient concentrations in cells can be debated. Therefore the quest for the identification of natural ligands continues. Several synthetic PPARγ ligands like the thiazolidinediones or 'glitazones' have been developed of which some are now widely used in the clinic (2). Of particular interest also, is the development of so called SPPARMs (selective PPARγ modulators) which function as partial PPARγ agonists. These ligands display insulin sensitization without increasing adipogenesis (19). SPPARMs bind to the PPARγ ligand binding domain in a distinc manner allowing tissue and promoter-selective gene expression. Also PPAR dual agonists are being developed as a way to improve treatment of type 2 diabetes. Many type 2 diabetic patients suffer from cardiovascular complications and several clinical trials have indicated beneficial effects of PPARγ and PPARα agonists in preventing cardiovascular risk (19). However, several PPARα/γ dual agonists have been implicated in increasing the risk of carcinogenicity. Therefore more research is required to elucidate the underlying mechanisms and possibly improve treatment of type 2 diabetes by (partial) PPAR agonists (20).

PPARγ-mediated transcription

Transcriptional regulation forms an essential part in regulating cellular homeostasis and responses to external cues. Nuclear receptors and other transcription factors play a key role in orchestrating the different dietary signals and regulate the various metabolic pathways in maintaining and establishing energy homeostasis (9). Genomic DNA in eukaryotes is tightly packed into chromatin, forming a barrier for transcription. Therefore, transcription initiation involves a process called "chromatin remodelling", in which different transcriptional complexes, including chromatin remodelling enzymes, are thetered towards the promoter region via transcription factor-mediated recruitment (21). Gene regulation is often thought of as a process composed of two opposites: on the one hand there is gene repression on the other there is gene activation. In recent years however it has become clear that this is an oversimplification of eukaryotic gene regulation. Instead gene expression is a well balanced process, more comperable to a "dance" of different protein complexes continously moving around promoter regions. Within these complexes there are enzymes that covalently modify the histone tails present within the promoter regions, thereby regulating the accesibility of the DNA. These modifications are key to functional gene regulation and found to be interconnected, meaning that modification of residue X results in modification of residue Y. Furthermore, the different histone modifications dictate the sequence of events taking place at a particular promoter region, allowing

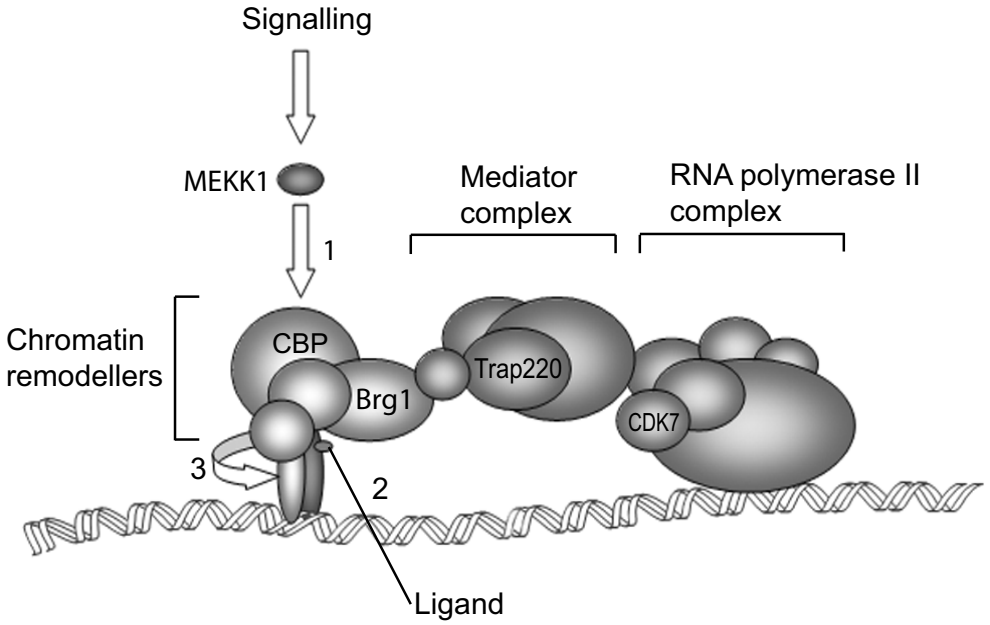

Figure 1. Different transcription complexes are involved in PPARγ mediated gene regulation. Transcription complexes can be grouped into three different classes as indicated. There are the chromatin-remodeling complexes which modify chromatin structure altering fluidity, folding and structure thereby making the promoter region accesible to other proteins. The second class contains the Mediator complex forming a bridge between the nuclear receptor and the third class of transcription complexes consisting of the RNA polymerase complex. PPARγ activity can be regulated via external cues (1) via ligand binding (2) and post-transcriptional modifications mediated by enzymes present within the transcription complex (3)(see text for details).

either rejection or attraction of certain protein complexes. Therefore the process of gene regulation can better be thought of as a very dynamic and ever changing process by which cells continously respond to extra- and intracellular signals (21;22). Transcription complexes can be grouped into three different classes as indicated in figure 1. The first class are the chromatin-remodeling complexes which modify chromatin structure by altering its fluidity, folding and structure, and thereby making the promoter region accesable to other proteins. The chromatin-remodeling complexes consist of two different groups; the first group comprises enzymes that alter histone proteins through post-transcriptional modifications like the acetyltransferases like CBP, p300 and Tip60 (see also chapter 4) and methyltransferases like PRMT5. The second group of chromatin remodellers are proteins that alter chromatin structure in an ATP-dependent way, including for example the SWI/SNF complex. The second class of transcriptional complexes is formed by the Mediator complexes that bridges many transcription factors to the general transcription machinery. An important subunit of the mediator complex is for example TRAP220 (TRansformation/tRanscription domain-Associated Protein) (23). The third class is formed by the general transcription machinery itself containing RNA polymerase II which is involved in the expression of virtualy any protein encoding gene.

The importance of the different chromatin remodelling complexes in adipogenesis and PPARγ mediated gene expression has become evident from various studies. Ribozyme mediated decrease of the histone acetyltransferases CBP and p300 expression, inhibited PPARγ mediated gene expression and adipogenesis (24). In accordance with these findings CBP-deficient as well as heterozygous mice show a reduced fat mass due to undeveloped white adipose tissue (11). Furthermore expression of dominant negative BRG-1 or BRM, components of the ATP-dependent chromatin remodelling SWI/SNF complex, also decreased PPARγ mediated gene activation (25;25). The main role of the TRAP complex, which is related to the yeast mediator proteins, lies in direct communication between the various coactivators and the general transcription complex. TRAP220 is an essential subunit of the TRAP complex, interacting with various nuclear receptors including PPARγ. By using TRAP220-/- fibroblasts it was shown that the TRAP complex plays an essential role in adipogenesis, acting as a PPARγ mediator complex (26). These data underscore the important function of coactivator complexes in PPARγ signalling and thereby regulation of energy metabolism. Therefore, further understanding of PPARγ activity is important since novel PPARγ modifiers could offer the possibility of therapeutic modulation and provide new ways of improving insulin sensitivity (see also chapter 2).

Aim of this thesis

Although several reports have pointed to the importance of coactivators in maintaining energy homeostasis, our current knowledge of the different transcription complexes proposed to play a part in PPARγ-mediated gene expression remains limited. Therefore, as described in this thesis, we set out to investigate the different levels at which coactivators function in PPARγ2 activation and also how mutant forms of PPARγ are affected in their associations with coactivator proteins (chapter 3). In an effort to identify novel regulators of PPARγ we have developed an immunoprecipitation assay coupled to mass spectrometry analysis (chapters 4 and 5) as well as a novel screening method based on PPARγ2 driven miRNA expression (chapter 6) for the use of high troughput siRNA library screens. Using these assays, several novel PPARγ2 interactors were identified.

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Chapter 2

Posttranslational modifications of PPARS: fine-tuning the metabolic master regulators

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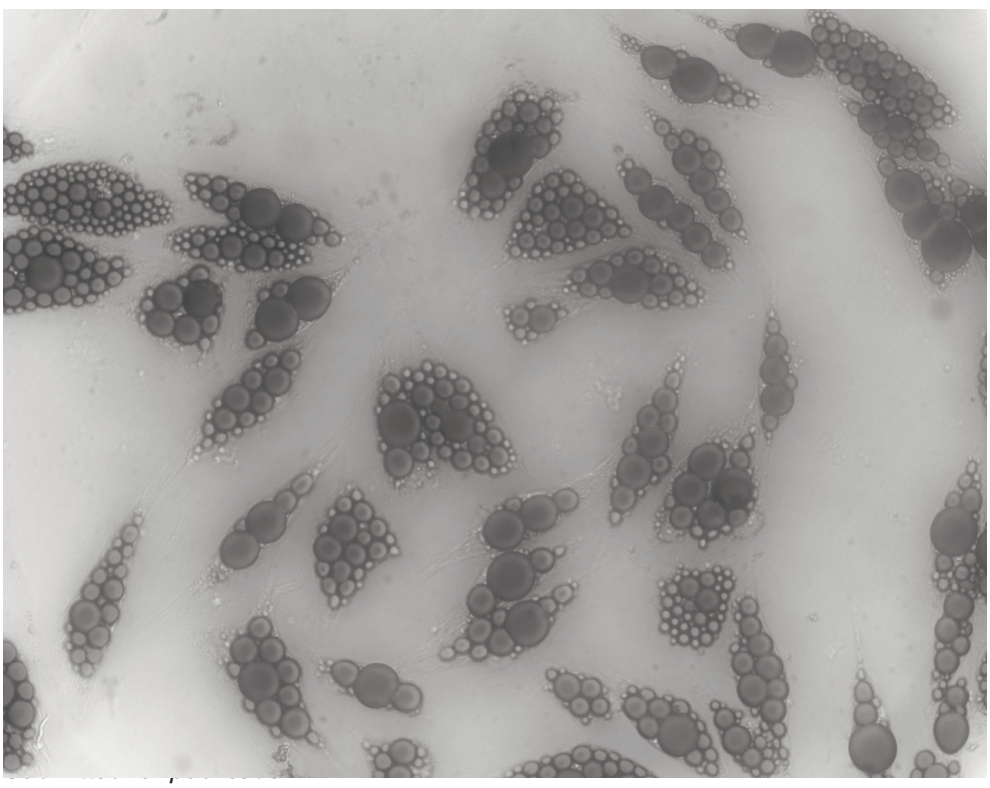

ABSTRACT

Peroxisome proliferator activator receptor (PPAR) –α, -β/δ and –γ are three closely related members of the nuclear receptor (NR) family of ligand-dependent transcription factors. Since PPARs are key regulators of glucose and lipid metabolism, they have received considerable attention as therapeutic targets for the treatment of various illnesses, including obesity and type 2 diabetes. Besides direct regulation of PPAR transriptional activity by ligands, their activity can also be regulated via post-translational modifications (PTMs), like phosphorylation, sumoylation, ubiquitination and probably acetylation. These different PTMs induced by various signalling cascades offer the possibility to fine-tune PPAR activity. Interestingly, different PTMs are often interdependent, allowing different pathways to communicate and merge at the intramolecular level. This review will focus on the emerging importance of PTMs in regulating the activity of these metabolic master regulators.

INTRODUCTION

Peroxisome proliferator activator receptors (PPARs) are members of the nuclear receptor family of ligand-dependent transcription factors which comprise a subgroup of three closely homologous genes PPARα (NR1C1), -β/δ (NR1C2) and -γ (NR1C3) (1-5). All PPARs bind to the PPAR responsive elements (PPRE) in the promoter regions of target genes as obligate heterodimers with retinoic acid X receptors (RXRs). Like other nuclear receptors, PPARs consist 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). Ligand binding stabilises the active conformation of the LBD, thereby serving as a "molecular switch" between activation and repression functions of the receptor (6;7). On the promoters of some target genes, unliganded PPARs recruit corepressors like N-CoR and SMRT, which are part of multiprotein complexes containing histone deacetylase (HDAC) activity that repress gene transcription (8-10). Upon ligand binding, these corepressor complexes are exchanged for activating coactivator complexes, including the SRC1/CBP and TRAP/DRIP/ARC complexes (11;12). Besides nuclear receptor ligands directly regulating nuclear receptor function, nuclear receptors are also regulated via the ligand independent AF-1 domain. In common with other NRs (13), the AF-1 domains of the different PPAR isotypes probably lack a stable secundary structure in aqueous solution, only to adopt a stable conformation upon interaction with other proteins.

Although all PPAR nuclear receptors play a part in lipid homeostasis and energy metabolism, the diffent PPAR proteins exhibit different physiological roles due to (i) distinct expression patterns, (ii) specific activation by different ligands and (iii) intrinsic functional differences between the different isotype proteins.

Of the different PPARs, PPARα was the first PPAR member to be identified as a protein activated by hypolipidemic synthetic fibrate drugs causing peroxisome proliferation in rodent livers (14). Subsequent cloning efforts identified the PPARγ and PPARβ/δ isotypes with tissue selective expression patterns different from PPARα. Besides synthetic fibrate drugs, various fatty acids and arachidonic acid metabolites (prostaglandins and leukotrienes) can serve as PPARα ligands. PPARα is mainly expressed in tissue with a high rate of lipid catabolism like liver, heart, kidney and brown adipose tissue. PPARα controls both lipid and glucose homeostasis in liver and skeletal muscle by targeting genes involved in peroxisomal and mitochondrial β-oxidation pathways and free fatty acid uptake. PPARα knock-out mice can not sustain long term food deprivation which is in line with the finding that fasting results in increased PPARα expression in the liver leading to upregulation of genes involved in β-oxidation (14;15).

Alternative splicing and differential promoter usage results in two PPARγ isoforms, PPARγ1 and PPARγ2, with the latter harbouring a 30 residue extension at its N-terminus (16-18). PPARγ1 is expressed in several tissues, including the lower intestine, macrophages and white adipose tissue, while PPARγ2 expression is almost exclusively restricted to white adipose tisssue. The endogenous ligands for PPARγ are not firmly established although some natural compounds, like polyunsaturated fatty acids, prostaglandin J2 derivatives (15-deoxy-$\Delta^{12,14}$-PGJ2) and nitro-oleic acid have been

shown to be able to activate PPARγ (15;19;20). Synthetic PPARγ agonists include the thiazolidinediones (TZDs)(21), which ameliorate insulin resistance and lower blood glucose levels in patients with type 2 diabetes (22). A huge amount of data indicates that PPARγ is one of the key players in the differentiation of fibroblast-like mesenchymal stem cells into adipocytes, a process known as adipogenesis. For example, PPARγ $^{+/-}$ mice lack most adipose tissue (23;24), while in vitro differentiation of fibroblasts into mature adipocytes can be induced by introduction of PPARγ (25). In addition, PPARγ is also essential for the maintenance of adipose tissue, since conditional knock-out of the Pparg gene resulted in reduced in vivo survival of mature adipocytes (26). Besides its role in adipocyte differentiation and maintenance, PPARγ directly regulates the expression of a number of genes involved in net lipid partitioning into mature adipocytes, underscoring the importance of PPARγ in glucose and lipid homeostasis. Compelling genetic evidence for this view comes from human Familial partial lipodystrophy subtype 3 (FPLD3, MIM 604367) patients, harbouring heterozygous mutations in the PPARG gene, as they are characterized by aberrant fat distribution and metabolic disturbances, including insulin resistance and dyslipidemia (1;27). Interestingly, PPARγ was recently shown to be involved in the inhibition of osteoblast differentiation and osteoclastogenesis (28), indicating that novel biological functions of this transcription factor remain to be identified beyond its role in lipid and glucose homeostasis.

PPARδ is the most ubiquitously expressed isotype with relatively high levels of expression in skeletal and cardiac muscles, brain and adipose tissue (2). While several fatty acids, including VLDL-delivered fatty acids, and naturally occurring eicosanoids serve as effective activators, the identitiy of the natural ligand(s) remains unsettled (15). The phsyiological function of PPARδ has remained elusive for a long time but studies in mice have also linked this receptor, like PPARγ and PPARα, to lipid metabolism. Although most PPARδ$^{-/-}$ embryos die in utero due to placental defects, mice that do survive show decreased adipose tissue (29;30). Studies in the pre-adipocyte cell line 3T3-L1 have shown that synergystic activation of PPARδ together with cAMP elevating agents signifigantly increases early expression of PPARγ itself and the PPARγ target gene FABP4/aP2. Because only modest effects on lipid accumulation were detected, it was suggested that PPARδ only plays a role in the early phases of fat cell differentiation (31). However, adipose tissue-specific deletion of the Ppard gene did not affect the fat mass, indicating that the main regulatory role of PPARδ in metabolism does not reside in adipose tissue (30). A more important function of PPARδ rather seems to lay in regulation of fatty acid oxidation in oxidative myofibers. Muscle specific overexpression of PPARδ in mice (“marathon mice”) results in a phenotype comparable to long term endurance excersise which is charactetrised by increment in oxidative myofiber number, increased muscle fatty acid oxidation and resistance to muscle fatigue and deleterious effects of a high fat diet (32;33).

While PPARs display a high degree in primary amino acid sequence homology in their LBD and DBD, both the N-terminal AF1 region and the hinge region are less well conserved between isotypes. The idea that these regions are therefore likely to contribute to the isotype-specific responses was recently supported experimentally. Using chimeric PPARγ-PPARβ/δ proteins, the AF1 region of PPARγ was shown to be essential for adipogenesis (34;35). Furthermore, gene expression profiling revealed

that the AF1 regions of the different PPAR family members are the main determinants of isotype-selective gene expression (35). It seems plausible that, in analogy with the AF2 domain, the activity of the AF1 region is dictated by the set of proteins with which this domain interacts. Only two AF1 interacting proteins have been identified so far which display isotype-specific interactions: PPARγ coactivator 2/SCAN domain protein 1 (PGC-2/SDP1), a PPARγ-specific co-activator which promotes adipogenesis (34;36), and Tip60, an adipogenic acetyltransferase, which stimulates the activity of PPARγ, but not PPARα or –β/δ ((37) and unpublished observations). Interestingly, the AF1 regions of PPARs are also subject to various posttranslational modifications (PTMs), and it seems likely that such PTMs regulate interactions with coregulators, and/or vice versa, ultimately controlling the transcriptional activity of the different PPAR isotypes. In this review we will therefore summarize the various PTMs reported for the different PPARs, evaluate the (possible) effects on co-regulator interactions and discuss their (potential) relevance for in vivo PPAR signalling.

Phosphorylation of PPARs

Phosphorylation of PPARα was first described by Shalev et al. (38) who immunoprecipitated PPARα from primary rat adipocytes stimulated with insulin. Subsequent experiments indicated that insulin treatment increased PPARα transcriptional activity via the first 92 N-terminal amino acid residues. Furthermore the effect of insulin is abolished in cells treated with p42/p44 inhibitor indicating the involvement of MAPK-mediated phosphorylation. In agreement with this, mutation of the consensus MAPK phosphorylation sites at positions 12 and 21, but not position 77, in the PPARα protein abolished the stimulatory effect of insulin on PPARα signalling (see also Figure 1). These data clearly indicate that the stimulatory effect of insulin on PPARα signalling resides within the AF1 region. The molecular mechanism however still remains unclear but it is temping to speculate that phosphorylation of serine 12 and/or 21 affects interactions with coactivator or corepressor proteins. Remarkably, phosphorylation of N-terminal serine residues has also been suggested to decrease PPARα activity. This was shown in a study on the function of PPARα in cardiac hypertrophic growth, a process that can be mimicked in culture by addition of the α_1-adrenergenic agonist PE to primary rat cardiac myocytes (39). Based on experiments with specific kinase inhibitors, in vitro phosphorylation experiments and reporter assays, the ERK-MAPK pathway was implicated in PPARα repression (39). It should however be noted that combinatorial mutants were used (serine residues 6, 12, 21, 73, 76, 77) in this study, and the exact ERK-MAPK phosphorylation site(s) remain(s) to be determined.

After these studies on the MAPK-mediated phosphorylation of serine 12 and 21 several additional phosphorylation sites as well as the kinases responsible for these modifications were identified. The stress-activated kinase p38 (mitogen-activated protein kinase 14) was found to phosphorylate serine 6 and 21, thereby increasing transcriptional activity independent of RXR heterodimerisation or DNA binding (40). Phosphorylation of these N-terminal serine residues resulted in an enhanced interaction with the PGC-1α coactivator , but not the SRC-1 and PBP coactivator proteins. Since there is no evidence suggesting a direct PGC-1α interaction with the AF-1 do-

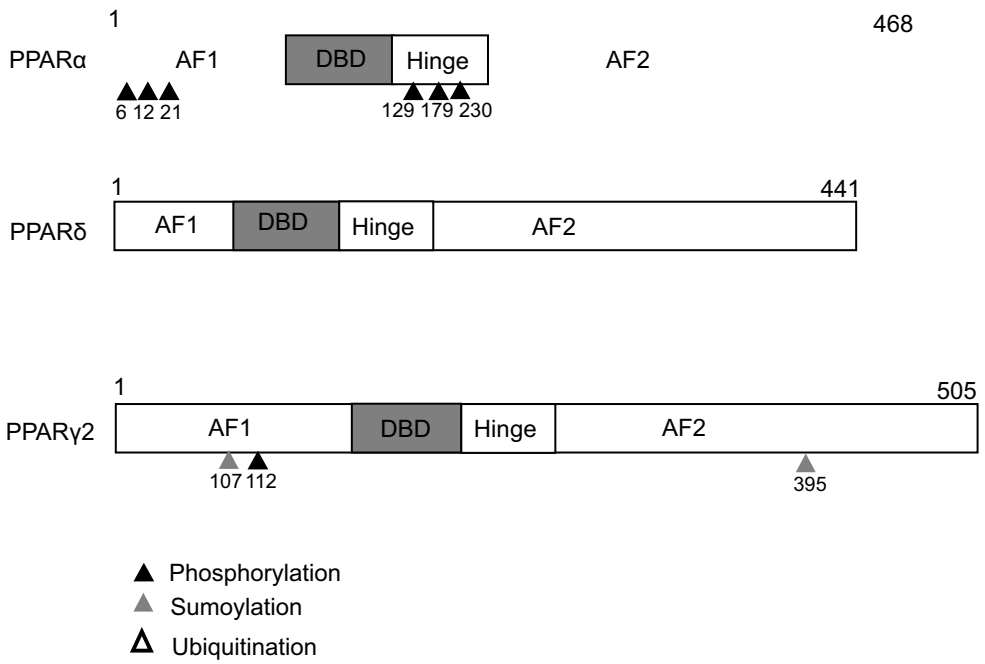

Figure 1. Schematic representation of the different PPARs. Indicated are the ligand-independent activation domain (activation function 1: AF1) the DNA binding domain (DBD) and the ligand-dependent domain (activation function 2: AF2). Different positions for which a modification is identified are indicated with a black arrow for phosphorylation and a grey arrow in case of sumoylation. PPARδ phosphorylation is mapped at a location between residue 44 to 55 this region contains multiple plausible phosphorylation sites which have not been verified yet.

main of PPARα, it was speculated that a yet to be identified PPARα/PGC-1 interacting protein could serve as an adaptor (40). More recently, the cdk7 kinase of the TFIIH complex was shown to phosphorylate serine 12 and 21, resulting in increased transcriptional activity (41).

The protein kinases A (PKA) and C (PKC) have been implicated in phosphorylation of PPARs outside their N-teminal AF1 regions (42;43). PKA activators were found to increase the transcriptional activity of PPARα as well as –β/δ and -γ. By using truncated versions of PPARα and GAL4DBD fusion proteins, PKA-mediated stimulation of PPARα activity was shown to depend on an intact DBD and AF2 domain, but the AF1 region was dispensable, as was for example also observed earlier for PKA-mediated activation of the nuclear receptors ERα and SF1 (44;45). In vitro experiments indicated that PKA-mediated phosphorylation mainly occurred in the DBD, possibly on the PKA consensus sites serine 142 and 163. PKA-mediated phosphorylation decreased DNA binding of the unliganded PPARα-RXR heterodimer, as was previously also observed for ERα (45), but stabilized binding of the liganded form, suggesting that this modification affects the function of not just the DBD but the protein as a whole. Furthermore, treatment with cholera toxin (CT), which activates PKA, increased the ligand-induced expression of the ACO gene but not the FABP gene, and the authors therefore concluded that ligand and PKA activation can converge in the

stimulation of specific PPARα target genes (43). The second kinase implicated in phosphorylation events outside the N-terminal AF1 region is PKC. Addition of PKC inhibitors or overexpression of dominant negative forms of PKC resulted in a loss of ligand-dependent PPARα activity in reporter assays (42). These effects were mapped to serines 179 and 230 in the hinge region, which were also the targets of PKC in in vitro kinase experiments. Interestingly, serine-to-alanine mutation of these residues enhanced the ability of PPARα to repress the finbrinogen-β promoter, indicating that phosphorylation induces a molecular switch allowing release of a yet to be identified repressor protein and subsequent activator recruitment (42).

As the first PTM for PPARγ2, phosphorylation has been studied extensively over the years. Phosphorylation of PPARγ2 was mapped to serine 112 (serine 82 in PPARγ1), a MAPK consensus site within the AF-1 region which is the only phophorylation site identified in PPARγ so far (see also Figure 1). Mutating this MAPK consensus site by changing either serine 112 or the adjacent proline at position 113 into alanine led to increased transcriptional activity in reporter assays (46-50). Furthermore overexpression of PPARγ S112A in mouse fibroblasts resulted in increased adipogenesis compared to cells overexpressing wild-type PPARγ (46). This indicates that phosphorylation of PPARγ represses its transcriptional and adipogenic function and indeed several kinase pathways have been identified that negatively regulate PPARγ transcriptional activity. Growth factors like EGF and PDGF stimulate PPARγ phosphorylation which is mediated by ERK1 and ERK2 (p42 and p44) as was shown both in vitro as well as in cells (48). Phosphorylation of PPARγ by these kinases has been shown as a inhibitory modification negatively affecting PPARγ-mediated gene expression and fat cell differentiation. Changing the serine 112 residue to alanine within the PPARγ protein indeed completly abolishes this inhibitory effect supporting a direct mechanism in growth factor induced PPARγ inhibition. Furthermore, other kinases like JNK and p38, which are part of different branches of the MAPK family, were both shown as PPARγ1 kinases that can inhibit PPARγ signalling via serine 112 (47;49). While growth factor- and/or stress kinase-induced phosphorylation generally leads to inhibition of PPARγ activtity, insulin was found to stimulate phosphorylation of PPARγ resulting in synergistic activation of target genes when combined with synthetic ligands (51). However, serine-to-alanine mutation of the MAPK at position 112 failed to abbrogate the stimulatory effect of insulin, suggesting that the primary phosphorylation target is not the PPARγ protein itself but possibly another protein in the transcription complex (51)

Phosphorylation of PPARγ represses its activity probably through multiple molecular mechanisms. Adams et al. showed that phosphorylation affected both ligand independent as well as ligand dependent transcription, based on reporter assays using full-length PPARγ proteins (47). In addition, their experiments with fusions of the AF1 region to a heterologous DNA binding domain (Gal4DBD) also revealed reduced transcriptional activity of the S112A mutant, suggesting either recruitment of a repressor protein to the AF1 region or release of an activator in a phosphorylation-dependent fashion (47). Subsequent studies showed that PPARγ phosphorylation affected ligand binding, and hence reduced transcriptional activity, indicative for AF1-LBD interdomain communication (52). Finally, phosphorylation of serine 112 has been associated with a repressive posttranslational modification named sumoylation, as will

be discussed below.
Although several kinases have been shown to affect PPARδ function, in most cases no phosphorylation sites have been identified (53). The only exception is the cdk7 kinase, which has been suggested to phosphorylate PPARδ between residues 44 and 55 ((41), see also below).

Sumoylation of PPARs

Recently, several research groups have reported another PTM that adds another layer of modulating PPAR activity: Sumoylation (54-57). The covalent attachment of small ubiquitin-like modifier (SUMO) peptides (SUMO-1, -2, -3 in mammals) to lysine residues involves an activating enzyme (SAE1/SAE2), a conjugating enzyme (Ubc9) and an E3 ligase (e.g. PIAS1)(58-61). This modification occurs on consensus sumoylation motifs ψKXE/D in the substrate proteins, were ψ represents a large hydrophobic residue followed by a lysine which is the SUMO acceptor site and X may be any residue. Mutation of either the lysine residue or the acidic residue at postion +2 has been shown to ablate sumoylation on these sites (62). Ligation of SUMO peptides, which are approximately 100 amino acids long, is linked to various cellular processes, including nuclear-cytoplasmatic transport, apoptosis and transcriptional regulation (58-61). Sumoylation of transcriptional regulators, like NRs (e.g. AR, ERα, MR, GR, SF1) and their coregulators (e.g. SRC1, GRIP1, p300, NCoR), mostly correlates with inhibition of transcription (63-65). Among PPARs, sumoylation has only been described for PPARγ and is probably restricted to this isotype since no consensus SUMO motif is present in the primary amino acid sequence of either PPARα or PPARδ. Indeed, no sumoylation was detected on PPARα or PPARδ under experimental conditions which allowed detection of SUMOylated PPARγ (54). Two functional sumoylation sites have been identified for PPARγ, lysine 107 in the AF1 region and lysine 395 in the AF2 region (lysine 77 and 365 in PPARγ1, respectively)(see also Figure 1). Conjugation of SUMO-1 or -2 to lysine 107 by the E3 SUMO ligases PIAS1 or –xβ modulates PPARγ activity in a negative manner, since (i) mutation of K107 itself or distortion of the sumoylation consensus motif by mutating glutamic acid at position 109 into alanine (E109A) increased PPARγ activity, and (ii) overexpression of a dominant negative form of the SUMO E2-ligase Ubc9 had the same effect. These data indicate that sumoylation of the AF1 domain induces active repression. Although K107 sumoylation has been reported to decrease the stability of the PPARγ protein (55), this finding has not been supported by other studies (54;56;57). The exact molecular mechanism behind sumoylation-mediated repression of PPARγ therefore remains to be established. Possibly the repressive effect involves specific binding of a repressor complex since Gal4DBD-AF-1 fusion proteins, which rule out DNA and ligand binding effects, show increased transcriptional activity upon mutation of K107 (54). SUMO-dependent binding of a repressor protein may require a SUMO interacting motif (SIM), present in several proteins (66). Although highly speculative, a possible mediator of SUMO-mediated inhibition of PPARγ activity could be the transcriptional repressor protein Daxx, an orphan nuclear receptor. This protein contains a SIM domain and was shown to supress transcription via recruitment of the histone deacetylase HDAC2 (67;68). Interestingly, Daxx has been reported to interact with

several nuclear receptors, like the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). Daxx-mediated repression of GR transcriptional activity required an intact SIM domain (69). Alternatively, the DEAD-box protein DP103/Ddx20 may function as a SUMO-dependent repressor of PPARγ activity. This protein, which lacks obvious SIM domains, has been shown to repress the activity of the nuclear receptor SF-1 in a SUMO-dependent fashion (70). Further research is required to establish whether SUMO-mediated repression of PPARγ activity involves Daxx, DP103 or another repressor protein.

Interestingly, lysine 107 sumoylation is to some extent linked to serine 112 phosphorylation. Lysine 107 and serine 112 are found to be part of a so called sumoyl-phospho switch (71) or PDSM motif (72), a conserved motif present in at least eigthy different proteins including several transcription factors and coactivators. The consensus site consist of the following motif: ψKxExxSP in which ψ is a hydrophobic residue, K is the sumo acceptor lysine, x is any amino acid and SP forms part of the downstream phosphorylation site. Between the different PPAR members this motif is only found in the AF-1 region of PPARγ and conserved from fish to humans, as shown in figure 2B. Mutation of the serine 112 to alanine, which ablates phosphorylation, significantly diminished lysine 107 sumoylation while the opposite held true for the PPARγ2 phospho mimic S112D which shows increased sumoylation (54). A similar interplay between phosphorylation and sumoylation has recently been reported for the nuclear receptors ERRα and –γ (73), which also contain a phospho-sumoyl switch (Figure 2B).

In contrast to lysine 107 sumoylation, conjugation of SUMO-1 to lysine K395 is not involved in the regulation of direct PPARγ target genes, but in the transrepression of inflammatory genes by PPARγ in macrophages, like the inos gene (74). Treatment with ligand results in sumoylation of K395, which in turn targets PPARγ2 to NCoR corepressor complexes that are bound to NFκB target genes prior to activation. These NCoR complexes are no longer cleared from the promoter once PPARγ2 is bound, resulting in sustained repression. A similar mechanism was recently reported for transrepression of NFκB target genes by the nuclear receptors LXRα and LXRβ, although in this case SUMO-2 or -3 and not SUMO-1 were conjugated to the receptor (75).

Ubiquitination of PPARs

Ubiquitination is the covelant attachment of ubiquitin, a 76 amino acid peptide to lysine residues in the substrate protein. While conjugation of ubiquitin chains linked through lysine 48 (polyubiquitination) primarily labels proteins for proteosomal degradation, attachment of single ubiquitin molecules or with lysine 63-linked chains (monoubiquitination) is linked to many different cellular processes (76-78). Similar to sumoylation, ubiquitination requires an activating enzyme (E1), a conjugating enzyme (E2) and an E3 ubiquitin ligase (HECT or RING protein). A growing body of evidence indicates that the RNA polymerase II machinery and the ubiquitin-proteasome system are intimately linked (79;80). Groundbreaking studies were perfomed by the Gannon and O'Malley laboratories, who showed that proteosomal degradation of the ERα protein is an essential step in the regulation of its target genes, possibly by enabling the sequential formation of protein complexes at the promoter region (81;82). Like ERα and many other

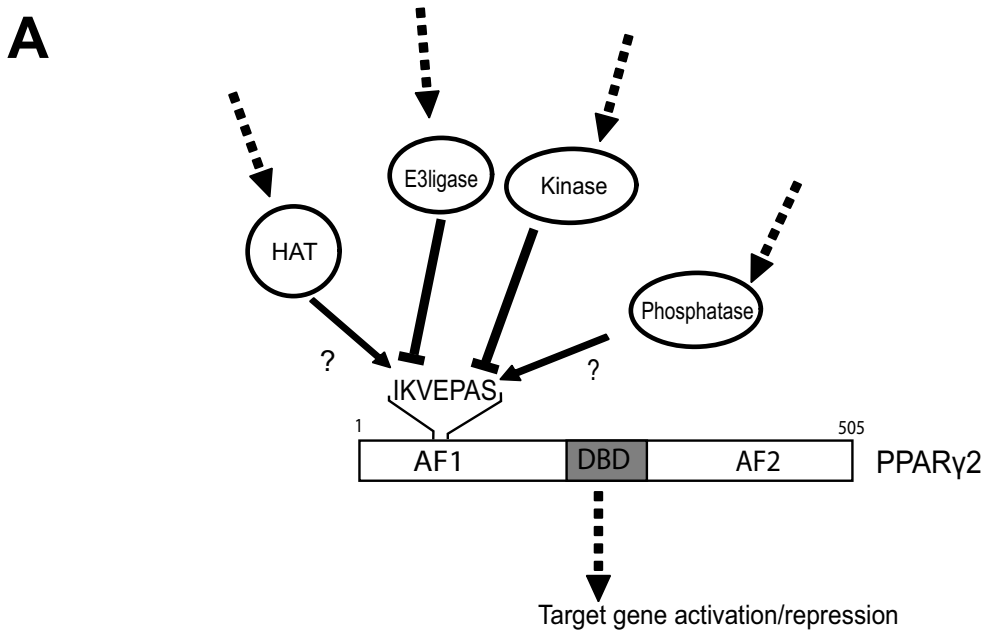

Figure 2 A. The cross-talk of different PTMs at the N-terminus of PPARγ2. Different modifications are induced via diverse signalling cascades enabling ligand independent regulation of the PPAR proteins. Acetylation and dephosphorylation has been described for other proteins containing the sumoyl-phospho switch but so far have not been shown for PPARγ. B. The sumoyl-phospho switch motif found in PPARγ1/2 is conserved between species ranging from fish to man. A functional sumoyl-phospho switch is conserved between the different ERR isoforms. Phosphorylation of the serine residue stimulates sumoylation, via an unknown mechanism, enabling intra-molecular cross-talk of kinase induced modifications and sumoylation. C. AR and ERα acetylation was mapped to the KxK(K) motif present in the C-terminal extension (CTE) of their DBDs. Similar motifs can be identified in the CTE of the different PPAR proteins.

NRs, PPARα and PPARγ were found to be poly-ubiquitinated and degraded by the proteasome (83;84). Heterodimerisation with RXR as well as coactivator binding increased transcriptional activity but also led to increased PPARα ubiquitination and turn-over rate. Corepressor binding on the other hand stabilized PPARα protein levels and decreased ubiquitation, indicating that PPARα-mediated gene expression requires degradation of PPARα (83;85). PPARγ2 ubiquitination is also strongly linked to ligand binding and, similar to PPARα, is increased by coactivator binding and decreased by corepressor binding. Furthermore, mutation of glutamic acid 499 to glutamine (E499Q) abrogated both ligand binding as well as ligand-induced degradation (84), similar to the situation in ERα (81). Interestingly, a strong correlation between phosphorylation and ubiquitination was shown for PPARα by the use of the phosphatase inhibitor okadaic acid: inhibition of phosphatase activity resulted in reduced PPARα ubiquitination and led to increased protein stabilisation (85). This crosstalk between phosphorylation and ubiquitination seems to be specific for PPARα since mutating the serine acceptor residue to alanine of the MAPK consensus site present within PPARγ AF1 did not effect ligand induced destabilisation (84).
Recently, ubiquitination was also reported as a regulatory mechanism in PPARδ-

mediated gene expression. In contrast to PPARα and PPARγ, ubiquitination of PPARδ was found to be inhibited upon ligand binding. It was suggested that this block of ubiquitination might be an essential step in PPARδ mediated gene activation, offering a way to overcome the short half-life of the PPARδ protein (86). However, Rieck and coworkers reported that the short half-life of the PPARδ protein is potentially an experimental artefact, since it was only observed when the protein was overexpressed (87). Under conditions of moderate PPARδ expression, the turnover rate was not affected by ligand (87). While several questions remain, these early studies do suggest a deviating role for ubiquitination in PPARδ signalling: unlike PPARγ and PPARα, PPARδ is not poly-ubiquitinated and rapidly degraded upon ligand binding. Future experiments employing moderate PPARδ overexpression or endogenous PPARδ protein are required to elucidate the exact function of PPARδ ubiquitination. Such studies would be aided by the use of ubiquitination mutants, but no ubiquitination acceptor lysines have been identified for any of the PPAR proteins yet. Current progress in mass spectrometry however holds the promise that ubiquitination sites will actually be identified for the different PPARs in the near future (88;89).

Acetylation

Reversible acetylation of N-terminal tails of histone proteins is an important step in transcription regulation (90-92). Together with other PTMs, like phosphorylation, methylation and ubiquitination, acetylation determines the accesibility of the DNA and subsequent activity of the affected genomic region. In addition, it creates specific docking sites for proteins (93), and contributes to the so-called “histone code”, a bar code which may be involved in the establishment of epigentic inheritance (90;91). Interestingly, reversible acetylation is not restricted to histone proteins, but also functions as an important regulatory mechanism for many non-histone proteins (94-96). Acetylation of non-histone proteins can result in either positive or negative effects on transcription by affecting for example protein-protein interactions (e.g. ACTR(97)), protein-DNA interactions (e.g. HMGI(Y) (98)), nuclear retention (e.g. HNF4 (99)) or protein halflife (e.g. E2F (100)). Among nuclear receptors, the androgen receptor (AR), estrogen receptor α (ERα) and thyroid hormone receptor (101) have been identified as acetylation substrates (102-106). AR and ERα acetylation was mapped to the KxK(K) motif present in the C-terminal extension (CTE) of their DBDs (see Figure 2C), while acetylation of the ERα hinge region has also been described (105). Acetylation-deficient AR mutants showed increased N-CoR corepressor binding and diminished ligand binding. For ERα, acetylation in the CTE region was shown to increase DNA binding and thereby transcriptional activity (104), while acetylation of the hinge region was suggested to inhibit receptor activity (105). The acetyltransferases responsible for the acetylation of AR were identified as Tip60 and CBP/p300 (102;103;106), while ERα is targeted by p300 (103;104). So far PPAR acetylation has not been reported, although the CTE region contains a conserved lysine-rich motif like the AR and ER (Figure 2C). Our preliminary data indicate that PPARγ is indeed subject to acetylation (Van Beekum and Kalkhoven, unpublished observations). Since acetylation and sumoylation have been described to compete

for the same lysine residue in a number of proteins (HIC1, PLAG1/2) (107;108), we mutated the N-terminal SUMO acceptor site in PPARγ2 (K107) and indeed found acetylation to be diminished (Van Beekum and Kalkhoven, unpublished observations). The acetyltransferase responsible for this remains to be identified, but is probably not the AF1-interacting acetyltransferase Tip60 (37). The two closely related acetyltransferases p300 and CBP are however good candidate proteins to fullfill this role, since both proteins are well established PPARγ interactors (109). Both acetyltransferases were reported to be essential for adipogenesis and are involved in activating PPARγ-mediated transcription (110;111). Further research is required to establish the function of PPARγ acetylation, to identify the responsible acetyltransferases and to assess the potential competition with sumoylation and acetylation of the PPARγ protein.

Physiological function of PPAR post-transcriptional modifications

With a few exceptions the in vivo relevance of PTMs of PPARs has not been adressed experimentally. In an elegant report by Rangwala et al., it was shown that homozygous PPARγ S1112A knock-in mice are no longer prone to develop insulin resistance when fed a high fat diet (112). No significant difference in percentage body fat was detected compared to wild-type mice. It was suggested that the underlying mechanism for this protection from obesity induced insulin resistance lies in decreased adipocyte size and secreted adipocytokines (112).
In contrast to the phenotype of PPARγ S112A knock-in mice, patients with a heterozygous PPARγ P113Q mutation develop severe obesity (113). Furthermore, in the same study three out of four patiens that were identified as having the PPARγ P113Q mutation also had developed type 2 diabetes mellitus (113). These findings suggest that PPARγ phosphorylation in humans serves a different function compared to mice.
Finally, Compe et al. reported decreased phosphorylation of PPARs in XPD/TTD deficient mice (41). XPD is a subunit of the basal transcription factor TFIIH which is involved in DNA repair. Mutations in XPD cause the rare autosomal recessive disorder xeroderma pigmentosum leading to predisposition for carcinogenesis caused by inceased photosensitivity. Besides sensitivity for UV radiation and a large range of other clinical symptoms, XPD patients show hypoplasia of adipose tissue (114;115). XPD mutant mice develop the same clinical features as found in patients, including a reduction of adipose mass. As described above, the cdk7 kinase, which is part of the TFIIH complex, is able to phosphorylate PPARα at serine 6 and serine 21 and PPARγ2 at serine 112. It is thought that mutations in the XPD protein weaken the interaction with other proteins in the TFIIH complex thereby explaining the loss of PPAR phosphorylation by cdk7. It was shown that decreased PPARγ phosphorylation led to increased PPARγ target gene expression in white adipose tissue and decreased PPARα target gene expression in liver tissue of XPD deficient mice. These results are in line with previous studies which showed that phosphorylation of serine 6 and 21 stimulated PPARα actvity, while serine 112 phosphorylation inhibited PPARγ activity (46-50). However, when PPARγ target gene expression was studied in brown adipose tissue, the levels were lower in XPD deficient mice when compared to wild-type

mice (41). This discrepancy between white and brown adipose tissue could point to a tissue-specific function of PPARγ phosphorylation. Alternatively, the outcome of PPARγ phosphorylation could somehow depend on the responsible kinase.

Future directions

Since Hu et al. reported in 1996 that PPARγ was a phospho-protein (46), numerous studies have followed showing that PPARs are subject to many different PTMs. While the modifying enzymes and substrate amino acids were identified in most cases, it is often still largely unknown how these modifications alter the function of a PPAR protein. The two most likely mechanisms are intramolecular, as was shown for serine 112 phosphorylation of PPARγ which reduces ligand binding (52), or intermolecular. In the latter case, modifications alter the PPAR protein to allow either recruitment of novel interactors or release of proteins that were bound to the unmodified receptor. An interesting parallel can be drawn with histone proteins, which are also subject to multiple modificiations (90;92). Multiple examples exits of specific docking of proteins to modified forms of histones, through specialized domains within these so-called effector proteins. For example, some effector proteins interact with acetylated histones through their bromodomains (116), while others specifically interact with tri-methylated lysine 4 of histone H3 through a Plant Homeodeomain(PHD) type zinc finger (117). Recently a similar mechanism has been proposed for microtubule formation. Within the cytoplasm different subpopulations of microtubules excist in which each population contains different PTMs that recruits different protein complexes thereby fullfilling different functions (118). It seems plausible that similar mechanisms are operational in the regulation of transcriptional activity of PPARs through PTMs, but most of the effector proteins remain to be identified.

Secondly, little attention has so far been paid to the fact that all the PTMs described here for PPARs can probably undergo de-modification by specific cellular enzymes. In the case of the histones, many de-modifying enzymes have been identified, which can deactylate (HDACs and SIRTs), de-ubiquitinate (DUBs) or de-phosphorylate (phosphatases) these proteins, thereby creating a highly dynamic and well-regulated control system. So far no demodifying enzymes have been identified for the PPARs, although these factors may play an extremely important role in balancing the transcriptional output.

Another area that is clearly understudied and needs further exploration is whether a given PTM of a PPAR protein alters the expression of all target genes or just a specific subset of target genes. The latter option may sound plausible, since it introduces another level of regulating the transcriptional output, but firm evidence supporting this hypothesis is currently limited. One example is the PKA-mediated phosphorylation of PPARα, which was shown to contribute to the induction of the ACO gene but not the FABP gene (43). Likewise, ERα phosphorylation of a conserved MAPK site at serine 118 is required for activation of some estrogen responsive genes but not others (119), but it is not clear how general this theme is. Furthermore, the sumoylation sites have been shown to be particularly important for the regulation of promoters harbouring multiple response elements (73;120). Recently, emerging technologies like ChIP on-chip (121;122) and ChIPseq (123) may help to address the intruiging link between PTMs and target gene specificity. It should however be noted that (i) these

methods require high quality antibodies that specifically recognize mono- or multi-modified PPAR proteins, (ii) the stochiometry of modification may be very low (e,g. sumoylation), and (iii) modified proteins are prone to demodification by specific cellular enzymes. In the case of SUMO–modified PPARγ for example, approaches like this may require prior inactivation or knock-down of SUMO isopeptidases in cells, since SUMOylated proteins are highly susceptible to demodification by isopeptidases. In additon, a great deal more needs to be done to investigate potential crosstalk between the different PTMs. Such crosstalk may be positive, as shown for the so-called phospho-sumoyl switch motif (71) or PDSM motif (72) in PPARγ2, whereby phophorylation of PPARγ2 serine 112 preceeds sumoylation of lysine 107 (54). Negative crosstalk, in which two modifying enzymes compete for the same substrate residue, has been described for many proteins (124;125). In the case of PPARγ, this may occur on lysine 107, which has been shown to be sumoylated (54;56;57), and possibly also acetylated (Van Beekum and Kalkhoven, unpublished observations). Crosstalk between PTMs poses the possibility of integrating multiple signalling pathways : the combination of different extracellular and intracellular signals could lead to a specific pattern of PPARγ modifications, which is “read” by the effector proteins, ultimately dictating the expression of a specific subset of target genes (Figure 2A).
Finally, at present the physiological relevance of PTMs of PPARs is clearly understudied. So far, only the in vivo effects of mutating the serine 112 phosphorylation site in PPARγ was studied (112), which has given interesting new insights in PPARγ action. It will therefore be essential for our understandig of PPAR action to extend such approaches to other posttranslationally modified residues.

Concluding remarks

Obesity and type 2 diabetes are ever increasing problems in industrialized countries, affecting millions of people world wide. The physiological roles of the different PPAR members in lipid and glucose metabolism has quickly led to the therapeutic use of fibrates, a class of synthetic PPARα ligands, to treat dyslipidemia and synthetic PPARγ ligands of the TZD class for the treatment of insulin resistance. However, the effectiveness of fibrates to treat dyslipidemia has recently been questioned (126). Furthermore, treatment of diabetic patients with synthetic PPARγ ligands of the TZD class has been linked to adverse side effects like undesired weight gain, fluid retention, peripheral edema and potential increased risk of cardiac failure (127;128). These adverse side effects may be due to the use of high doses of full PPAR agonists, suggesting that “activation in moderation” may be a more sensible approach (18). This may be achieved through the use of compounds displaying partial agonism, so-called selective PPAR modulators (SPPARMs)(129;130). Alternatively, modulating the PTMs on PPAR proteins may present a novel approach in this respect. Therefore increasing our knowledge of PPAR PTMs, the modifying and de-modifying enzymes involved, the effector proteins, the specific transcriptional output and the cellular and in vivo effects can be of great value for the development of novel pharmaceutical approaches to fight the illnesses in which the PPAR family plays a role.

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Impaired PPARγ function through mutation of a conserved salt bridge (R425C) in Familial Partial Lipodystrophy

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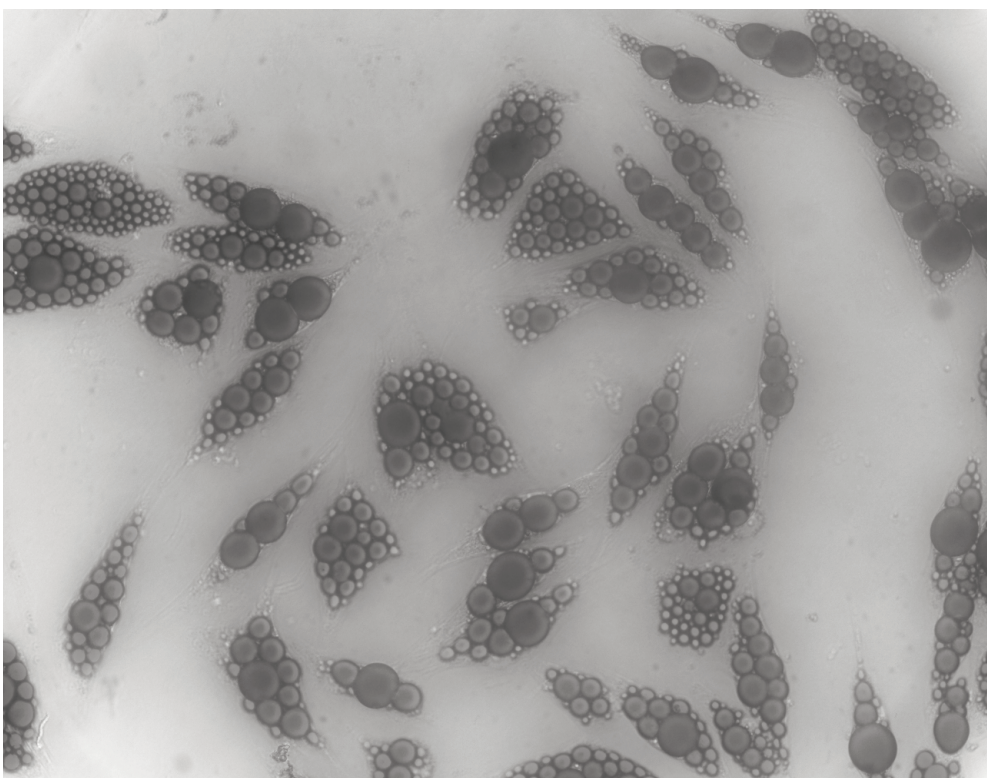

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ABSTRACT

The nuclear receptor PPARγ plays a key role in the regulation of glucose and lipid metabolism in adipocytes by regulating their differentiation, maintenance and function. A heterozygous mutation in the PPARG gene, which changes an arginine residue at position 425 into a cysteine (R425C), has been reported in a patient with familial partial lipodystrophy subtype 3 (FPLD3). The strong conservation of arginine 425 among nuclear receptors that heterodimerise with RXR prompted us to investigate the functional consequences of the R425C mutation on PPARγ function. Here we show that this mutant displayed strongly reduced transcriptional activity compared to wild type PPARγ, irrespective of cell type, promoter context or ligand, whereas transrepression of NF-κB activity remained largely intact. Our data indicate that the reduced transcriptional activity of PPARγ R425C is not caused by impaired corepressor release, but due to reduced dimerisation with RXRα in combination with reduced ligand binding and subsequent coactivator binding. As a consequence of these molecular defects, the R425C mutant was less effective in inducing adipocyte differentiation. PPARγ R425C did not inhibit its wild type counterpart in a dominant negative manner, suggesting a haploinsufficiency mechanism in at least some FPLD3 patients. Using molecular dynamics simulations, substitution of R425 with cysteine is predicted to cause the formation of an alternative salt bridge. This structural change provides a likely explanation how mutation of a single conserved residue in a patient with FPLD3 can disrupt the function of the adipogenic transcription factor PPARγ on multiple levels.

Introduction

The prevalence of obesity and overweight rapidly increases worldwide (1). This is a major health concern, because adiposity is highly associated with insulin resistance, type 2 diabetes, dyslipidemia, hypertension and atherosclerosis (components of the metabolic syndrome). Interestingly, the selective loss of adipose tissue (lipodystrophy) is also frequently associated with marked insulin resistance and complications such as type 2 diabetes, dyslipidemia and hypertension (2). Thus, normal amounts of adipose tissue and normal fat distribution appear to be critical for the optimal regulation of lipid and energy metabolism.

Lipodystrophies represent a heterogeneous group of diseases characterized by altered amounts and/or distribution of body fat and major metabolic complications. The main forms can be classified according to their origin, either genetic or acquired, and subclassified according to the clinical pattern (3;4). Whereas acquired lipodystrophies, like the metabolic syndrome and lipodystrophy due to highly active anti-retroviral therapy (HAART) (5) are fairly common, inherited forms of lipodystrophy are rare. Familial partial lipodystrophy (FPLD) is an autosomal dominantly inherited disorder, characterized by gradual loss of subcutaneous fat from the extremities and an accumulation of excess fat in the intra-abdominal regions (3;4). FPLD subtype 3 (FPLD3, MIM 604367) is associated with mutations in the PPARG gene (MIM 601487)(6;7). The majority of FPLD3 patients have profound metabolic disorders, in particular severe insulin resistance (IR) and early onset diabetes mellitus, with polycystic ovarium syndrome seen in female subjects as a direct consequence of IR and marked or extreme dyslipidemia, characterized by high triglycerides and low HDL cholesterol. Some subjects also suffer from hypertension and hepatic steatosis (6;7).

The PPARG gene encodes the PPARγ protein, a member of the nuclear receptor superfamily of ligand inducible transcription factors (8-10). Differential promoter usage and alternative splicing of the PPARγ gene generates four mRNA species (PPARγ 1-4), but just two different receptor proteins, PPARγ1 and PPARγ2. The PPARγ2 isoform contains 28 additional amino acids at its N-terminus and its expression is restricted to adipose tissue, while PPARγ1 is more widely expressed (e.g. in adipose tissue, lower intestine and macrophages). PPARγ plays a key role in glucose and lipid metabolism in adipocytes (8-10). Studies in murine cell lines have established that liganded PPARγ is both essential and sufficient for adipogenesis (11), while PPARγ knockout mice fail to develop adipose tissue (12-14). In addition, PPARγ directly regulates the expression of a number of genes involved in net lipid partitioning into mature adipocytes. Besides PPARγ, two closely related receptors have been identified, named PPARa and PPARb/d, which are encoded by different genes (10;15). These three related receptors all bind to the PPAR responsive elements (PPRE) in the promoter regions of target genes as obligate heterodimers with retinoic acid X receptors (RXRs), but exhibit different physiological roles due to their distinct expression patterns and specific activation by different ligands. Like other nuclear receptors, PPARγ consists of distinct functional domains including a constitutively active transactivation domain (AF-1), at the N-terminus, a centrally located highly conserved DNA-binding domain (DBD) composed of two zinc finger motifs, and a C-terminal ligand-binding domain (LBD) that contains a powerful ligand-dependent

transactivation function (AF-2). Ligand binding stabilises the active conformation of the PPARγ LBD, thereby serving as a "molecular switch" between activation and repression functions of the receptor (16;17). On some promoters, unliganded PPARγ recruits corepressors like N-CoR and SMRT, which are part of multiprotein complexes containing histone deacetylase (HDAC) activity that repress gene transcription (18-20). Upon ligand binding, these corepressor complexes are released and replaced by coactivator complexes, including the SRC1/CBP and TRAP/DRIP/ARC complexes, that are involved in transcriptional activation (21;22). The endogenous ligands for PPARγ are not firmly established although some natural compounds, like polyunsaturated fatty acids and prostaglandin J2 derivatives (15-deoxy-$\Delta^{12,14}$-PGJ2) have been shown to be able to activate PPARγ (23;24). Synthetic PPARγ agonists include the thiazolidinediones (TZDs)(25) and tyrosine-based agonists (26), which ameliorate insulin resistance and lower blood glucose levels in patients with type 2 diabetes (8). At least part of this response is thought to occur through indirect regulation of gene expression by PPARγ, e.g. by transrepression of TNFα-induced NF-κB activity (27;28).

Seven PPARγ missense mutations have been described in patients suffering from FPLD3, which are located either in the DNA binding domain (C142R, C159Y, C190W (PPARγ2 nomenclature)) or in the ligand binding domain (V318M, F388L, R425C, P495L (PPARγ2 nomenclature)) (29-32). In addition, 2 nonsense mutations (R385X, Y355X) and 2 frameshift/premature stop mutations have been reported (32-34). Except for R425C, all mutations have been shown to result in receptors with reduced transcriptional activity and some, but not all mutants inhibited their wild-type counterpart in a dominant negative manner (see also Discussion). The functional consequences of the R425C FPLD3-associated missense mutation, which changes arginine 425 into cysteine, are currently unknown. Interestingly, a structure based sequence alignment of nuclear receptors revealed that this arginine residue is part of a structural signature, which defines nuclear receptors that form heterodimers with RXRα, including the thyroid hormone receptor b (TRb) (35). Within this subclass of proteins, named class II receptors, R425 is involved in the formation of a conserved salt bridge with a negatively charged residue in helix 4/5 (E352 in PPARγ2). In contrast, the homodimeric class I receptors lack this conserved arginine residue and the internal salt bridge (35).

The strong conservation of arginine 425 in PPARγ2 among class II nuclear receptors together with the lack of functional data on the natural R425C mutation motivated us to investigate the functional consequences of this FPLD3-associated mutant. Here we combine detailed molecular analysis with structural modelling, using molecular dynamics simulations, to show that the R425C mutation found in a patient with FPLD3 causes aberrant salt bridge formation and thereby abrogates PPARγ transcriptional activity leading to an inhibition of adipocyte differentiation.

MATERIALS AND METHODS

Materials

Rosiglitazone was purchased from Alexis. Pioglitazone, Ciglitazone, Troglitazone and 15-deoxy-$D^{12,14}$-PGJ_2 were from Cayman Chemical Company. GW1929 and TNFa were from Sigma-Aldrich. [^{3}H]-Rosiglitazone was purchased from American Radiolabeled Chemicals, Inc. FugeneÒ6 transfection reagent was from Roche Applied Biosciences. Anti-PPARγ (sc-7273), anti-RXRα (sc-553) and anti-Gal4 (sc-510) antibodies were from Santa Cruz Biotechnologies. Anti-tropomyosin antibody (T2780) was purchased from Sigma-Aldrich and Roche Applied Biosciences, respectively. Anti-rabbit-HRP (111035144) and anti-mouse-HRP (115035146) were from Jackson Immunoresearch Laboratories Inc. Oil-Red-O was purchased from Sigma-Aldrich (O-0625). Crystal violet was from Chroma-Gesellschaft Schmid&Co.

Plasmids

All recombinant DNA work was performed according to standard procedures (58). The pCDNA3-PPARγ1, pCDNA3-PPARγ2 and pCDNA3-PPARβ/d constructs were kind gifts from Dr. V.K.K. Chatterjee (48). pCDNA3.1-PPARa expression plasmid was a kind gift from Dr. S. Ali. The reporter containing the human aquaporin promoter (-681/+11) was a kind gift from Dr. N. Maeda (38). The reporter plasmid 2kB-luciferase was a kind gift from Dr. E. Burstein (59). The retroviral pMSCV-vector (Clontech) containing wild type murine PPARγ2 was a kind gift from Dr. B.M. Spiegelman and Dr. R.G. Roeder (60). The bacterial expression vector for GST-SMRT (61) and the 3xPPRE-tk-Luc reporter (23) were kind gifts from Dr. R.M. Evans. The bacterial expression vector for GST-SRC1 (amino acids 570-780) (62), GST-RelA (63) and the 5xGal4-AdMLTATA-Luc (64), 5xGal4-TK-Luc (64) and 3xPPRE-tk-Luc (23) reporters have been described earlier.

The pCDNA-Gal4DBD-PPARγ LBD (amino acids 173-475) expression construct was generated by cloning a BamH1/Xba1 fragment from pSG424-PPARγLBD (gift from Dr. V.K.K Chatterjee (48) into the respective sites of pCDNA3-Gal4DBD (64). The expression vector containing the complete coding region of human RXRα was a kind gift from Dr. J.D. Baxter (65). pCDNA3.1-RXRα was generated by PCR amplification of RXRα from this vector using primers containing XbaI and HindIII-sites and cloned into the respective sites of pCDNA3.1(-). PGEX2TK-GST-CBP was generated by cloning the BamHI/EcoRI fragment (amino acids 1-452) from pCDNA3.1-CBP (64) into the pGEX2TK vector. To generate a pCDNA3-Gal4DBD-SMRT-ID1 construct, the SMRT-ID1 fragment (amino acids 2302-2352) was amplified from the GST-SMRT construct (61) by PCR using primers containing BamH1and XbaI-sites respectively and cloned into the pCDNA3-Gal4DBD construct. PCDNA3-VP16 was constructed by amplification of the VP16 from a psG5-VP16 expression construct (66) by PCR using a forward primer containing a HindIII-site and a reverse primer containing a stop-codon and BamH1-site. The VP16 cDNA was inserted in the corresponding sites of pCDNA3. PCDNA3-VP16-PPARγLBD was generated by PCR amplification of VP16 activation domain using primers containing HindIII and BamH1-sites respectively and

insertion into the respective sites of pCDNA-Gal4DBD-PPARγLBD, after removal of the Gal4DBD sequence. All mutations were generated by Quickchange mutagenesis (Stratagene) and verified by sequencing.

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 mg of penicillin/ml and 100 mg streptomycin/ml (Gibco Life Technologies). U2OS cells used for NF-kB-luciferase reporter assays were maintained in DMEM Glutamax (Dulbecco) containing 5% dextran-coated charcoal stripped foetal calf serum (67) (Gibco Life Technologies), 100 mg of penicillin/ml and 100 mg streptomycin/ml (Gibco Life Technologies). The murine NIH-3T3 cell line was cultured in the same media but now with 10% bovine serum (Gibco Life Technologies), 100 mg of penicillin/ml and 100 mg streptomycin/ml (Gibco Life Technologies). For luciferase reporter assays cells were seeded in 24-wells plates and transiently transfected using the calcium-phosphate precipitation method. Each well was cotransfected with a reporter construct, PPAR expression constructs and 2 µg pCMV-Renilla (Promega) or 12,5 µg TK-Renilla (Promega) as indicated in the figure legends. The next day, cells were washed twice with HBS (pH 7.05) and subsequently maintained in medium in absence or presence of PPAR ligands for 24 hours. After incubation cells were washed twice with phosphate-buffered saline (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 Centro LB 960 luminometer (Berthold Technologies). For western blot analyses of the different PPARγ proteins, U2OS cells were transiently transfected with 2 mg PPARγ expression construct using FugeneÒ6 transfection reagent and treated as described as above. Cells were lysed in Laemli sample buffer and subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore). α-PPARγ antibody was used to probe for PPARγ protein and ECL (Amersham Biosciences) was used for detection.

GST-pull downs

Recombinant PPARγ2 cDNAs (wild type or mutants) in the pCDNA3 expression vector were transcribed and translated in vitro in reticulolysate in the presence of [^{35}S]methionine according to manufacturer's protocol (TNT T7 Quick Coupled Transcription/Translation Kit, Promega). Rosetta pLysS competent bacteria (Novagen) were transformed with GST expression plasmids. GST fusion proteins were induced and purified as described earlier (62). [^{35}S]–labelled proteins were incubated with GST fusion proteins in NETN-buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0,5% NP40) containing protease inhibitors (Complete, Roche Applied Biosciences). Samples were subsequently washed and subjected to SDS-PAGE. Gels were enhanced with Amplify (Amersham) and dried and the [^{35}S]-labelled proteins were visualized by fluorography. For each assay, at least three independent pull downs were performed.

EMSA

Double-stranded DNA oligomers, containing the PPRE from the rat acyltransferase-coenzyme A oxidase promoter (36), were labelled with [a-^{32}P]dATP using Klenow enzyme and purified using Probe Quant™ G-50 Micro Colums (Amersham Biosciences). In vitro translated PPARγ (wild type or mutants) and/or in vitro translated RXRα proteins were pre-incubated for 15 minutes at room temperature in absence or presence of 10-fold cold probe (wild type or mutant) in a buffer containing 10 mg BSA, 1 mg poly dI:dC, 40 mM HEPES (pH 7.4), 100 mM KCl, 2 mM DTT and 20% glycerol. For supershift experiments 1 µg of α-RXR or α-PPARγ or α-Gal4 antibodies were added to the pre-incubation mix. Following pre-incubation, purified [^{32}P]-labelled probe was added and incubated for 30 minutes at room temperature, followed by 30 minutes at 4°C. Receptor-DNA complexes were separated from unbound DNA on non-denaturing polyacrylamide gels and visualized by autoradiography. At least three independent experiments were performed.
The complete probe sequences used for binding and competition analysis were as follows: PPRE-wild-type, 5'-CCGGGGACCAGGACAAAGGTCACGAAGCT-3' and PPRE-mutant, 5'-CCGGGGGACCAGCACAAAGCACACGAAGCT-3'

Ligand-binding assay

Filter binding assays were performed as described by Adams et al. (68) with minor modifications. Bacterially expressed and purified GST-PPARγ2 fusion proteins were incubated with 10 or 100 nM [^{3}H]-Rosiglitazone in binding buffer (50mM HEPES pH 7.9, 100 mM KCl, 2 mM DTT, 10% glycerol) followed by incubation with an 200-fold excess of competing cold ligand (rosiglitazone). Bound ligand was separated from unbound ligand by passage through a filter membrane (Millipore HA filters, 0.45mm) under vacuum followed by three washes with binding buffer. Filters were then transferred into tubes containing scintillation fluid and counted in a g-counter. Binding experiments were performed independently three times.

Differentiation assay

Retrovirus was produced by Fugene transfection of Phoenix Ampho cells with the parental retroviral pMSCV-vector, pMSCV containing either wild type murine PPARγ2, or PPARγ2 R425C and collection of virus supernatants. NIH-3T3 fibroblasts were infected overnight with the virus supernatants in medium containing 4 µg/ml polybrene and infected cells were selected by addition of 2 mg/ml puromycine to the culture medium. Selected cells were grown to confluency and after two days incubated with culture medium containing dexamethasone (250 nM), IBMX (500 µM), insulin (170 nM) and rosiglitazone (2.5 nM) for two days. At day 3, medium was changed for culture medium supplemented with insulin (170 nM) and Rosiglitazone (2.5 nM) and left for a week. Cells were subsequently washed and triglycerides were stained by Oil-Red-O and the cell nuclei by crystal violet. Stained cells were photographed with a light microscope (Zeiss Axiovert 40 CFL) coupled to a digital camera (Canon DSC Powershot G5). Two independent retroviral transductions were performed.

For western blot analyses, differentiated NIH-3T3 cell lines (empty vector, PPARγ wild type or PPARγ2 R425C) were lysed in Laemli sample buffer and samples were subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore). α-PPARγ and a-tropomyosin antibodies were used to probe for PPARγ protein and tropomyosin protein respectively. ECL (Amersham Biosciences) was used for detection.

Quantitative RT PCR

Transduced NIH-3T3 fibroblasts were differentiated as described above. For each cell line (empty vector, PPARγ2 wild type or PPARγ2 R425C), total RNA from three wells was isolated independently using TriPure reagent (Roche Applied Science) and reverse transcribed using MMLV-RT Rnase H Minus Point Mutant (Promega), according to manufacturer's protocol. Transcription levels were determined by real-time PCR using the LightCycler (Roche Applied Science). Briefly, the relative expression of the housekeeping gene Hprt1 (hypoxanthine-guanine phosphoribosyltransferase 1) was used to calculate the relative expression level of the PPARγ2 target genes Fabp4, Aqp7 and Gyk respectively, according to Vandesompele et al. (69). The PCR reactions were performed using the DNA Master SYBR-green 1 kit (Roche Applied Science) and contained 5.0 µl 1:40 diluted cDNA, 0.25 pmol/µl. primer, DNA master SYBR-green I solution, and $MgCl_2$ (3.5 mM). Reactions were carried out in triplicate for each sample.
The sequences of the primers are as follows: murine Hprt1 sense primer; 5'-TCCTCCTCAGACCGCTTTT-3'; anti-sense primer, 5'-CCTGGTTCATCATCGCTAATC-3'; murine Fabp4 sense primer 5'-GAAAACGAGATGGTGACAAGC-3'; anti-sense primer 5'-TTGTGGAAGTCACGCCTTT-3'; murine Aqp7 sense primer, 5' GGCTTCTCCCTTCCTCTAGTTT-3'; anti-sense primer, 5'- AAGGCCACTGAGGAAGTCATT-3'; murine Gyk sense primer, 5'- TTCCAGGAAATAATAACTTTGTCAAG-3'; anti-sense primer, 5'- CACTGCACTGAAATACGTGCT-3'.

MD simulations

Molecular dynamics simulations were performed with the GROMACS 3.1.4 package (70;71), using the GROMOS96 43A1 force field (72). The starting structure of the wild type protein was obtained from the protein data bank (PDB) (73), entry 1FM6 (16), chain D or chain X and entry 1FM9 chain D; the mutant protein structure was obtained by replacing Arginine 425 by a Cysteine (for 1FM6 chain D) or by an Alanine (for all three chains). Cofactors and ligands were removed. Each simulation was run for 5.0 ns. A total of seven simulations was thus performed: three for wild type, three for the R425A mutant and one for the R425C mutant.
The starting structure was solvated in a cubic box with a minimum solute-box distance of 14Å, filled with approximately 25000 SPC water molecules (74) and 5 (wild type) or 6 (mutant) additional Na^+ ions to electro-neutralize the system, respectively. Periodic boundary conditions were applied.
Each system was first energy minimized using 1.000 steps of Steepest Descent algorithm. After this, equilibration was performed in five 20 ps phases during which the

force constant of the position restraints term for the solute was decreased from 1000 to 0 KJ mol^{-1} nm^{-2} (1000, 1000, 100, 10, 0 KJ mol^{-1} nm^{-2}, respectively). The initial velocities were generated at 300K following a Maxwellian distribution.
Solute, solvent and counterions were independently weakly coupled to reference temperature baths at 300 K (τ = 0.1 ps) (75). The pressure was maintained by weakly coupling the system to an external pressure bath at one atmosphere except for the first 20 ps equilibration part which was performed at constant volume (NVT). The LINCS algorithm (76) was used to constrain bond lengths, allowing an integration time step of 0.002 ps (2 fs) to be used. The non-bonded interactions were calculated with a twin-range cut-off (77) of 0.8 and 1.4 nm. The long-range electrostatic interactions beyond the 1.4 nm cut-off were treated with the generalized reaction field model (78) using a dielectric constant of 54. The non-bonded interaction pair list was updated every 5 steps. Trajectory coordinates and energies were stored at 0.5 ps intervals. The analysis was performed using the set of programs within GROMACS. Figures were generated using MolScript (79) and Raster3D (80).

RESULTS

The FPLD3-associated PPARγ2 R425C displays reduced transcriptional activity

As a first step in our analysis of the FPLD3-associated R425C mutation, its effect on the ability of PPARγ2 to activate transcription was tested in reporter assays. For this the human osteosarcoma cell line U2OS was used, which lacks endogenous PPARγ expression and displayed the most significant reporter activation by liganded PPARγ among several human cell lines tested. Cells were transfected with expression vectors encoding wild type PPARγ2 or R425C mutant together with a reporter construct containing three copies of the PPRE found in the rat acyl CoA promoter (36). While wild type PPARγ2 readily activated this 3xPPRE-tk-Luc reporter approximately 120-fold upon addition of the synthetic ligand rosiglitazone (1 μM), PPARγ2 R425C only activated this reporter 20-fold (Figure 1A). Figure 1B shows that PPARγ2 R425C was functionally defective at all concentrations of rosiglitazone tested, including saturating amounts. Cotransfection with RXRα did not increase the transcriptional activity of either wild type or mutant PPARγ, indicating that RXRα is not limiting under these experimental conditions (data not shown). Similar results were obtained in 293T cells,

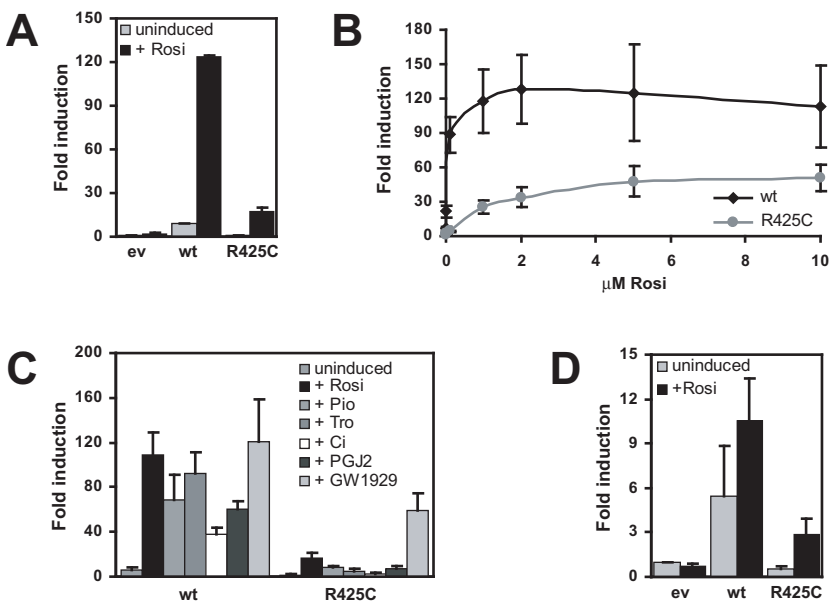

Figure 1 The FPLD3-associated R425C mutation reduces the transcriptional activity of PPARγ2. A, U2OS cells were transfected with expression vector encoding PPARγ2 wild type (wt) or PPARγ2 R425C respectively, and a 3XPPRE-tk-Luc reporter. Activation of the luciferase reporter, in the absence or presence of 1 μM rosiglitazone, is expressed as fold induction over that with empty vector (ev) in the absence of ligand, after normalisation for Renilla luciferase activity. Results are averages of at least three independent experiments assayed in duplicate ± standard error of the means. B, Dose-response curve of U2OS cells transfected as in panel A. C, Reporter assay as outlined in panel A using the PPARγ ligands, pioglitazone (Pio; 2 μM), troglitazone (Tro; 1.5 μM), ciglitazone (Ci; 6 μM), 15-deoxy-Δ12,14-PGJ2 (PGJ2; 14 μM) and GW1929 (1 mM), respectively. D, U2OS cells were transfected as in A, but with the human adipose aquaporin (AQP7)-Luc reporter instead of the 3xPPRE-tk-Luc reporter.

indicating that the reduced activity of the R425C mutant is not cell type specific (data not shown).

Since PPARγ can be activated by various ligands (23-26), reporter assays were also performed using several other synthetic and one natural PPARγ ligand. All the TZDs tested (rosiglitazone, pioglitazone, troglitazone and ciglitazone) and the natural ligand 15-deoxy-$D^{12,14}$-PGJ_2 showed similar differences in transcriptional activity between wild type and mutant PPARγ2 (Figure 1C). Interestingly, the impaired activity of the R425C mutant was partially restored by the tyrosine-based agonist GW1929, a strong agonist that is also capable of activating the FPLD3 associated V318M and P495L mutants of PPARγ2 (37).

The reporter construct used above contains three copies of a functional PPRE. In order to test the effect of PPARγ2 R425C on a more “natural” promoter, reporter assays were performed using a luciferase reporter construct containing the promoter of the human adipose aquaporin gene (AQP7), which contains a single functional PPRE (38). As shown in Figure 1D, activation of this reporter by wild type PPARγ2 was less pronounced (6- and 12-fold in the absence and presence of rosiglitazone, respectively), compared to the 3xPPRE reporter (Figure 1A). However, mutation of R425 into cysteine reduced the ability of PPARγ2 to activate this natural promoter in a similar fashion (Figure 1D).

Amino acid sequence alignment of PPARs revealed that the arginine at position 425 in PPARγ2 lies within a region that is highly conserved between species and different PPAR family members (Figure 2A). To investigate the effect of the R425C mutation on other PPAR isoforms, analogous mutations were generated in PPARγ1

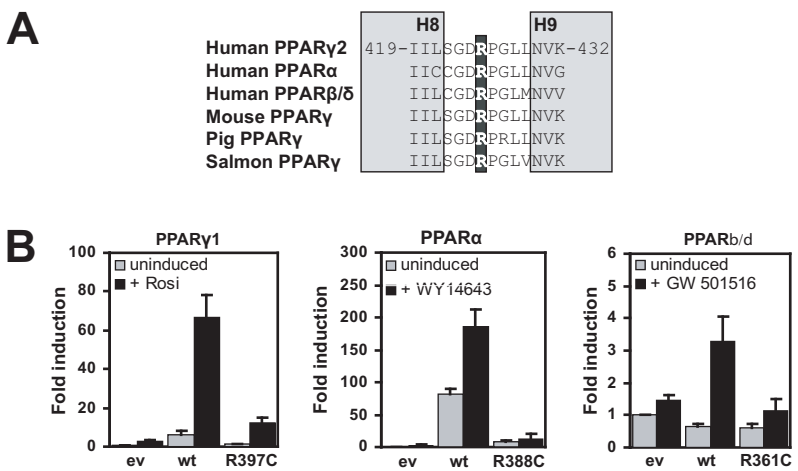

Figure 2. Mutations analogous to R425C in PPARγ2 have similar effects on transcriptional activity of different PPAR proteins. A, Alignment of the amino acid sequence surrounding R425 in human PPARγ2 (CAA62153), with human PPARα (AAB32649), human PPARβ/δ (AAH02715), mouse PPARγ (AAO45098), pig PPARγ (CAA07225) and salmon PPARγ (CAC02968). Species in which the sequence surrounding R425 was identical to human and mouse (e.g. rat (AADA0119), zebrafish (AAY85274), Xenopus (AAH60474), chicken (AAL85323)) were omitted for reasons of clarity. Also indicated are the boundaries of helix 8 and 9, as defined in the crystal structure of the PPARγ LBD (17). B, U2OS cells were transfected with expression vectors encoding PPARγ1 wt or R397C mutant (left panel), PPARα or R388C mutant (middle panel) or PPARδ or R361C mutant (right panel). Activation of the 3xPPRE-tk-Luc reporter in the absence or presence of rosiglitazone (1 μM) for PPARγ1, WY14643 (100 μM) for PPARα or GW501516 (2.2 nM) for PPARδ, are presented as described in Figure 1A.

(R397C), PPARa (R388C) and PPARb/d (R361C) and the proteins were subsequently tested for their ability to activate a 3xPPRE reporter construct in U2OS cells. Wild type PPARγ1 activated the 3xPPRE-tk-Luc reporter approximately 70-fold upon addition of rosiglitazone, while the R397C mutant only activated this reporter 15-fold (Figure 2B, left panel). As reported earlier (39), wild type PPARα displayed significant activity in the absence of ligand, which was further increased by addition of the synthetic PPARα-specific ligand WY14643. Mutation of PPARα R388 into cysteine almost completely inhibited transcriptional activity, both in the absence and presence of ligand (Figure 2B, middle panel). Finally, assays performed with PPARb/δ showed that this receptor also activated the reporter upon addition of a specific PPARβ/δ ligand (GW501516), albeit significantly less than the PPARγ isoforms or PPARα. Nevertheless, the weak transcriptional activity of PPARb/δ was clearly inhibited by mutating R361 into cysteine (Figure 2B, right panel).
Collectively, these results suggest that the FPLD3-associated R425C mutation results in reduced transcriptional activity of PPARγ2, independent of ligand type, ligand concentration and promoter used. Furthermore, the reduced transcriptional activity of R425C is not limited to the PPARγ2 protein, since similar effects were observed when analogous mutations were generated in the PPARγ1 isoform as well as in the related PPARα and PPARb/δ proteins.

Corepressor binding and -release of PPARγ2 R425C is impaired

Mutations in the thyroid hormone receptor b (TRb) are found in patients with resistance to thyroid hormone (RTH)(40). One RTH-associated mutation changes an arginine at position 383 into a histidine (R383H), resulting in impaired release of the corepressor SMRT (41). Since R383 in TRb is analogous to R425 in PPARγ2 (Figure 3A), the effect of the R425C mutation in PPARγ2 on binding and release of SMRT was investigated. For this, bacterially expressed and purified GST or GST-SMRT fusion proteins were incubated with in vitro translated [^{35}S]methionine-labelled PPARγ2 proteins (wild type or R425C) in the absence or presence of rosiglitazone. As shown in Figure 3B, wild type PPARγ2 interacted with SMRT in the absence of ligand and this interaction was reduced upon addition of rosiglitazone (Figure 3B). The PPARγ2 R425C mutant interacted with SMRT less efficiently, both in the absence and presence of ligand and did not show an obvious impairment of SMRT release in response to ligand (Figure 3B).
To substantiate these in vitro results in living cells we performed mammalian two-hybrid assays. For this, an expression vector consisting of the interaction domain 1 (ID1) of SMRT fused to the DNA-binding domain of Gal4 (Gal4DBD) were used, together with an expression vector encoding a fusion protein of PPARγLBD with the activation domain of VP16 (VP16). In the absence of ligand, wild type PPARγLBD clearly interacted with the SMRT-ID1, as demonstrated by the activation of the 5xGal4-TK-Luc reporter (Figure 3C). Upon addition of rosiglitazone this interaction decreased in a dose dependent manner. In contrast, PPARγLBD R425C showed limited interaction with SMRT-ID1, either in the absence or presence of ligand. In contrast, the FPLD3-associated P495L mutant of PPARγ2, which was previously shown to exhibit impaired corepressor release (37), showed a comparable level of transcriptional activity at all

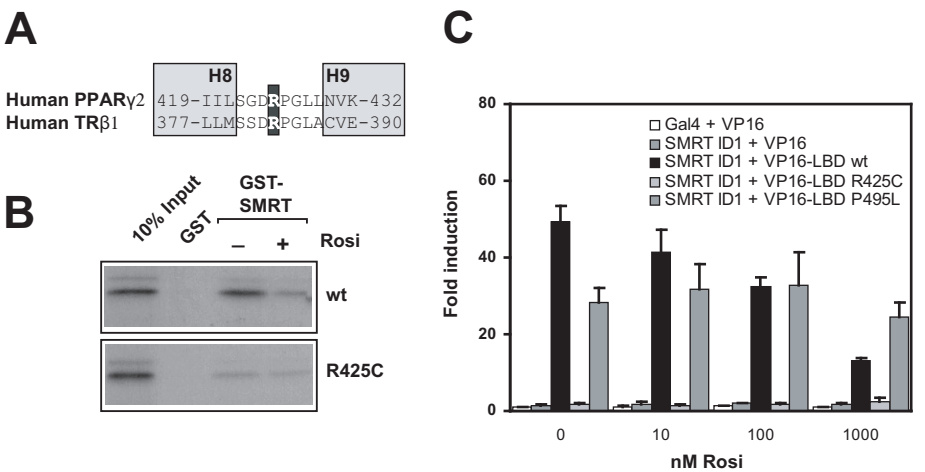

Figure 3. The PPARγ2 R425C mutation affects corepressor binding but not release. A, Alignment of the amino acid sequence surrounding R425 in human PPARγ2 and human TRb1 (AAI06930). B, GST fusion proteins coupled to Sepharose beads were incubated with in vitro translated [35S]-labelled PPARγ2 proteins (wt or mutant) in the absence or presence of rosiglitazone (1 μM). After extensive washing, samples were boiled and separated on SDS-polyacrylamide gels. Gels were fixed and dried and the labelled proteins were detected by fluorography. The input lanes represent 10% of the total lysate used in each reaction. C, U2OS cells were transfected with Gal4DBD or Gal4DBD-SMRT ID1 (50 ng), VP16 or VP16-PPARγLBD (wt or mutant; 50 ng), 5xGal4-TK-Luc reporter (500 ng) and incubated with different concentrations of rosiglitazone as indicated. Activation of the luciferase reporter, in the absence or presence of rosiglitazone, is expressed as fold induction over that with Gal4DBD+VP16 in the absence of ligand, after normalisation for Renilla luciferase activity. Results are averages of at least three independent experiments assayed in duplicate ± standard error of the means.

concentrations of rosiglitazone. The negative controls, in which VP16 protein was co-expressed with either Gal4DBD or Gal4DBD-SMRT-ID1, displayed negligible luciferase activity.

Thus, our data indicate that the R425C mutation in PPARγ2 does not impair corepressor release, but rather reduces the affinity for the corepressor. We conclude therefore that in contrast to the natural R383H mutant of TRβ, the reduction in PPARγ-mediated transcription caused by the R425C mutation is unlikely to be caused by impaired corepressor release.

The PPARγ2 R425C mutation results in reduced heterodimerisation with RXRα

The crystal structure of PPARγ-LBD reveals 13 a-helices and a small four-stranded b-sheet (16;17). The arginine at position 425 lies within a highly conserved sequence that is located in a loop between helix 8 and 9, and some of the residues surrounding arginine 425 (G423, D424 and P426) are involved in heterodimerisation with RXRα. This prompted us to investigate whether the PPARγ R425C mutation affected dimerisation with RXRα. First, we studied the effect of this mutation and mutations of the other highly conserved loop residues on in vitro heterodimerisation by performing GST-pull down assays. For this, in vitro translated [^{35}S]methionine-labelled PPARγ2 proteins (wild type or mutants) were incubated with bacterially expressed

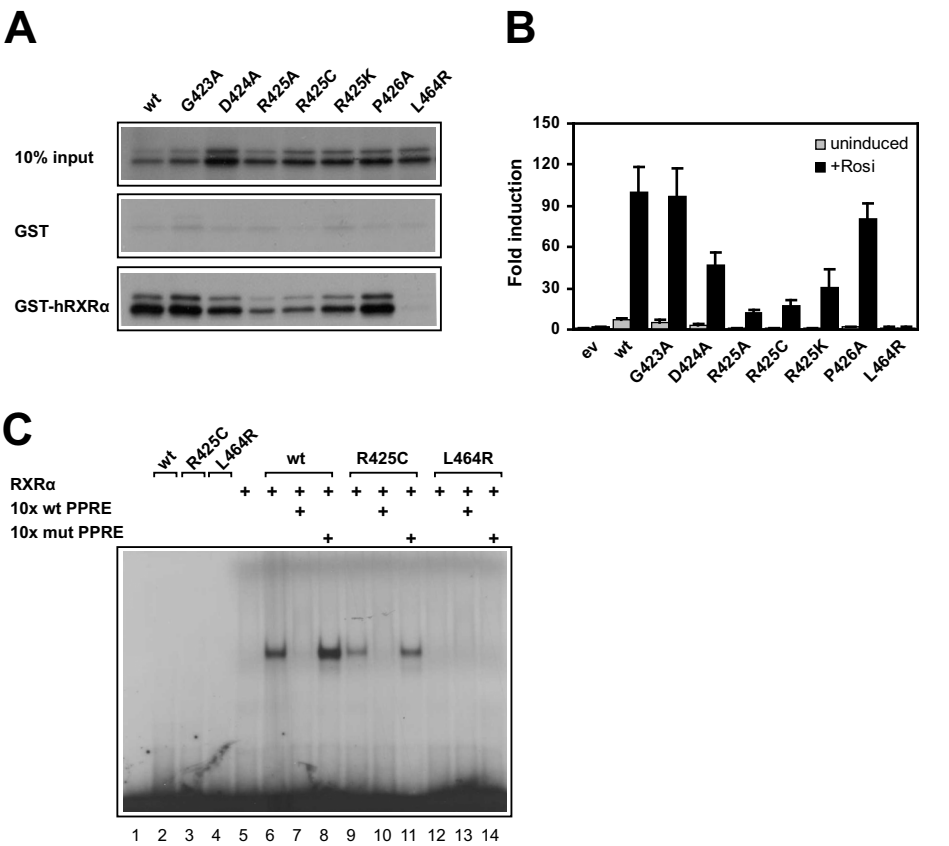

Figure 4. Heterodimerisation of PPARγ2 R425C with RXRα and subsequent DNA binding is impaired. A, Bacterially expressed and purified GST or GST-RXRα proteins were incubated with in vitro translated [35S]methionine-labelled PPARγ2 proteins (wt or mutant). Bound proteins were visualised as described in Figure 3B. B, U2OS cells were transfected with various PPARγ2 mutants. Experiments were performed and data are presented as described in Figure 1A. Expression of the different PPARγ2 proteins was confirmed by western blot analysis using an antibody directed against PPARγ. C, In vitro translated RXRα or PPARγ2 (wt or mutant) proteins were incubated with [32P]-labelled probe in absence or presence of 10X unlabelled probe (wt or mutant) as indicated. Protein-DNA complexes were separated from unbound DNA on non-denaturing SDS-polyacrylamide gels and visualized by autoradiography of dried gels.

and purified GST-RXRα. While wild type PPARγ2 efficiently interacted with RXRα, the R425C mutant protein displayed reduced binding (Figure 4A). Mutation of arginine 425 into alanine (R425A) or lysine (R425K) also decreased the PPARγ2-RXRα interaction. PPARγ2 D424A showed a slightly reduced binding compared to wild type PPARγ2, whereas mutation of glycine 423 or proline 426 into alanine (G423A and P426A, respectively) did not have an appreciable effect on in vitro dimerisation with RXRα. As a control, a PPARγ2 mutant was generated (L464R), analogous to the homodimerisation defective mouse ER mutant (L511R) (42). This L464R mutant completely failed to dimerise with RXRα in these in vitro assays (Figure 4A).
Next, the effects of the mutants described above on the transcriptional activity of PPARγ2 were studied. Like the R425C mutant (Figure 1), the R425A and R425K mu-

tants displayed strongly reduced ligand-dependent activation of a 3xPPRE-tk-Luc reporter in U2OS cells (Figure 4B). The D424A mutant showed slightly reduced transcriptional activity, while the activity of the G423A and P426A mutants was similar to wild type PPARγ2. Transcriptional activity of the heterodimerisation defective mutant L464R was completely abolished. The expression of all PPARγ proteins was confirmed by Western blot analysis (Figure 4B, lower panel). In general, the observed differences in transcriptional levels between the PPARγ2 mutants reflected the differential effects on heterodimerisation of these mutants (Figure 4A), confirming that heterodimerisation is a prerequisite for the transcriptional activity of the PPARγ2 protein. In addition, mutation of the positively charged arginine at position 425 into a cysteine (R425C), into the uncharged residue alanine (R425A) or into a lysine residue (R425K), which is also positively charged, all affected the in vitro RXRα heterodimerisation capacity as well as the transcriptional activity of PPARγ2. These findings point to the specific requirement of the arginine residue at position 425 for optimal function of PPARγ2 as a transcription factor.

Since PPARγ can only bind to PPRE sequences in the DNA upon heterodimerisation with RXRα, we investigated the effect of the R425C mutant on DNA binding. Electrophoretic mobility shift assays were performed in which in vitro translated RXRα and PPARγ2 proteins (either wild type or mutant) were incubated with a [^{32}P]-labelled PPRE sequence. A specific PPARγ2-RXRα heterodimeric complex was formed on the PPRE, as formation of this protein-DNA complex could be diminished by addition of an excess of unlabelled wild type PPRE, but not by an excess of mutant PPRE (Figure 4C). The R425C mutant protein also displayed specific DNA binding in the presence of RXRα, albeit 2.3 fold less than wild type, as determined by densitometric analysis. A similar difference in binding capacity was observed with increasing amounts of PPARγ2 protein (wild type or R425C) (see Supplementary material, Figure S1). Specific antibodies against PPARγ and RXRα supershifted both the wild type and mutant protein-DNA complexes, confirming the heterodimeric composition of both complexes (see Supplementary material, Figure S2). The dimerisation-defective L464R mutant failed to bind the PPRE in the presence of RXRα (Figure 4C). Taken together, these results indicate that heterodimerisation with RXRα and subsequent DNA binding of PPARγ2 is significantly, but not completely impaired by the R425C mutation.

Ligand binding and subsequent coactivator binding of PPARγ2 R425C is reduced

Since heterodimerisation with RXRα and subsequent DNA binding of PPARγ2 R425C is not completely impaired, this suggests that additional molecular defects contribute to the impaired function of this FPLD3-associated mutant. To investigate this, we performed reporter assays in which PPARγ driven transcription is independent of heterodimerisation with RXRα. For this, the mutations described above were introduced into a chimeric Gal4DBD-PPARγ LBD receptor and expressed together with a Gal4 reporter gene in U2OS cells. Both the wild type Gal4-PPARγLBD receptor and the dimerisation defective L464R mutant stimulated transcription from the reporter up to 350-fold in the presence of rosiglitazone, showing that transcriptional activation is in-

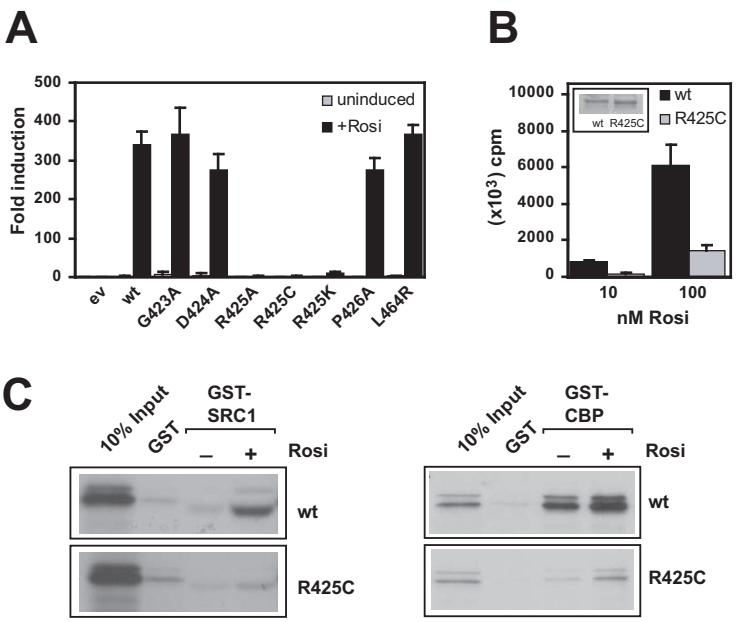

Figure 5. Ligand binding and subsequent coactivator binding of PPARγ2 R425C is reduced. A, U2OS cells were transfected with 5xGal4-AdMLTATA-Luc reporter (1 µg), empty vector or Gal4DBD-PPARγLBD expression vectors (wt or mutant; 200 ng) and incubated with or without rosiglitazone (1 µM) as indicated. Data are presented as described in figure 1A. Comparable amounts of the different GAL4 DBD fusion proteins were detected by western blot analysis using a GAL4 antibody. B, GST-PPARγ2 (wt or mutant) was incubated with different concentrations of [3H]-rosiglitazone. Bound ligand was measured by a g-counter. Data are indicated as mean of three separate experiments performed in duplicate. Inset, bacterially expressed and purified PPARγ2 proteins (wt or mutant) were subjected to SDS-PAGE and visualized by Coomassie brilliant blue staining. C, In vitro translated [35S]-labelled PPARγ2 proteins (wt or mutant) were incubated with bacterially expressed and purified GST or GST-SRC1 in the absence or presence of increasing concentrations of rosiglitazone (0.1, 1 or 2 µM). Bound proteins were visualised by fluorography of dried gels. D, In vitro translated [35S]-labelled PPARγ2 proteins (wt or mutant) were incubated with bacterially expressed and purified GST or GST-CBP in the absence or presence of rosiglitazone (1 µM). Bound proteins were visualised by fluorography of dried gels.

deed independent of heterodimerisation with RXRα in this assay (Figure 5A). In contrast the activity of the R425C mutant, as well as the R425A and R425K mutants, was strongly reduced. The G423A, D4242A and P426A mutants displayed levels of transcription similar to wild type PPARγ. As a control, Western blot analysis was performed and showed comparable amounts of GAL4DBD fusion proteins (Figure 5A, lower panel). These results indicate that the transcriptional defect in the PPARγ2 R425C protein is not only due to reduced heterodimerisation with RXRα.

In order to identify additional defects in the R425C protein, we first performed ligand binding assays. For this, bacterially expressed and purified GST-PPARγ2 fusion proteins (wild type or R425C) were incubated with either 10 nM or 100 nM of [^{3}H]-labelled rosiglitazone. Whereas wild type PPARγ2 protein displayed specific binding of rosiglitazone, the mutant protein showed reduced binding capacity for the ligand at both concentrations tested (Figure 5B). The amounts of the bacterially produced receptors were similar (Figure 5B; inset), indicating that the lower binding capacity for rosiglitazone displayed by the mutant protein was not caused by differences in pro-

tein levels. Since a coactivator binding surface is generated in the LBD of PPARγ upon ligand binding, we next examined whether the reduced affinity for rosiglitazone displayed by the R425C protein also affected interaction with coactivators. For this, bacterially expressed and purified GST-fusion proteins of the coactivators CBP and SRC1 were incubated together with in vitro translated [^{35}S]methionine-labelled PPARγ2 wild type or PPARγ2 R425C proteins in the absence or presence of rosiglitazone. As observed before (43-45), wild type PPARγ2 bound to SRC1 in the presence of rosiglitazone in a dose-dependent manner (Figure 5C), while the interaction between PPARγ2 and CBP was less dependent on the presence of ligand (Figure 5D). Although PPARγ2 R425C also displayed specific binding to SRC1 and CBP under these conditions, these protein-protein interactions were significantly weaker compared to wild type PPARγ2.
In summary, these experiments indicate that in addition to impaired heterodimerisation (Figure 4), the R425C mutation also abrogates ligand binding compared to the wild type protein and as a consequence diminishes ligand-dependent interaction with transcriptional coactivators.

Transrepression of NF-κB by PPARγ R425C remains largely intact and is independent of heterodimerisation with RXRα

In order to examine the effect of the R425C mutation on PPARγ-mediated repression of NF-κB activity, we performed reporter assays in which U2OS cells were transfected with expression vectors encoding wild type PPARγ2 or mutant PPARγ (R425C or L464R) or an empty vector together with a 2kB-luciferase reporter. Upon addition of TNFα, this reporter was activated up to 10-fold and cotransfection of increasing amounts of either wild type PPARγ2 or R425C both reduced this activation in a dose dependent manner. Addition of rosiglitazone enhanced this inhibitory effect to some extent (Figure 6A). Since the R425C mutant displayed reduced dimerisation with RXRα (Figure 4), these results suggest that monomeric forms of the receptor may be sufficient for transrepression of NF-κB activity, as was also shown for other nuclear receptors (46). To investigate this, we employed the PPARγ2 L464R mutant, which displays a complete loss of dimerisation activity (Figure 4). A dose dependent decrease in reporter activation by TNFa was observed upon addition of increasing amounts of L464R mutant, corroborating the hypothesis that PPARγ mediated transrepression of NF-kB is independent of heterodimerisation with RXRα.
In addition, since transcriptional interference between PPARγ and NF-κB occurs through the RelA (p65) subunit of NF-κB (47), we assessed this protein-protein interaction by incubating bacterially expressed GST-RelA fusion proteins with in vitro translated PPARγ2 proteins (either wild type, R425C or L464R). As shown in figure 6B, all three PPARγ2 proteins were able to bind to GST-RelA and this interaction was largely rosiglitazone independent.
In summary, although the FPLD3-associated R425C mutation results in reduced transcriptional activity of PPARγ (Figure 1), PPARγ-mediated transrepression of NF-κB activity remains largely intact, especially in the presence of ligand. Furthermore, our data suggest that the PPARγ-mediated transrepression is largely independent of heterodimerisation with RXR.

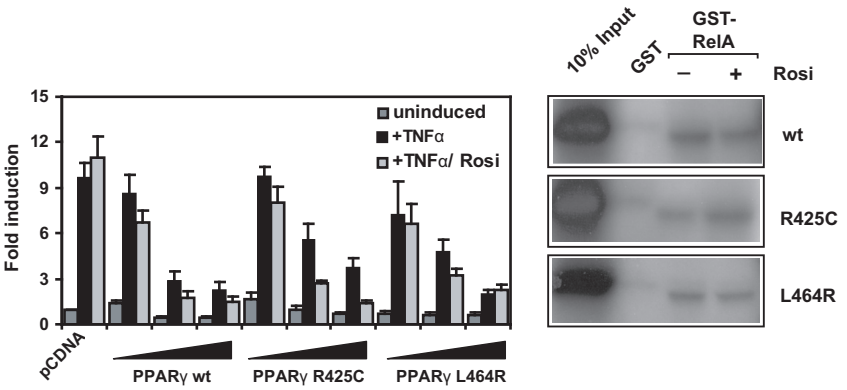

Figure 6. The FPLD3-associated PPARγ R425C mutation is able to transrepress NF-kB activity. A, U2OS were transfected with empty vector (10 ng), wild type PPARγ2, PPARγ2 R425C and PPARγ2 L464R respectively (10, 50 or 100 ng) and 2κB-luciferase reporter (300 ng). Activation of the luciferase reporter in the absence or presence TNFα (250 units) or TNFα in combination with rosiglitazone (1 μM), respectively, is expressed as fold induction over that of empty vector in the absence of TNFα and rosiglitazone, after normalisation for Renilla luciferase activity. Results are averages of three independent experiments performed in duplicate ± standard error of the means. B, In vitro translated [35S]-labelled PPARγ2 proteins (wt, R425C or L464R) were incubated with bacterially expressed and purified GST or GST-RelA in the absence or presence of rosiglitazone (1 μM). Bound proteins were visualised by fluorography of dried gels.

Molecular dynamics simulation predicts an alternative salt bridge in PPARγ2 R425C

Since the arginine residue on position 425 in PPARγ2 is involved in the formation of an internal salt bridge (16), the effects of substituting this amino acid by cysteine was examined using several 5 nanosecond (ns) molecular dynamics simulations of wild type protein and R425A and R425C mutants starting from various crystal structures of the LBD domain of PPARγ (see Material and Methods). Positional root mean square deviation (RMSD) from the starting structures as well as the various energy terms reached equilibrium in a few ns for all the simulations (see Supplementary material Figure S3 and S4). When analysing the trajectories resulting from the different simulations, there was a clear and consistent difference between wild type PPARγ2 and PPARγ2 R425C with respect to salt bridges that are present. In the wild type protein, R425 (located in the loop between helix 8 and 9) forms a salt bridge with E352 (in helix 4/5), and R471 (in helix 10) forms a salt bridge with D424 (Figure 7A, left panel). In the R425C mutant, however, the salt bridge between R425 and E352 obviously cannot be formed, and this resulted, unexpectedly, in a switch from the R471-D424 salt bridge to a salt bridge between R471 and E352 (Figure 7A, right panel, and Supplementary material Figure S5). A similar change was observed when R425 was mutated into alanine (data not shown). We also compared various structural parameters (RMSDs, angles between helices, solvent accessibility of surface residues). Although in some simulations helix 10 seemed to be deformed in the R425C mutant, we could not find consistent differences between wild type and mutant simulations. The difference in salt bridge pattern was consistently found between on the one hand the three wild type simulations and on the other hand four mutant

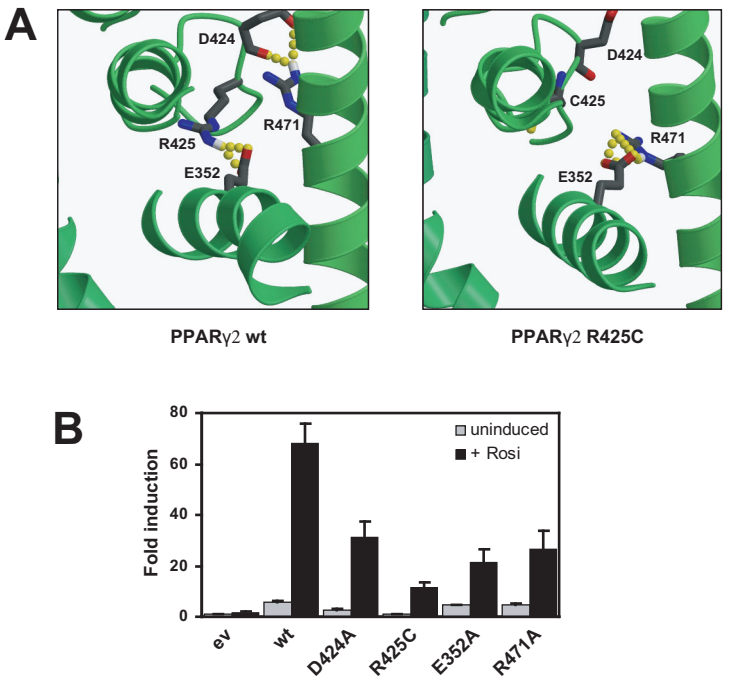

Figure 7. An internal salt bridge in PPARγ2 is disturbed by the substitution of arginine 425 with cysteine. A, Snapshots from Molecular Dynamics simulations for wild type (left panel) and R425C (right panel) mutant PPARγ. A close-up view of the salt bridges discussed in the text is provided, with residues involved represented in ball-and-stick. B, U2OS cells were transfected with vectors expressing various mutants of PPARγ2. Experiments were performed and data are presented as described in Figure 1A. Expression of the different PPARγ2 proteins was confirmed by western blot analysis using an antibody directed against PPARγ.

simulations (both R425A and R425C). The fact that the same rearrangement is observed in independent simulations starting from various crystal structures, make the observed structural difference between wild type and mutant protein highly significant.

To test the importance of those salt bridges for transcriptional activity, we mutated the individual residues involved in the salt bridge formation to alanine residues and performed reporter assays in U2OS cells. Like R425C, the salt bridge mutants D424A, E352A and R471A all displayed reduced transcriptional activity compared to wild type PPARγ2 (Figure 7B). Western blot analysis was performed as a control and showed comparable expression levels for all PPARγ proteins (Figure 7B, lower panel). These findings suggest that the internal salt bridges between R425 and E352 and between D424 and R471 play an important role in PPARγ2-mediated transcription by maintaining the structural integrity of the ligand binding domain of this receptor.

PPARγ2 R425C and PPARγ1 R397C do not display dominant negative behaviour

All of the FPLD3-associated PPARγ mutations are heterozygous and some of the resulting PPARγ mutant proteins have been shown to inhibit their wild type counterpart

in a dominant negative manner (29;37). We therefore examined whether the R425C mutant of PPARγ2 and the same mutant in the context of the PPARγ1 protein (R397C) would inhibit the activity of the respective wild type receptors. For this, reporter assays were performed, using a 1:1 ratio between wild type and mutant receptors and different concentrations of ligand. As shown in figure 8A, PPARγ1 R397C failed to display dominant negative behaviour independent of ligand concentration while the FPLD3-associated PPARγ1 P467L inhibited wild type PPARγ1 at low concentrations of ligand, as reported earlier (37). In addition, the artificial PPARγ1 L468A/E471A mutant displayed a strong dominant negative activity against its wild type protein independent of ligand concentration, as shown earlier (48).
When tested in the context of the PPARγ2 protein, the R-to-C mutant also lacked dominant negative activity, whereas PPARγ2 L496A/E499A was able to inhibit wild type PPARγ2 at all concentrations of rosiglitazone (Figure 8B). Interestingly, whereas PPARγ1 P467L displayed a dominant negative effect over its wild type counterpart (Figure 8A), the same mutant in the context of PPARγ2 (P495L) was not able to inhibit wild type receptor in a dominant negative manner (Figure 8B). In addition, the PPARγ1 P467L mutant was dominant negative over wild type PPARγ2, but PPARγ2 P495L did not display dominant negative activity over wild type PPARγ1 (data not shown). Similar results were obtained in 293T cells, indicating that the observed effects were not restricted to U2OS cells (data not shown).
In summary, these data show that unlike the artificial PPARγ double mutant, the FPLD3-associated PPARγ R-to-C mutant does not exert dominant negative activity over the wild type receptor, whereas dominant negative activity of the FPLD3-associated PPARγ P-to-L mutant appears to be limited to the PPARγ1 isoform under our experimental conditions.

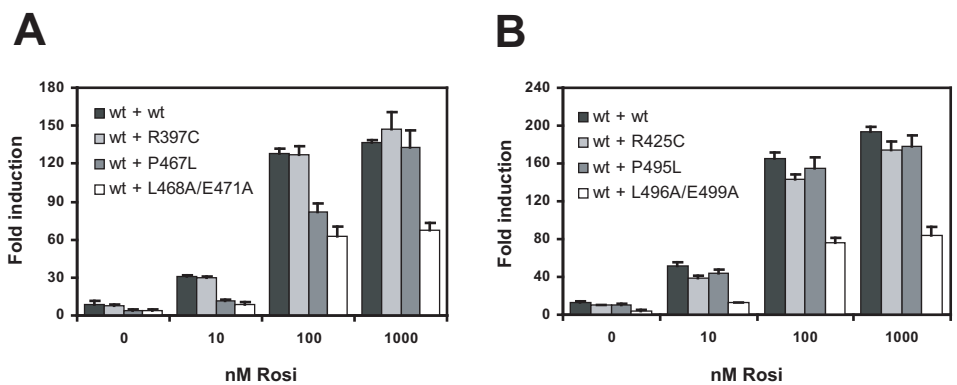

Figure 8. FPLD3-associated R-to-C mutants fail to display dominant negative activity towards wild type PPARγ isoforms. A, U2OS cells were transfected with equal amounts of wild type and mutant PPARγ1 (both 100 ng), 3xPPRE-tk-Luc reporter (500 ng) and incubated with different concentrations of rosiglitazone, as indicated. B, U2OS were transfected as in panel A, but with PPARγ2 expression vectors. Results are averages of at least three independent experiments assayed in duplicate ± standard error of the means.

The R425C mutation reduces the ability of PPARγ2 to induce adipocyte differentiation

Having established by in vitro and cell-based assays that PPARγ function is affected on multiple levels we investigated the effect of this mutation in vivo on adipocyte differentiation. NIH-3T3 fibroblasts were transduced with virus obtained from cells transfected with the parental retroviral pMSCV vector, pMSCV-PPARγ2 or pMSCV-PPARγ2 R425C and stable cell lines were selected. Approximately 20% of the NIH-PPARγ2 cells differentiated into adipocytes, as assessed by staining of triglycerides with Oil-red-O (Figure 9A). Virtually no adipocyte differentiation could be detected in NIH-vector or NIH-PPARγ2 R425C fibroblasts under our experimental conditions. To independently confirm adipocyte differentiation, the mRNA expression of three PPARγ target genes, fatty acid binding protein 4 (Fabp4), adipose aquaporin (Aqp7) and glycerol kinase (Gyk), was determined on the same cell lines by quantitative RT-PCR assays. While a 34-fold increase in Fabp4 mRNA expression was observed for NIH-PPARγ2 cells, mRNA expression of this gene in NIH-PPARγ2 R425C cells was similar to NIH-vector cells (Figure 9B). The PPARγ2 R425C protein also failed to induce mRNA expression of Aqp7 and Gyk, both of which were significantly

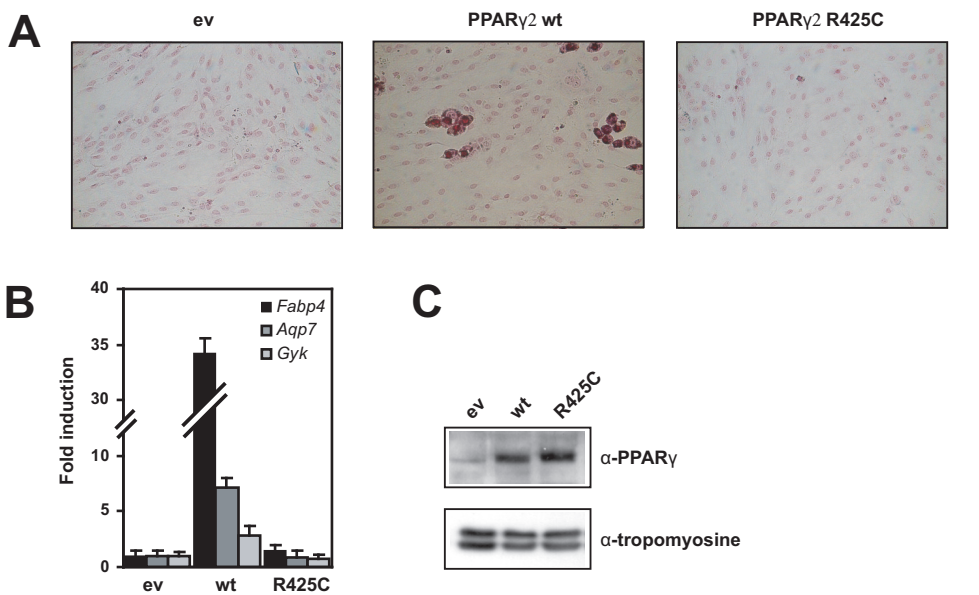

Figure 9. Reduced potential of the PPARγ2 R425C to induce adipocyte differentiation. A, NIH-3T3 fibroblasts were transduced with retrovirus obtained form cells transfected with pMSCV (empty vector; ev), pMSCV-PPARγ2 or pMSCV-PPARγ2 R425C. After a week of incubation with differentiation medium, triglycerides were stained with Oil-Red-O and cell nuclei with crystal violet. Pictures are representative for three independent experiments. B, mRNA expression of the Fabp4, Aqp7 and Gyk genes in NIH-3T3 cell lines described in panel A after differentiation. Expression levels are indicated as fold induction over that with NIH-empty vector cells (ev), after normalisation for the housekeeping gene Hprt1. Results are averages of at least three independent experiments assayed in duplicate ± standard deviation. C, Western blot analysis of lysates of the NIH-3T3 cell lines described in panel A with antibodies against PPARγ and tropomyosine (internal control).

upregulated by the wild type receptor (Figure 9B). As shown by Western blot analysis, the PPARγ protein expression levels for wild type and R425C proteins were similar (Figure 9C).
These findings show that the R425C mutation not only compromises the ability of the PPARγ2 protein to activate transiently transfected reporter genes (Figure 1), but also reduces its potential to activate endogenous target genes (Fabp4, Aqp7, Gyk) and promote adipogenesis (Figure 9).

Discussion

The nuclear receptor PPARγ plays a key role in the regulation of glucose and lipid metabolism in adipocytes by regulating their differentiation, maintenance and function (9;10). Compelling genetic evidence for this view comes from human FPLD3 patients, harbouring heterozygous mutations in the PPARG gene, as they are characterized by aberrant fat distribution and metabolic disturbances, including insulin resistance and dyslipidemia (6;7). In this report, we demonstrate that the FPLD3-associated PPARγ2 R425C mutant displays multiple molecular defects. These defects probably do not involve impaired corepressor release, but comprise a combination of reduced heterodimerisation with RXRα, lower ligand binding affinity and subsequent reduced binding of coactivators, resulting in reduced transcriptional activity and eventually in an impaired ability of the R425C mutant to induce adipocyte differentiation. Interestingly, like the dimerisation defective L464R mutant, the R425C mutant could still transrepress TNFα-induced NF-κB activity. These results are reminiscent of the transrepression of NF-κB activity by monomeric forms of other nuclear receptors, like the glucocorticoid receptor (46), and indicate that activation and transrepression critically depend on different regions of the receptor (49). Recently, a structure based sequence alignment revealed two sets of differentially conserved residues that divided the entire nuclear receptor superfamily in two classes related to their oligomeric behaviour (35). Nuclear receptors that form homodimers belong to class I, while nuclear receptors that form heterodimers with RXRα, like PPARγ, belong to class II. A differentially conserved arginine (R425 in PPARγ2) defines the signature of these class II receptors, and is involved in the formation of a salt bridge with a negatively charged residue in H4/H5 (E352 in PPARγ2) (16;17). Moreover, the crystal structure of PPARγ LBD revealed an additional internal salt bridge between two different conserved “class II” residues, D424 (L8/9) and R471 (H10). Our molecular dynamics simulations predicted that substitution of PPARγ2 R425 with a cysteine residue would disrupt both internal salt bridges leading to the formation of an alternative salt bridge between R471 and E352. The conserved “salt bridge residues” are structurally important in defining the conformation of the LBD of PPARγ2: both D424 and R471 are involved in dimerisation with RXRα (16), while R425 and E352 form hydrogen bonds with Y505 at the C-terminus of helix 12 leading to the stabilisation of H12, which is important for cofactor binding (39). In addition, destabilisation of H12 could in turn affect heterodimerisation, since an interdimer salt bridge is formed between Y505 (H12) of PPARγ2 and K431 of RXRα (16). Finally, crystallographic studies on PPARα together with a corepressor peptide indicate that the two hydrophobic residues immediately preceding E352 in PPARγ2 are probably part of the corepressor interaction surface (50). Therefore, conformational changes resulting from the simultaneous disruption of both internal salt bridges through the R425C mutation provide a likely explanation for the impaired interactions with cofactors and RXRα, as well as the reduction in ligand binding, despite the fact that R425 is not in the direct vicinity of the ligand binding pocket. The partial restoration of PPARγ2 R425C activity by the tyrosine-based agonist GW1929 might be explained by the benzophenone group in this type of ligand, which is not present in TZDs. This group can make additional hydrophobic interactions with residues in H3, H7 and H10 (16), which could contribute

to the active conformation of the LBD structure and thereby partially restore the activity of PPARγ2 R425C, as was observed specifically with this ligand.
Studies on four other mammalian class II nuclear receptors, vitamin D receptor (VDR), TRβ, RXRα and retinoic acid receptor α (RARα), have pointed to the importance of the conserved salt bridge residues for their function. In keeping with our results on the E352A mutation in PPARγ2, the analogous mutant in VDR (E269A) displayed reduced ligand binding and transcriptional activity (51). Unlike the PPARγ2 R425C mutant, however, mutation of the analogous residue in TRβ, as exemplified by the RTH-associated R383H mutant, did not affect activation of target genes, but resulted in impaired corepressor release (41). Furthermore, the R339A mutation reduced the transcriptional activity of RARα, but heterodimerisation was unaffected (35), which is in contrast to the analogous PPARγ2 mutant (R425A). Finally, the D384A mutant of RXRα (D424A in PPARγ2) displayed reduced ability to homodimerise, but heterodimerisation with RARα was unaffected (52). These findings therefore clearly underscore the important role of the internal salt bridges in maintaining the structural integrity of the LBDs of class II receptors, but also show that the molecular defect(s) caused by their disruption can differ between individual nuclear receptors.
Although the PPARγ2 R425C mutant displays some similarities with other FPLD3-associated PPARγ mutants, the molecular defects are clearly not identical. Based on functional characterisations in combination with studies on dominant negative activity of these different PPARγ mutants, PPARG mutations can be divided in at least three different subgroups (29-34;37). The first group comprises the missense DNA binding mutants and ligand binding domain (LBD) truncation mutants (C142R, C159Y, C190W, FS343X, R385X). These mutants have no DNA binding capacity, but are still able to recruit coactivators and can therefore compete for common cofactors (“squelching”), which can explain their dominant negative activity. The second group consists of missense mutations located in the LBD (V318M, P495L), which inhibit PPARγ activity through an alternative mechanism. These mutants are transcriptionally inactive due to impaired ligand and coactivator recruitment, but are able to compete for DNA binding with wild type PPARγ. As a consequence the activation of PPARγ target genes is reduced, which could explain the dominant negative activity of these mutants. The third group, represented by the PPARγ R425C mutant, displays both impaired heterodimerisation with RXRα and subsequent DNA binding, as well as impaired ligand and cofactor binding, the combination of which could account for the absence of dominant negative activity. In line with this, addition of an artificial mutation in TRb, that attenuated heterodimerisation with RXRα and subsequent DNA binding, abrogated the dominant negative activity of three different natural LBD mutants (53;54). This suggests that the combination of either intact DNA binding together with impaired cofactor binding, or intact cofactor binding in combination with impaired DNA binding is a prerequisite for dominant negative activity of TRβ and PPARγ mutants. Like the R425C mutant analysed here, the FPLD3-associated PPARγ2 F388L mutant (30) and the recently published nonsense mutation, which truncates PPARγ2 after residue 355 (Y355X) (33) also lack dominant negative behaviour. Together with the FPLD3-associated PPARγ4 promoter (-14A>G) mutation (55), which only reduces the expression of the PPARγ protein, these findings indicate that in some FPLD3 patients PPARG mutations act through a haploinsufficiency mechanism rather than dom-

inant negative activity of the affected gene product.
When the FPLD3-associated PPARγ P-to-L mutant was tested in the context of the PPARγ1 protein (P467L), this protein inhibited wild type PPARγ1 and -γ2 in a dominant negative fashion at low concentrations of rosiglitazone, as described earlier (37). Interestingly, the same amino acid substitution in the context of the PPARγ2 protein (P495L) resulted in a protein that lacked this capacity, indicating that at least under our experimental conditions the dominant negative activity of the PPARγ P-to-L mutant is isoform specific. These findings suggest that the effect of dominant negative PPARγ mutants might be more pronounced in tissues with a high PPARγ1/-γ2 ratio, but it is currently unknown whether this ratio differs between body fat depots. An important challenge for the future will be to establish how heterozygous PPARG mutations can lead to depot-specific effects on adipose tissue, as exemplified by the characteristic aberrant fat distribution in patients suffering from FPLD3 and mice expressing PPARγ mutants (56;57).

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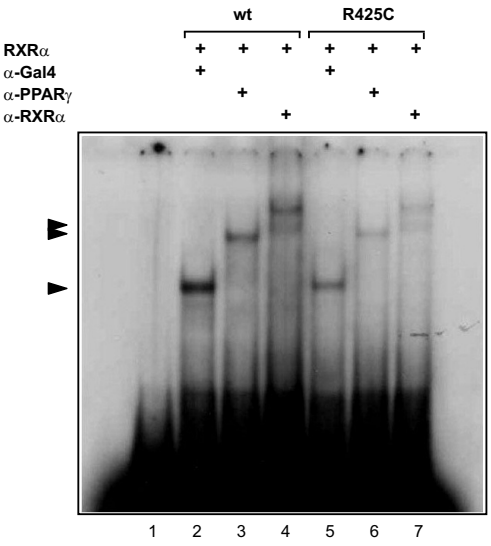
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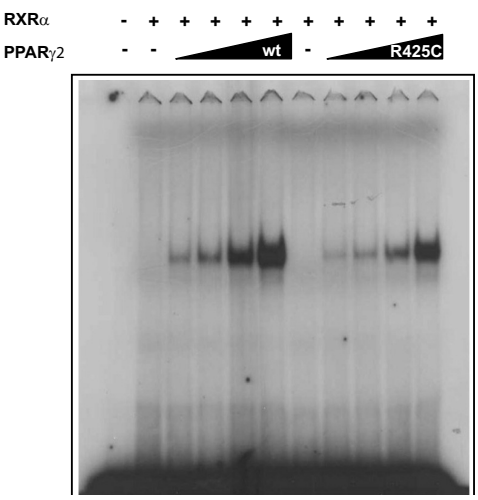
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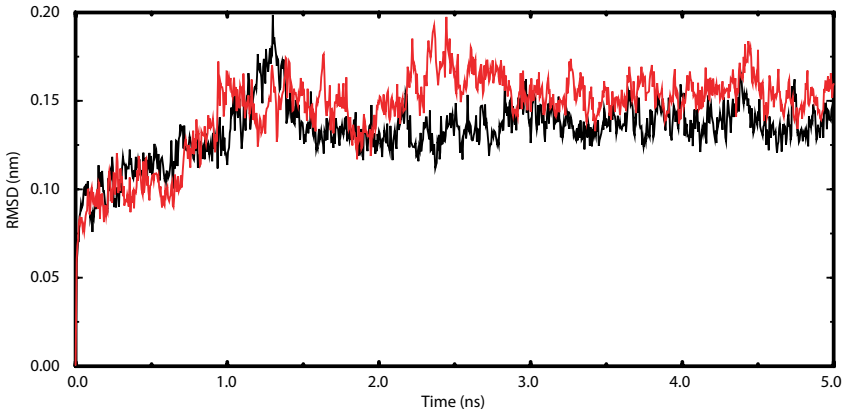
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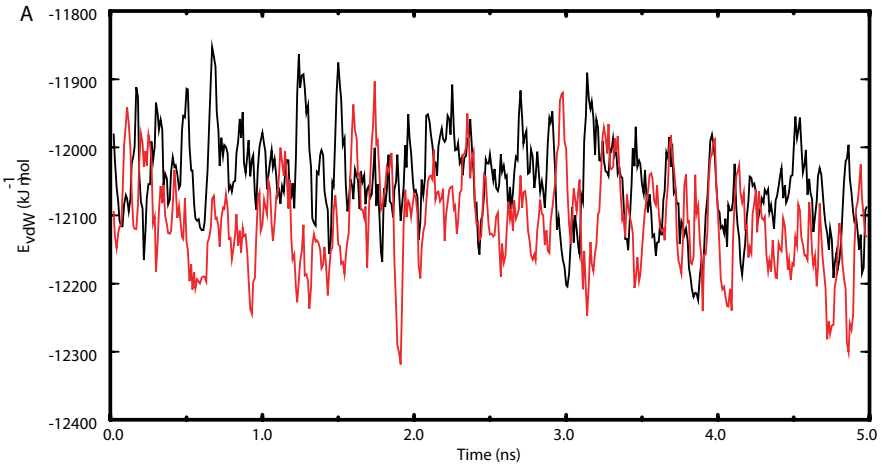
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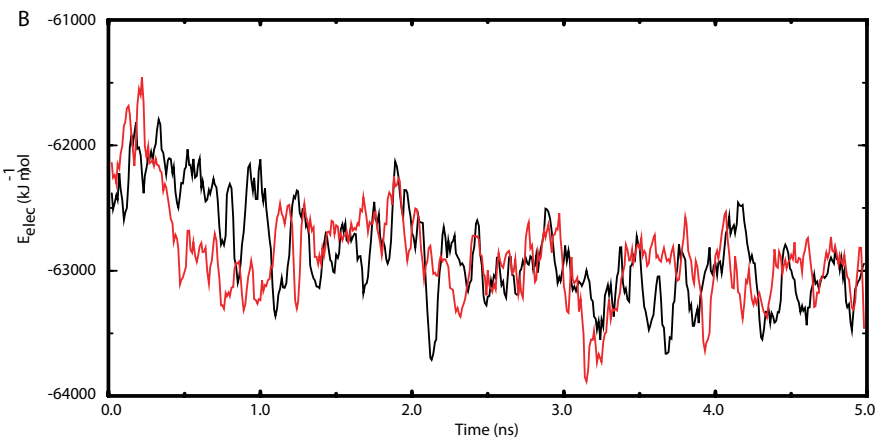
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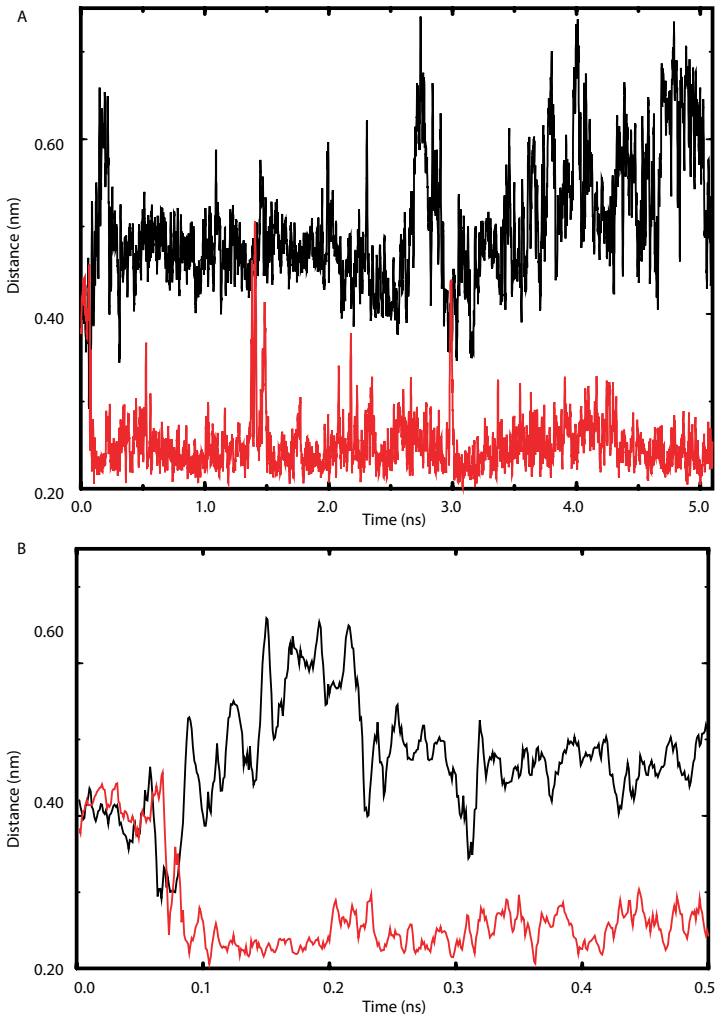
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Chapter 4

The adipogenic acetyltransferase Tip60 targets activation function 1 of PPARγ

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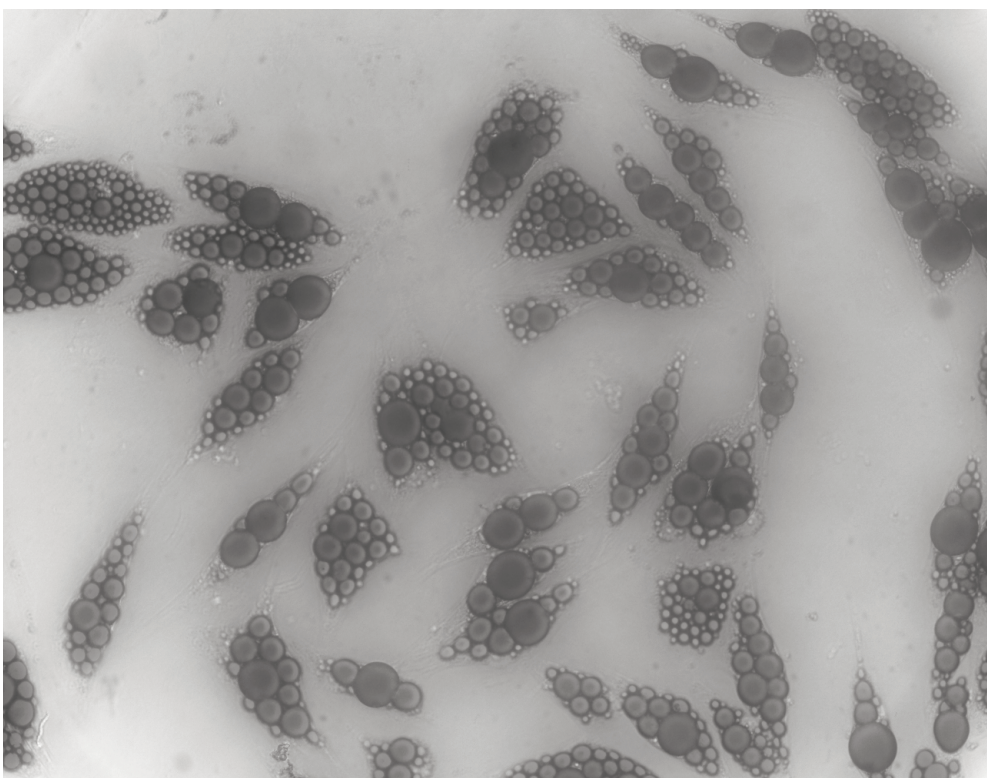

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ABSTRACT

The transcription factor Peroxisome Proliferator-Activated Receptor γ (PPARγ) plays a key role in the regulation of lipid and glucose metabolism in adipocytes, by regulating their differentiation, maintenance and function. The transcriptional activity of PPARγ is dictated by the set of proteins with which this nuclear receptor interacts under specific conditions. Here we identify the HIV-1 Tat interacting protein 60 (Tip60) as a novel positive regulator of PPARγ transcriptional activity. Using tandem mass spectrometry we found that PPARγ and the acetyltransferase Tip60 interact in cells, and through use of chimeric proteins we established that coactivation by Tip60 critically depends on the N-terminal activation function 1 of PPARγ, a domain involved in isotype-specific gene expression and adipogenesis. Chromatin immunoprecipitation experiments showed that the endogenous Tip60 protein is recruited to PPARγ target genes in mature 3T3-L1 adipocytes, but not in pre-adipocytes, indicating that Tip60 requires PPARγ for its recruitment to PPARγ target genes. Importantly, we show that in common with disruption of PPARγ function, siRNA-mediated reduction of Tip60 protein impairs differentiation of 3T3-L1 pre-adipocytes. Taken together, these findings qualify the acetyltransferase Tip60 as a novel adipogenic factor.

Introduction

Peroxisome proliferator activator receptors (PPARs) are members of the nuclear receptor (NR) family of ligand-dependent transcription factors that play essential roles in lipid homeostasis and energy metabolism (1-4). The three mammalian isotypes, named PPARα, -β/δ and -γ, all bind to PPAR responsive elements (PPRE) in the promoter regions of target genes as obligate heterodimers with retinoic acid X receptors (RXRs). However, each receptor is associated with distinct biological effects, which may partly be explained by their tissue specific expression. An important site of PPARγ action is adipose tissue, where it is a key player in the differentiation, maintenance and function of these cells (5-7). Its relevance for the differentiation of fibroblast-like mesenchymal stem cells into adipocytes, a process known as adipogenesis, is clearly exemplified by PPARγ $^{+/-}$ mice, which lack adipose tissue (8-10). In addition, in vitro differentiation of fibroblasts into mature adipocytes can be induced by introduction of PPARγ, showing that PPARγ is not only necessary but also sufficient for adipogenesis (11). Compelling genetic evidence for this view comes from the Familial partial lipodystrophy subtype 3 (FPLD3, MIM 604367), an autosomal dominantly inherited disorder, characterized by gradual loss of subcutaneous fat from the extremities and an accumulation of excess fat in the intra-abdominal regions, which is caused by heterozygous mutations in the PPARG gene (4;12).

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). Furthermore, two isoforms of PPARγ exist, named PPARγ1 and PPARγ2, which differ by the presence of an additional N-terminal 30 amino acids in the PPARγ2 isoform. The crystal structure of the PPARγ-LBD reveals 13 a-helices and a small four-stranded b-sheet (13;14). Upon ligand binding, the AF-2 helix (also referred to as helix 12) is stabilized in an active state, resulting in a charge clamp pocket consisting of a conserved lysine residue in helix 3 and a glutamate in the AF-2 helix (K329 and E499 in PPARγ2, respectively)(13;14). This pocket serves as a binding site for coactivator complexes, including the SRC1/CBP and TRAP/DRIP/ARC complexes, which interact with the LBD through the LXXLL motifs (in which L is leucine and X is any amino acid) present in these coactivators (15;16). Interestingly, thiazolidinediones (TZDs), drugs that ameliorate insulin resistance in humans, are able to initiate such a sequence of events since they are ligands for PPARγ (17).

While numerous studies have focussed on the ligand-dependent AF2 function of PPARs, recent studies indicate that the AF1 region also plays an important regulatory role in PPAR signalling. Using chimeric PPARγ-PPARβ/δ proteins, the AF1 region of PPARγ was shown to be essential for adipogenesis (18;19). Furthermore, gene expression profiling revealed that the AF1 regions of the different PPAR family member are the main determinants of isotype-selective gene expression (19). It seems plausible that, in analogy with the AF2 domain, the activity of the AF1 region is dictated by the set of proteins with which this domain interacts. Despite its emerging importance, only a limited number of proteins have been described so far which interact with and regulate the function of the N-terminal region of PPARγ. These AF1 inter-

acting proteins include PPARγ coactivator 2//SCAN domain protein 1 (PGC-2/SDP1) (18;20) and the transcriptional coactivator with PDZ-binding motif TAZ (21). PGC-2 interacts specifically with and increases the activity of PPARγ, and not PPARα or PPARβ/δ (18), although studies on the human homologue SDP1 showed binding to PPARα and –β/δ as well (20). Interestingly, overexpression of PGC-2 in 3T3-L1 preadipocytes resulted in increased adipogenesis, thereby qualifying this protein as an adipogenic cofactor (18). Since PGC-2 lacks intrinsic transactivation potential (18), the molecular mechanism behind coactivation of PPARγ by PGC-2 remains to be established. TAZ, which is a positive regulator of the osteogenic transcription factor Runx2, inhibits the activity of PPARγ, but also in this case the mechanism is unknown (21). The acetyltransferases CREB binding protein (CBP) and p300 can also interact with the AF1 region of PPARγ, but the relative importance of this interaction is unclear since CBP and p300 also bind to the LBD in a ligand-dependent fashion (22). Interestingly, the AF1 region is also subject to phosphorylation at serine residue 112 (23) and SUMOylation at lysine residue 107 (24-26), and it seems likely that such post-translational modifications regulate interactions with coregulators, and/or vice versa, ultimately controlling the output of the AF1 region.

To identify novel regulators of PPARγ activity we performed a mass spectrometry analysis of PPARγ-associated proteins and by this means found the HIV-1 Tat interacting protein 60 (Tip60). Tip60 is a member of the MYST family of acetyltransferases, named after its founding members MOZ, Ybf2/Sas3, Sas2 and Tip60, which share a highly conserved MYST acetyltransferase domain, but display limited homology outside this region (27;28). Tip60 is part of a large multi-protein complex (29-31), implicated in many cellular processes like DNA damage repair, cell cycle control and apoptosis (32-34). We show here that Tip60 targets the AF1 region of PPARγ, a region of the protein implicated in isotype-selective gene expression and adipogenesis (19). Furthermore, siRNA-mediated knock down of Tip60 expression resulted in inhibition of the differentiation of 3T3-L1 cells into adipocytes. These findings qualify the MYST acetyltransferase Tip60 as a novel adipogenic factor.

MATERIALS AND METHODS

Materials

Rosiglitazone was purchased from Alexis (San Diego, CA). FugeneÒ6 transfection reagent and protease inhibitor tablets (11697498001) were purchased from Roche Applied Biosciences (Indianapolis, IN). PEI (#23966) was purchased from Polysciences Inc. (Warrington, PA). The following antibodies were used: anti-PPARγ (sc-7196), anti-Tip60 (sc-5725) and anti-Fabp4 (sc-18661) Santa Cruz Biotechnologies (Santa Cruz, CA); anti-FLAG(M2)-HRP (A8592), anti-HA (H9658), anti-goat-HRP (A9452) and anti-Tropomyosin (T2780), Sigma-Aldrich (St. Louis, MO); anti-rabbit-HRP (111035144) and anti-mouse-HRP (115035146), Jackson Immunoresearch Laboratories Inc. (West Grove, PA); anti-Brd8 (BL 1231), Bethyl Laboratories (Montgomery, TX). Anti-FLAG(M2) agarose beads (A02220) and Oil-Red-Ọ (O-0625) were purchased from Sigma-Aldrich, Crystal violet was from Chroma-Gesellschaft Schmid&Co (Köngen, Germany). Formaldehyde solution (4%) was purchased from Klinipath (Duiven, the Netherlands), RNAiMAX was purchased from Invitrogen (Carlsbad, CA). Purified Drososphila core histones were a kind gift from G. Chalkley (Erasmus University Medical Centre, Rotterdam, The Netherlands).

Plasmids and siRNA oligonucleotides

All recombinant DNA work was performed according to standard procedures (35). pCDNA-FLAG-MOZ (36), pCDNA-HA-HBO-1 (37) and pCDNA-HA-Tip60 (38) were kind gifts from Drs. D.M. Heery, (University of Nottingham, UK) Z. Sun (Stanford University School of Medicine, CA) and D. Trouche (UMR5099, Toulouse, France), respectively. pGEX4T1-PPARγ2 deletion constructs (39) were a kind gift from Dr. S. Kato (University of Tokyo, Japan). pSport mMOF was purchased from RZPD (clone IRAVp968c0560D). Expression vectors pcDNA-hPPARγ2/a and pcDNA-hPPARa/g were generated by site directed mutagenesis of pcDNA-PPARα and -PPARγ2 generating NheI restriction sites at codon 147 of PPARγ2 and codon 108 of PPARα, and subsequent exchange of the relevant fragments. To generate the Fabp4 promoter reporter construct, the HindIII sites surrounding the 7.9 kb mouse Fabp4 promoter were used to clone the Fabp4 promoter into pBSK and from there subcloned into pGL3-Basic (Promega) using SmaI and KpnI sites. The TK-Luc reporter was generated by insertion of a BamHI fragment from 5xGal-TK-Luc (40) into a BamHI-BglII digested pGL3 basic plasmid (Promega). All mutations were generated by Quickchange mutagenesis (Stratagene) and verified by sequencing. All other plasmids have been described before (41). Control (D-001210-01-20) and mTip60 (M-057795-00) smartpool siRNA oligonucleotides or individual siRNA duplexes (MQ-057795-00) were purchased from Dharmacon.

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), penicillin and streptomycin (both 100 mg /ml; Gibco Life Technologies). The murine 3T3-L1 cell line was cultured in the same media but now with 10% bovine serum (Gibco Life Technologies), penicillin and streptomycin (both 100 mg /ml; Gibco Life Technologies). Reporter assays were performed in 24-well plates with 1 µg 3xPPRE-tk-Luc reporter construct, 2 ng PPAR expression construct, 100 ng Tip60 expression construct (or empty vector) and 2 ng pCMV-Renilla (Promega) as described before (41).

Tandem Mass Spectrometry and Western blotting

For immunoprecipitation experiments, U2OS cells or 293T cells were grown in 15 cm dishes and transiently transfected with PPARγ2 or Tip60 expression vectors (10 µg) using either FugeneÒ6 or PEI transfection reagent. At day 2, cells were put on 2µM rosiglitazone and harvested at day 3 in 500 µl RIPA buffer (without SDS) per dish. Immunoprecipitation of FLAG-PPARγ2 and HA-Tip60 were performed at 4˚C for at least 4 hours, using anti-FLAG(M2) agarose beads or HA antibodies precoupled to agarose beads, respectively. For tandem mass spectrometry, overexpressed FLAG-PPARγ2 and interacting proteins were isolated from 20 dishes (15 cm). Proteolytic digestion of proteins and LC-tandem MS were performed as described (42). For Western blotting, whole cell lysates (directly lysed in 2x SDS-PAGE sample buffer) or immunoprecipitated proteins were subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore). ECL (Amersham Biosciences) was used for detection, after incubation with primary and secondary antibodies.

GST-pull down assays and histone acetylation assays

Recombinant Tip60 cDNA in the pCDNA3 expression vector was transcribed and translated in vitro in reticulocyte lysates in the presence of [^{35}S]-methionine according to manufacturer's protocol (TNT T7 Quick Coupled Transcription/Translation Kit, Promega). Rosetta pLysS competent bacteria (Novagen) were transformed with GST expression plasmids. GST fusion proteins were purified as described earlier (43). [^{35}S]–labelled proteins were incubated with GST fusion proteins in NETN-buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0,5% NP40) containing protease inhibitors (Complete, Roche Applied Biosciences). Samples were subsequently washed and subjected to SDS-PAGE. Signals were enhanced with Amplify (Amersham Biosciences), gels were fixed and dried and the [^{35}S]-labelled proteins were visualized by fluorography.
Histone acetylation assays were performed exactly as described (40), using immunopurified HA-tagged Tip60 from transiently transfected 293T cells and purified Drosophila core histones.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation assays were performed as decribed (44). In short, 3T3-L1 preadipocytes and differentiated 3T3-L1 adipocytes were crosslinked with 1% formaldehyde in PBS and quenched with glycin (final concentration 0.125M). Cells were washed two times with ice cold PBS, collected in PBS and resuspended in lysis buffer (0.1% SDS, 1% Triton X-100, 0.15M NaCl, 1mM EDTA and 20mM TrisHCl pH 8.0). Chromatin was fragmented using the Bioruptor (Diagenode) and cellular debris was removed by centrifugation. Chromatin was diluted in lysis buffer and incubated with antibody against PPARγ or Tip60 and incubated overnight with Protein A or Protein G agarose slurry (Amersham Biosciences) in presence of BSA (1 µg/µl). Immunoprecipitates were washed twice with buffer 1 (0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 0.15M NaCl, 1mM EDTA and 20mM TrisHCl pH 8.0), one time with buffer 2 (0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 0.5M NaCl, 1mM EDTA and 20mM TrisHCl pH 8.0) and buffer 3 (0.25M LiCl, 0.5% NaDOC, 0.5% NP-40, 1mM EDTA and 20mM TrisHCl pH 8.0) and twice in TE. Immuprecipitated chromatin was eluted with 400 µl 1%SDS and 0.1M NaHCO3 for 30 minutes followed by addition of NaCl to a final concentration of 0.2M and then decrosslinked overnight at 65°C. DNA was purified with a phenol/chloroform extraction and precipitated at -20°C with sodiumacetate pH 5.2 and ethanol in the presence of glycogen. The precipitated DNA was dissolved in water and analyzed by qPCR with primers against mouse Fabp4 PPRE (5'-GAGAGCAAATGGAGTTCCCAGA; 5'-TTGGGCTGTGACACTTCCAC) and an intergenic region on mouse chromosome 15 as control (5'-TGGTAGCCTCAGGAGCTTGC; 5'-ATCCAAGATGGGACCAAGCTG).

Differentiation assays, quantitative RT PCR and siRNA transfections

Differentiation assays on mouse 3T3-L1 cells, Oil-Red-O and crystal violet staining were performed as described earlier (41). Three independent samples of total RNA were isolated at different time points and subjected to quantitative PCR analysis (41). The expression of the reference gene Hprt1 was used to calculate the relative expression levels according to Vandesompele et al. (45). The sequences of the primers are as follows: murine Hprt1 sense primer; 5'-TCCTCCTCAGACCGCTTTT-3'; antisense primer, 5'-CCTGGTTCATCATCGCTAATC-3'; murine Fabp4 sense primer 5'-GAAAACGAGATGGTGACAAGC-3'; anti-sense primer 5'-TTGTGGAAGTCACGCCTTT-3', murine Tip60 (isoform2) sense primer 5'-AGCCTCGGTTTTCCCTCA-3'; anti-sense primer 5'-TCTGAGCTGTCCTGAGAATCC-3'; murine PPARg (isoform 2) sense primer 5'-CGCTGATGCACTGCCTATGA-3'; anti-sense primer 5'-AGAGGTCCACAGAGCTGATTCC-3'.

For siRNA experiments, cells were grown to 80% confluency and transfected with siRNA oligonucleotides using RNAiMAX according to the manufacturer's protocol. siRNA transfections were repeated every 3 days during the differentiation assay. At least 3 independent siRNA transfections followed by differentiation assays were performed. In parallel, cells were lysed in 2x SDS-PAGE sample buffer and subjected to Western blot analysis.

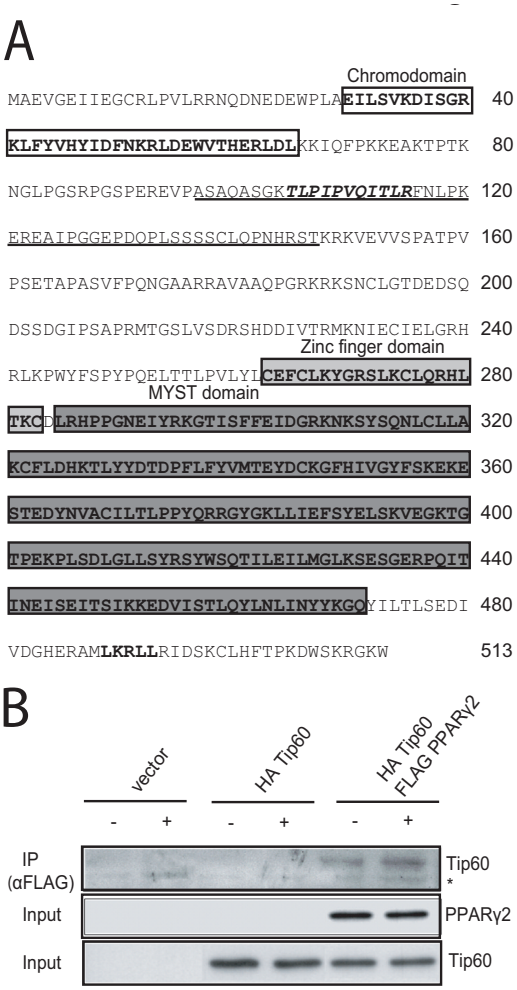


Figure 1. The MYST acetyltransferase Tip60 is a novel PPARγ-interacting protein. (A) Amino acid sequence of Tip60 (isoform 2). Indicated are the chromodomain, zinc finger domain (light grey) and MYST-acetyltransferase domain (dark gray). The amino acid region unique to isoform 2 is underlined with the peptide identified by mass spectrometry in italics; the nuclear receptor box (LXXLL motif; amino acid 488-492) is in bold. (B) FLAG-PPARγ2 and HA-Tip60 were overexpressed in U2OS cells in the presence (+) or absence (-) of 2 μM rosiglitazone. After lysis in RIPA buffer, PPARγ2 was immunoprecipitated using FLAG beads. Immunoreactive proteins were detected on Western blots by anti-FLAG and anti-HA antibodies. An aspecific band is indicated (*).

RESULTS

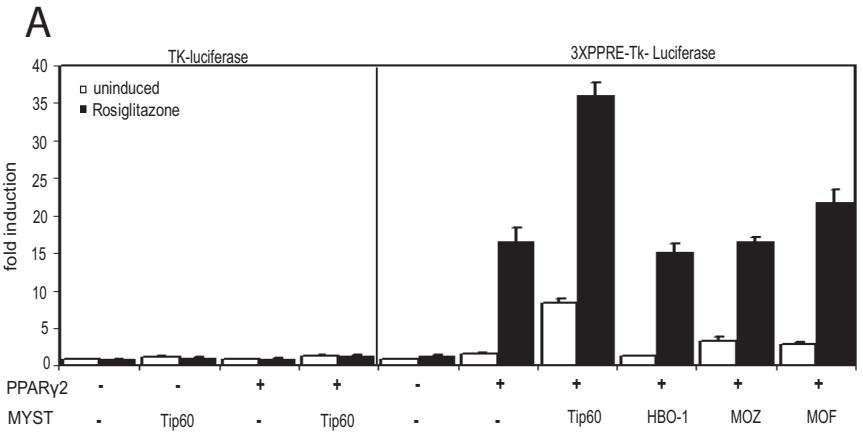
Identification of Tip60 as a novel coactivator for PPARγ2

In order to identify novel PPARγ2 interacting proteins, ectopically expressed FLAG-tagged PPARγ2 was immunopurified from HEK 293T cells and the associated proteins determined by mass spectrometry analysis. One of the peptides we identified corresponded to amino acid TLPIPVQITLR (position 104-114) of the Tip60 protein, a member of the MYST family of histone acetyltransferases (Fig. 1A). At least three Tip60 isoforms exist in man and mouse, as a result of alternative splicing (33). Isoform 1 results from the translation of intron 1, while the shorter isoforms 2 and 3 (also referred to as Tip60α and –β, or Tip60a and –b, respectively) are identical apart from the absence of the internal exon 5 in isoform 3. The peptide detected here is encoded by exon 5, indicating that Tip60 isoform 1 and/or 2 were immunopurified. Because mRNA and protein expression of isoform 2 is much higher than isoform 1 in several cell types, including NIH-3T3 mouse fibroblasts (46), all subsequent experiments were performed with Tip60 isoform 2.

To confirm the interaction between PPARγ2 and Tip60 in living cells, HA-tagged Tip60 was over-expressed in U2OS cells in the presence of FLAG-tagged PPARγ2 with or without the synthetic ligand rosiglitazone. As is shown in Figure 1B, Tip60 co-immunoprecipitated with FLAG tagged PPARγ2, both in absence and presence of rosiglitazone. As controls, immunoprecipitations were performed on cell lysates in which neither of the proteins or only Tip60 was present. No co-immunoprecipitations were observed in these cases (Fig. 1B, lanes 1-4).

To investigate whether Tip60 functions as a transcriptional coactivator for PPARγ2, the two proteins were over-expressed in U2OS cells together with a reporter construct containing three copies of the PPRE found in the rat acyl CoA oxidase promoter (47) in the absence or presence of the synthetic ligand rosiglitazone (1 μM) and luciferase activity was subsequently measured. As shown in Figure 2A, unliganded PPARγ2 activated this 3xPPRE-tk-Luc reporter only marginally but Tip60 further potentiated this activity 4-6 fold. The activity of liganded PPARγ2 was increased approximately 2-3 fold by Tip60 (see also Discussion). Since the interaction between cellular proteins and Tip60 often maps to the 52 amino acids encoded by exon 5 (28), we also performed these assays with isoform 3 of Tip60 and obtained similar results (data not shown). PPARγ2, Tip60 or the combination of these proteins failed to activate a reporter which lacks functional PPREs (TK-luc), indicating that coactivation by Tip60 critically depends on DNA-bound PPARγ (Fig. 2A).

Tip60 is a member of the MYST family of acetyltransferases, which share a highly conserved MYST acetyltransferase domain, but display limited homology outside this region (Fig. 2B). To investigate whether other MYST acetyltransferases could also upregulate PPARγ activity, the HBO-1, MOF and MOZ proteins were tested under the same conditions. The HBO-1 protein, which has been reported to interact with several other nuclear receptors (37;48), as well as the MOF and MOZ proteins displayed negligible ability to potentiate PPARγ2 activity (Fig. 2A). As a control, Western blot analysis was performed to show that all 4 MYST proteins were efficiently expressed under our experimental conditions (Fig. 2C). Finally, the effect of Tip60 on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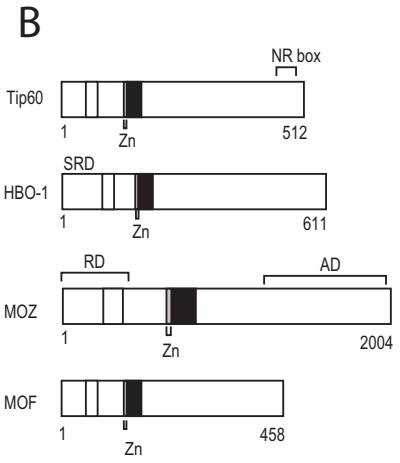
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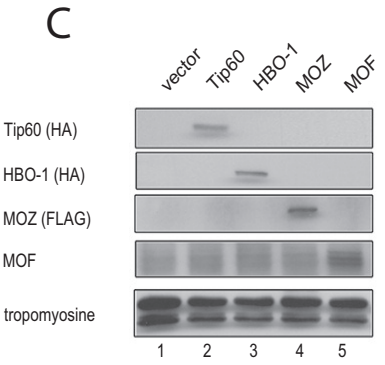
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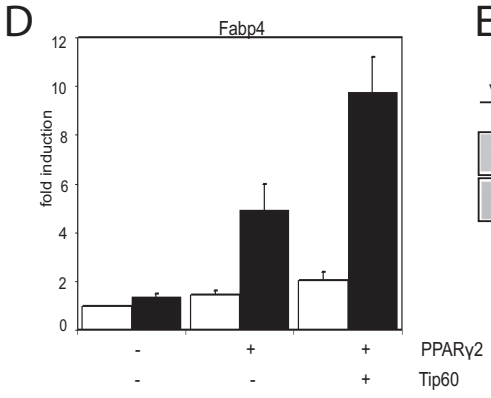
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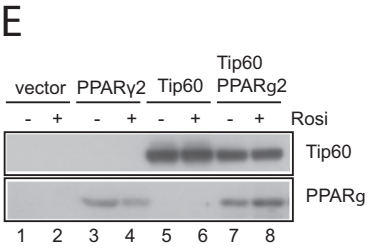
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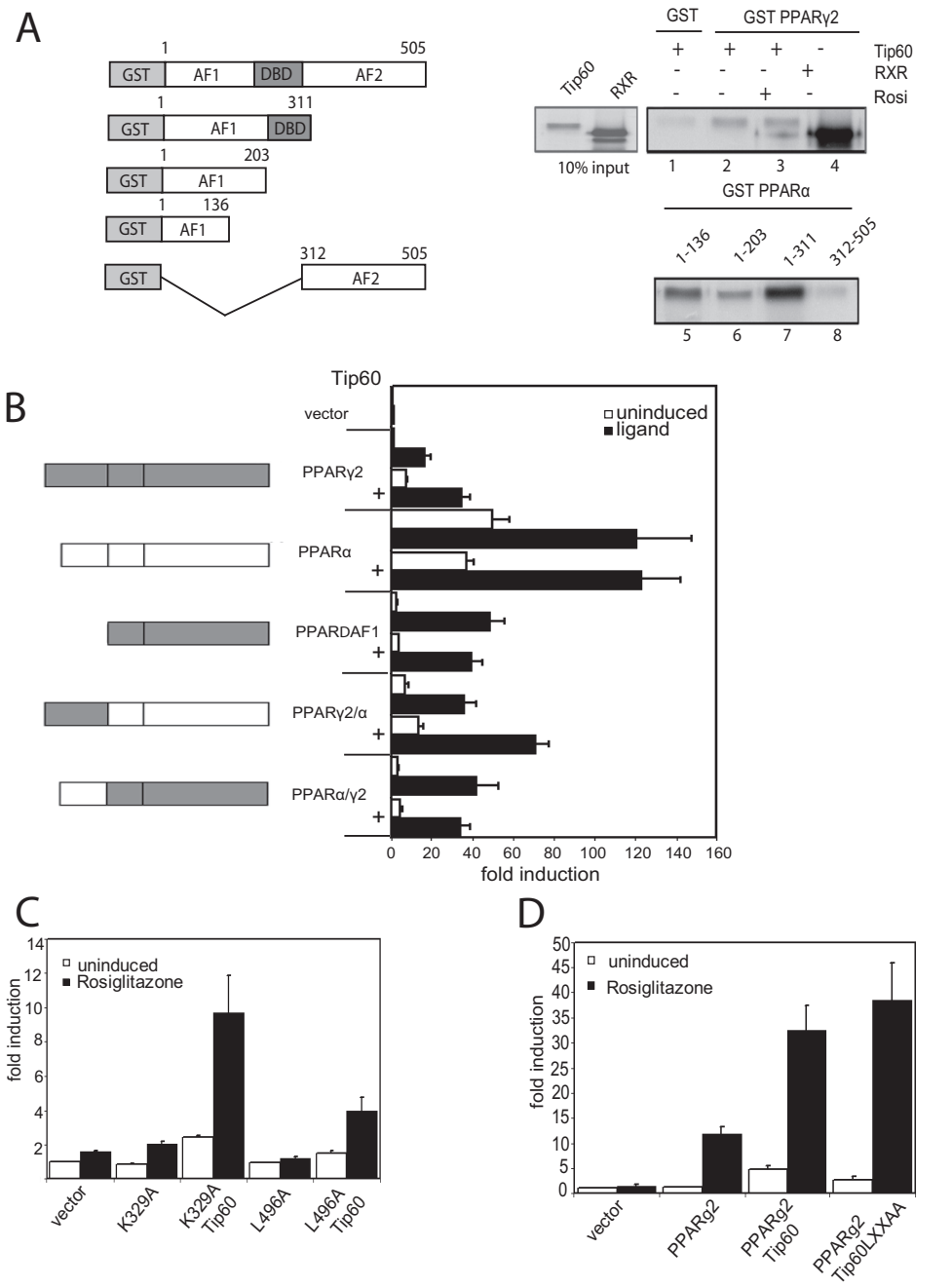
← **Figure 2**. PPARγ2 activity is specifically poteniated by the MYST acetyltransferase Tip60. (A) U2OS cells were transfected with an expression vector encoding PPARγ2 wild type (wt), expression vectors encoding MYST acetyltransferases HA-HBO-1, MOF, HA-Tip60 or FLAG-MOZ and a TK-Luc Reporter or a 3XPPRE-TK-Luc reporter. Activation of the luciferase reporter, in the absence or presence of 1 mM rosiglitazone, is expressed as fold induction over that with empty vector in the absence of ligand, after normalisation for Renilla luciferase activity. Results are averages of at least three independent experiments assayed in duplicate ± standard error of the means. (B) Schematic representation of the different MYST members. CD, chromodomain; Zn, zinc finger domain; HAT, histone acetyltransferase domain; NR, nuclear receptor box; SRD, serine rich domain; RD, repression domain; PHD, plant homeodomain type zinc finger; AD, activation domain. (C) Overexpression of different MYST proteins was assessed by Western blot analysis. (D) U2OS cells were transfected as in A, but with a mouse fatty acid binding protein 4 (Fabp4)-Luc reporter instead of the 3xPPRE-tk-Luc reporter. (E) Expression of PPARγ2 and HA-Tip60 proteins in U2OS cells, as assessed by Western blot analysis using anti-PPARγ2 or anti-HA antibodies.

PPARγ-mediated transcription was tested on a more "natural" promoter. Reporter assays using a luciferase reporter construct containing the promoter of the murine fatty acid binding protein 4 gene (Fabp4, also referred to as A-FABP or aP2), which contains several functional PPREs approximately 5.5. kb upstream of the transcription start site(49;50). As shown in Figure 2D, PPARγ-mediated activation of this reporter was also potentiated by Tip60. Western blot analysis revealed that Tip60 did not affect the expression of PPARγ protein, or vice versa (Fig. 2E), indicating that Tip60 stimulates PPARγ activity and not protein expression. Taken together, these findings establish Tip60 as a novel and specific coactivator for PPARγ.

Tip60 coactivates PPARγ via the AF1 domain

We next determined the specific domains required for the PPARγ-Tip60 interaction. For this, bacterially expressed and purified GST or a series of GST-PPARγ2 fusion proteins were incubated with in vitro translated [^{35}S]-methionine-labelled Tip60 protein. As a positive control in vitro translated RXR, the heterodimeric partner of PPARγ2, was used. Consistent with our co-immunoprecipitation experiments (Fig. 1B), Tip60 interacted with full length PPARγ2 independently of the presence of ligand (Fig. 3A). Moreover, the N-terminal 136 amino acids (Fig. 3A, lane 5) were indispensable for this interaction, whereas the C terminal region, containing the LBD, was not required (Fig. 3A, lane 8). Coomassie staining of the gels showed equal amounts of GST fusion proteins used (data not shown). These data indicate that the interaction between Tip60 and PPARγ2 is mediated through the AF1 domain and not the LBD of this NR. To corroborate these findings in living cells, an AF1 deletion mutant (ΔAF1) was tested in the luciferase reporter assay described above. As observed before, deletion of the AF1 region of PPARγ protein resulted in significantly higher activity in the presence of ligand compared to the wild type protein, probably as a result of loss of repressive functions encoded by the AF1 domain (11). In contrast to the wild type PPARγ2 protein, the activity of this ΔAF1 protein was only stimulated marginally by co-expression of Tip60, suggesting that coactivation of PPARγ by Tip60 critically depends on the AF1 region. To substantiate these findings, we made use of the PPARα protein, which displays extensive homology with PPARγ in its DBD and LBD, but not in its AF1 domain. This NR harbours significant ligand-independent activity that can still be increased by addition of the synthetic ligand WY14643 (41;51). As shown in Figure 3B, Tip60 failed to potentiate either the ligand –independent or ligand–de-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← **Figure 3.** Association and coactivation of PPARγ by Tip60 specifically requires the AF1 region. (A) GST-fusion proteins of PPARγ2 full-length or various deletion fragments, depicted schematically in the left panel, were overexpressed in E.coli. PPARγ2 proteins were isolated by gluthatione sepharose precipitation and incubated with [35S]-labelled Tip60. Samples were subjected to SDS-PAGE and labelled protein was visualized by fluorography. (B) U2OS cells were transfected with expression vectors encoding PPARγ2, PPARα, PPARγ2/α or PPARα/γ, either alone or in combination with Tip60 expression vector (indicated with +). Activation of the 3xPPRE-tk-Luc reporter in the absence or presence of rosiglitazone (1 mM) for PPARγ2, PPARγΔAF1 and PPARα/γ2, or WY14643 (100 mM) for PPARα and PPARγ/α are presented as described in Figure 2C. (C) U2OS cells were transfected with expression vector encoding the PPARγ2 mutants K329A or L496A, either alone or in combination with an expression vector encoding wild type Tip60. Activation of the 3xPPRE-tk-Luc reporter in the absence or presence of rosiglitazone (1 mM) is presented as described in Figure 2C. (D) U2OS cells were transfected with expression vector encoding PPARγ2 either alone or in combination with an expression vector encoding wild type Tip60 or an LXXAA mutant. Results are presented as described in Figure 2A.

pendent PPARα activity. Next, chimeric PPARγ2-PPARα constructs were generated, in which the AF1 domains of these receptors were exchanged. We found that the activity of a PPARα protein with the AF1 domain of PPARγ2 (PPARγ2/α) was stimulated by Tip60, whereas a PPARγ2 protein harbouring the AF1 domain of PPARα (PPARα/γ) was insensitive to Tip60 (Fig. 3B). Tip60 was however able to stimulate the activity of the PPARγ1 isoform (data not shown), which lacks the first 30 amino acids present in PPARγ2, indicating that Tip60 specifically requires the AF1 region between amino acid 31 and 136 of PPARγ2 for coactivation.

To verify whether the AF1 region of PPARγ2, and not the LBD, is indeed the predominant interaction interface, two additional approaches were taken. Firstly, two LBD mutants of PPARγ2 were used in which AF2 activity is affected, while ligand binding is intact (52). Mutation of the conserved lysine in helix 3 (K329A), which is part of the charge clamp, or one of the conserved leucines in helix 12 (L496A) resulted in markedly reduced transcriptional activity, either in the absence or presence of synthetic ligand (Fig. 3C). However, simultaneous over-expression of Tip60 clearly stimulated reporter activity of both mutant proteins; this effect was most pronounced in the presence of ligand (Fig. 3C). Secondly, we tested whether disruption of the nuclear receptor box present in the C terminal region of Tip60 (see Fig. 1A and 2A), which was shown to be required for the interaction between Tip60 and the LBD of the androgen receptor (53), affected the coactivation of PPARγ by Tip60. Mutation of the LXXLL motif in Tip60 (amino acid 488-492) to LXXAA had no appreciable effect on the ability of Tip60 to stimulate PPARγ2-mediated transcription (Fig. 3D), indicating that indeed interactions between the LBD and the LXXLL motif play a minor role in the interaction between PPARγ2 and Tip60. Collectively, these results strongly suggest that the interaction between PPARγ2 and Tip60 and coactivation of PPARγ2 by Tip60 critically depend on the AF1 region, and not the LBD, of this NR.

The acetyltransferase activity of Tip60 contributes to coactivation of PPARγ2

Since Tip60 contains no intrinsic transcriptional activity (54) but can acetylate histones and non-histone proteins (32;33), we determined if this function is necessary for its role as a PPARγ coactivator. As described before (55), Tip60 acetylates core histones with a preference for H3 and H4 (Fig. 4A). Mutation of a conserved glycine residue in the MYST domain of Tip60 into glutamic acid (G380E), analogous to the

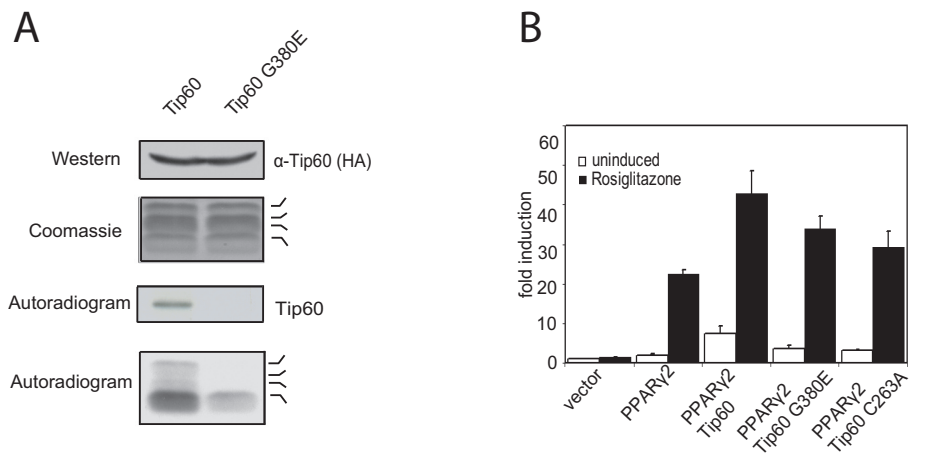

Figure 4. The acetyltransferase activity of Tip60 contributes to potentiation of PPARγ2 activity. (A) Immunoprecipitated HA-Tip60 or the G380E mutant were incubated with purified Drosophila histones together with [14C]-Acetyl CoA. Reaction samples were separated by SDS-PAGE and stained with Coomassie brilliant blue to verify the presence of equal amounts of histones in each lane. Gels were subsequently dried and labelled proteins were visualized by fluorography. An aliquot from the same immunoprecipitation was used for Western blot analysis with an anti-HA antibody. (B) U2OS cells were transfected with expression vector encoding PPARγ2 either alone or in combination with an expression vector encoding wild type Tip60, the G380E mutant or the C263A mutant. Activation of the 3xPPRE-tk-Luc reporter in the absence or presence of rosiglitazone (1 mM) is presented as described in Figure 2A.

catalytic mutation of the Drosophila MYST family member mof (56), abrogates most of the histone acetyltransferase activity as well as auto-acetylation activity (Fig. 4A). We tested this Tip60 mutant for its ability to activate a PPAR responsive reporter and found that Tip60 mediated activation of this mutant was decreased, indicating that the acetyltransferase activity of Tip60 plays a role in the coactivation of PPARγ2 (Fig. 4B). In addition, mutation of the zinc finger (C263A), which plays a role in the binding of some substrates (57;58), also resulted in reduced coactivation (Fig. 4B). These findings indicate that coactivation of PPARγ2 by Tip60 depends at least in part on the ability of this protein to acetylate histone and/or non-histone proteins.

Tip60 is essential for adipogenesis

The essential role of PPARγ2 in adipogenesis prompted us to investigate whether Tip60 would also play a role in this process. As a first experiment the protein expression of Tip60 was studied during differentiation of 3T3-L1 cells into adipocytes. As is shown in Figure 5A, the expression levels of Tip60 decreased from two days before the start of the differentiation assay up to day zero, possibly caused by growth inhibition as a result of contact inhibition. Between day 1 and 9 after initiation of differentiation a progressive increase in Tip60 levels was detected. A similar pattern of protein expression was detected for the Brd8 protein (Fig. 5A), which is a stable component of the Tip60 complex (29-31). Re-probing of the Western blots with an antibody against PPARγ showed that this protein was expressed at detectable levels around three days after addition of the differentiation medium. Interestingly, quanti-

tative RT-PCR experiments revealed unaltered expression of Tip60 mRNA during adipogenesis, indicating that the increase in Tip60 protein levels was not paralleled by increased mRNA expression (Fig. 5B). As a control, the mRNA expression of Pparg itself and Fabp4, an established PPARγ target gene, was determined, and both genes were clearly induced during adipocyte differentiation (Fig. 5B).

We next investigated whether endogenous Tip60 protein was recuited to the promoter region of PPARγ target genes in intact adipocytes. For this, chromatin immunoprecipitation (ChIP) experiments were performed in 3T3-L1 pre-adipocytes and adipocytes. As shown in Figure 5C, PPARγ and Tip60 could readily be detected in the region of functional PPREs in the Fabp4 promoter in mature 3T3-L1 adipocytes (day 6). Little PPARγ and Tip60 were detected on this promoter in undifferentiated cells (Fig. 5C; day 0), in which PPARγ expression is low (Fig. 5A). Similar results were obtained for a second PPARγ target gene, perilipin (data not shown). As a control, ChIP assays were performed on an intergenic region on chromosome 15, to which neither protein bound (Fig. 5C). Since endogenous Tip60 protein could be detected on the Fabp4 promoter in mature adipocytes, which express PPARγ, but not in pre-adipocytes, whick lack detectable PPARγ expression (Fig. 5A), these data suggest that Tip60 requires the PPARγ protein for its recruitment to PPARγ target genes.

To address the relevance of Tip60 in adipogenesis, the expression of this protein was reduced using siRNA-mediated knocked-down. Two independent siRNA oligonuleotides (siRNA oligonucleotides #1 and #2) reduced the expression of the Tip60 protein, with siRNA oligonucleotide #2 being the most efficient, while siRNA oligonucleotide #4 hardly affected Tip60 protein levels when compared to cells treated with non-targeting siRNA oligonucleotides (Fig. 5E). Knock-down of Tip60 expression with siRNA oligonucleotides #1 and #2 resulted in impaired differentiation of 3T3-L1 cells into adipocytes, as illustrated by staining of triglycerides with Oil-red-O at day 6 of differentiation (Fig. 5D). Similar findings were obtained with an siRNA oligonucleotide pool directed against Tip60 (data not shown). To confirm this reduction in adipocyte differentiation independently, the protein expression of Fabp4 was determined. While Fabp4 protein was clearly induced at day 6 of differentiation (Fig. 5E, lanes 1 and 2), treatment of cells with siRNA oligonucleotide #2 and to a lesser extent siRNA oligonucleotide #1, blunted this repsonse (Fig. 5E). Treatment of cells with siRNA oligonucleotide #4, which failed to reduce Tip60 expression (Fig. 5E), had no effect on triglyceride accumulation (Fig. 5D) or Fabp4 protein expression (Fig. 5E). Since our experiments indicate that Tip60 contributes to the adipogenic action of PPARγ by targeting the N-terminal AF1 domain (Fig. 2) and that the protein levels of this MYST acetyltransferase are rate-limiting during adipogenesis (Fig. 5), we conclude that Tip60 qualifies as a novel adipogenic factor.

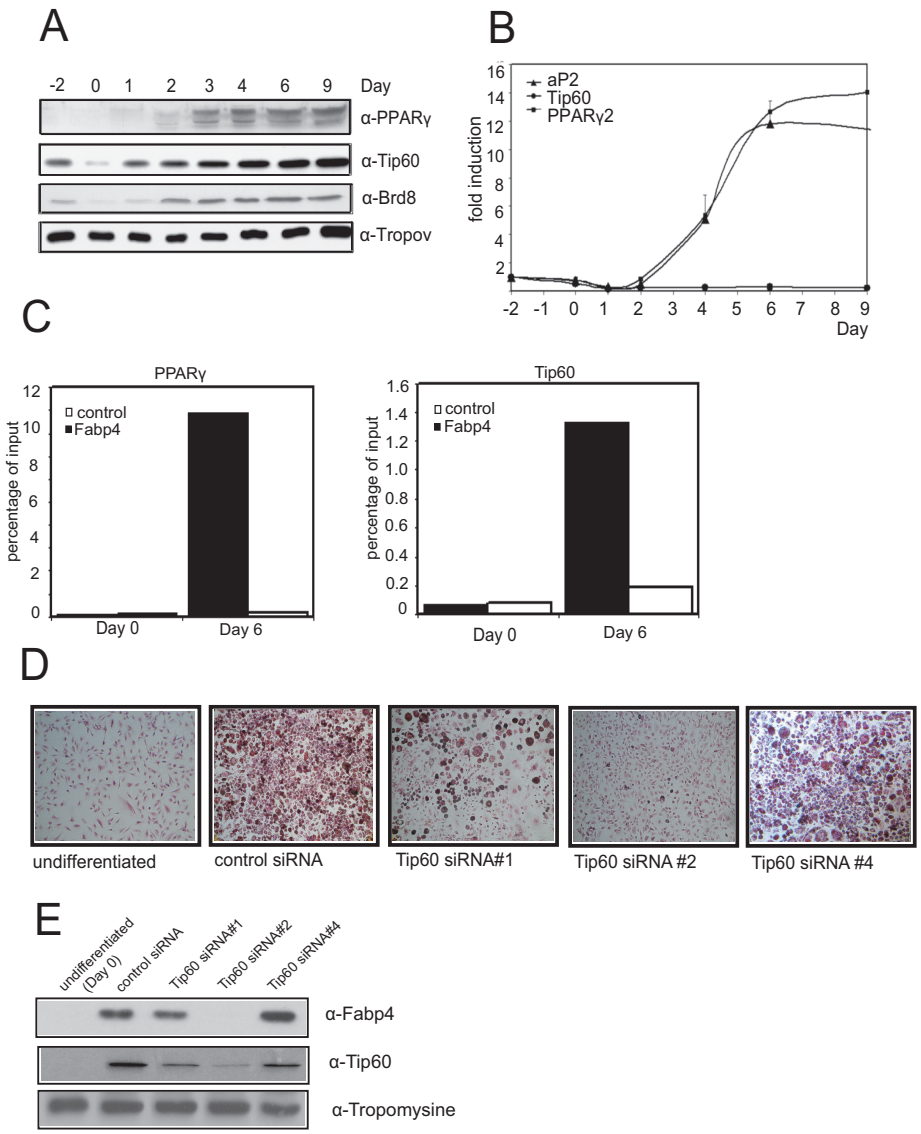

Figure 5. Tip60 plays an essential role in 3T3L1 adipocyte differentiation. (A) Mouse 3T3L1 preadipocytes were differentiated into mature adipocytes and samples were taken at different time points during differentiation. Protein expression levels of Tip60 and Brd8 were determined by Western blot analysis. As control for differentiation PPARγ2 protein levels were analysed. (B) Tip60 mRNA levels were analysed by quantitative PCR. As control for differentiation Pparg mRNA levels were analysed together with mRNA expression of a common PPARγ target gene, Fabp4. (C) Recruitment of PPARγ and Tip60 to the Fabp4 promoter was assessed by ChIP analysis in 3T3-L1 pre-adipocytes and adipocytes. Chromatin was prepared on day 0 and day 6 of differentiation and subsequently subjected to IP using antibodies against PPARγ (left panel) and Tip60 (right panel). Enriched DNA was analyzed using quantitative PCR with primers located at the Fabp4 PPREs (~5500 bp from transcription start site). Primers corresponding to an intergenic region on mouse chromosome 15 were used as a negative control. Results are shown as percent recovery relative to chromatin input. Results are representative of at least 3 independent experiments. (D) 3T3L1 cells were transfected with either control or Tip60 siRNA oligonulceotides 2 days prior to differentiation initiation. siRNA transfection was repeated every 3 days. Cells were fixed at day 6 and stained for triglycerides using Oil-red-O, nuclei were stained with crystal violet. Pictures are representative for three independent experiments. (E) Western blot analysis of PPARγ2 in differentiated 3T3L1 cells. At day 6, lysates of 3T3L1 cells treated with control siRNA or Tip60 siRNA were subjected to Western blot analysis, using antibodies against Tip60, Fabp4 and tropomyosin.

Discussion

The activity of nuclear receptors like PPARγ is dictated by the set of proteins with which this NR is able to interact. Here we report that the MYST acetyltransferase Tip60 functions as a novel and specific coactivator for PPARγ. Our experiments show that, unlike most transcriptional coregulators, Tip60 interacts with the N-terminal AF1 domain of PPARγ. Several lines of evidence indicate that this AF1 region, which is poorly conserved between the PPARα, -γ and -β/δ proteins, plays an essential role in determining the specific gene expression profile elicited by the different isotypes (19;44;59). Firstly, gene expression studies in cell lines expressing chimeric PPARγ-PPARδ proteins have revealed that the AF1 regions are responsible for PPAR-selective gene expression (19). Secondly, the AF-1 regions restrict the transcriptional response, since deletion of these regions resulted in less selective gene expression profiles (e.g. activation of PPARδ-specific target genes by PPARγΔAF1)(19). Finally, exchanging the AF1 region of PPARδ with the corresponding region of PPARγ converts this protein from a non-adipogenic to an adipogenic transcription factor (18;19). Since Tip60 targets the AF1 domain, this acetyltransferase is, together with PGC-2 (18), among the first proteins identified to date that may play a role in determining PPAR isotype-selective gene expression.

Tip60 has previously been implicated in signalling through other nuclear receptors, including the androgen receptor (AR), glucocorticoid receptor (GR), estrogen receptor (ER) and RORα (53;60-63). In the case of AR, GR and ER, Tip60 was reported to interact directly with these class I nuclear receptors through their LBDs and the interactions required the presence of the respective ligands as well as an intact LXXLL motif in the Tip60 protein (53). The failure to detect interactions between Tip60 and the LBDs of several class II nuclear receptors (Thyroid hormone receptor (TR), vitamin D receptor (VDR) and retinoid X receptor (RXR)), together with the lack of up-regulation of these receptors by Tip60 led to the hypothesis that Tip60 was a coactivator specific for class I nuclear receptors (53). Our experiments now indicate that Tip60 can potentiate the activity of the class I nuclear receptor PPARγ, but that this occurs through an alternative molecular mechanism. In contrast to the Tip60-class I NR interactions, the Tip60-PPARγ interaction can occur in the absence of ligand and is independent of the interaction interface defined by the charge clamp in the LBD and the LXXL motif in the coactivator molecule. Furthermore, using chimeric NRs and in vitro protein-protein interaction assays, we demonstrate that Tip60 targets the N-terminal AF1 region of PPARγ. We conclude therefore that the same coactivator (or coactivator complexes) can play a role in the activation process of closely related transcription factors through distinct molecular mechanisms. Our data suggest that Tip60 is at least in part responsible for releasing the repressive functions encoded by the AF1 domain of PPARγ (11).

Tip60 is part of a large multi-protein complex, which is conserved between yeast and humans (27;29-31). Our results indicate that the acetyltransferase activity of Tip60 plays a role in PPARγ-mediated transcription, since a catalytic mutant of Tip60 (G380E) displayed reduced ability to potentiate the transcriptional activity of PPARγ. Tip60 can acetylate both histones and non-histone proteins (27;33), including the AR in its hinge region (60). Whether histones, PPARγ itself or other proteins in the tran-

scription complex are the main acetylation substrates in our experimental setting remains to be established. The Tip60 complex also contains the Brd8 protein, a coactivator for PPAR/RXR, which contains LXXLL motifs (64;65). The Brd8 protein may stabilize interactions between the Tip60 complex and the PPAR/RXR heterodimers by binding to the AF2 region. Such additional protein-protein interactions may account for the increase in ligand-dependent activity of PPARγ by Tip60, while we found the protein-protein interaction between PPARγ and Tip60 to be independent of ligand. Alternatively, Tip60 may cooperate with other coregulators outside the Tip60 complex, like TIF2 (66), to potentiate PPARγ-mediated transcription in a ligand-dependent fashion.

While lysine modifications are clearly important mechanisms to regulate protein function (67), very little is known about the mechanism that regulates the activity and/or expression of the modifying enzymes, like acetyltransferases. As is shown here, Tip60 protein levels increase during adipogenesis and are clearly not regulated at the mRNA level, but are probably controlled post-translationally via a yet unidentified mechanism. Furthermore, it has been published that Tip60 acetyltransferase activity is regulated during the cell cycle by phosphorylation (46;68), and it will therefore be of much interest to investigate whether the intrinsic acetyltransferase activity of Tip60 is also regulated in cellular processes like adipogenesis.

In conclusion, besides ligand binding, modulation of AF1 activity through interactions with coregulators and/or post-translational modifications provides an important extra layer in the regulation of PPARγ activity. Given the central role of PPARγ in glucose and lipid metabolism, the enzymes involved in regulating PPARγ activity can be considered as potential therapeutic targets for the treatment of type 2 diabetes. This underscores the importance of further elucidating the molecular mechanisms that regulate the transcriptional activity of PPARγ.

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The serine/threonine phosphatase PPM1B (PP2Cβ) regulates PPARγ activity

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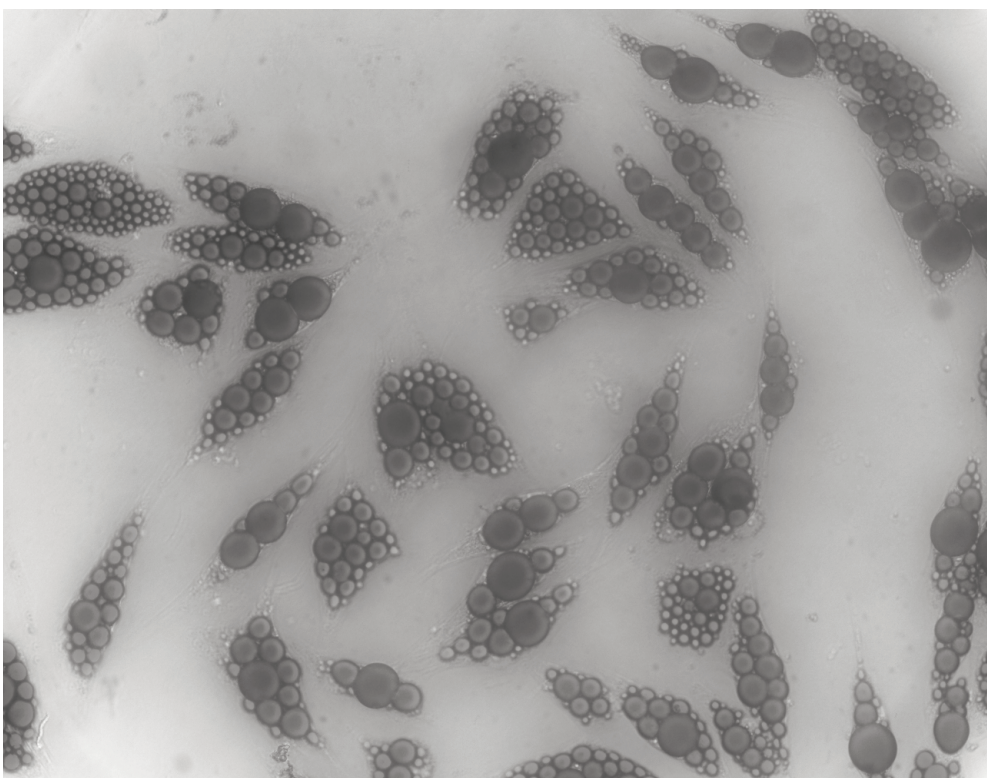

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ABSTRACT

Reversible phosphorylation is a widespread molecular mechanism to regulate the function of cellular proteins, including transcription factors. Phosphorylation of Peroxisome Proliferator Activated Receptor γ (PPARγ) on a conserved N-terminal serine residue (S112) results in reduced transcriptional activity of this member of the nuclear receptor superfamiliy of transcription factors. So far, no cellular enzymatic activity 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 PPARγ2 interacting protein. Furthermore we show that PPM1B can directly dephosphorylate PPARγ2, both in intact cells and in vitro. In addition PPM1B increases PPARγ2 mediated transcription via dephosphorylation of serine 112. These findings qualify PPM1B as the first nuclear receptor phosphatase.

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 (1-4). 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 stabilises the active conformation of the PPARγ LBD, thereby serving as a "molecular switch" between activation and repression functions of the receptor (5). It has however become increasingly clear that post-translational modifications (PTMs) represent an additional important molecular mechanism to regulate the activity of nuclear receptors, including PPARγ (1;6-8). The first PTM described for PPARγ was phosphorylation on a single serine residue (serine 112 in PPARγ2, 82 in PPARγ1) by various kinases like ERK1, p38, JNK and cdk7 (9-14). Phosphorylation of PPARγ impairs its transcriptional activity by either inhibition of ligand binding (15) or stimulation of a repressive PTM named SUMOylation (16;17), or a combination of both.
Although protein phosphorylation events are generally reversible and many nuclear receptors are subject to phosphorylation (18-20), no cellular enzymatic activity has been described so far which can dephosphorylate nuclear receptors. 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 (21-23). 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. 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 (24;25). The PPP family includes PP1, PP2A and PP2B whereas the PPM family consists of the Mg^{2+} or Mn^{2+} -dependent phosphatases. The latter subfamily comprises the PP2C isozymes and pyruvate dehydrogenese phosphatase (26). PPM family members have been reported to function in the regulation of different cellular processes including apoptosis, cell cycling and differentiation (27-29). To conform with 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 in order to identify novel PPARγ associated proteins. We describe the serine/threonine phosphatase PPM1B as a novel PPARγ2 interacting protein. Furthermore we show that PPARγ2 functions as a direct substrate in PPM1B mediated dephosphorylation, both in intact cells and in vitro. In addition PPM1B increases PPARγ2 mediated transcription via dephosphorylation of serine 112. As such, PPM1B is the first nuclear receptor phosphatase described so far.

MATERIAL AND METHODS

Material

Rosiglitazone was purchased from Alexis. FugeneÒ6 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-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. Anti-FLAG M2 agarose beads (A02220), Oil-Red-Ọ (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 (30). pCMV Renilla was purchased from Promega. All mutations were generated by Quickchange mutagenesis (Stratagene) and verified by sequencing. pSport PPM1B1 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 (31). All other plasmids have been described before (32;33).

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 mg of penicillin/ml and 100 mg 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 mg of penicillin/ml and 100 mg streptomycin/ml (Gibco Life Technologies). Reporter assays were performed in 24-well plates with 1 µg 3xPPRE-tk-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 (32). 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 FugeneÒ6 or PEI transfection reagent, as described before (33).

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 (33;34), except that proteins were not only subjected to trypsin, but also chymotrypsin and Arg-C digestion.

Western blot analysis

For detection of phosphorylated PPARγ2 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 (32).

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.

RESULTS

In order to identify novel PPARγ interacting proteins, ectopically expressed FLAG-tagged PPARγ2 was immunopurified from HEK 293T cells in the presence of the synthetic ligand rosiglitazone (1μM). Associated proteins were determined by mass spectrometry analysis. Several peptides corresponding to the serine/threonine phosphatase PPM1B (also named PP2Cβ) were repeatedly identified in independent experiments (Figure 1A). At least five different isoforms of human and murine PPM1B have been described so far, which differ in the N- or C-terminal region of the protein by length or sequence (35)(See also Supplementary figure S1). 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 exclusively present in isoform 1. These findings suggest that PPM1B isoform 1 is a PPARγ2 interacting protein.

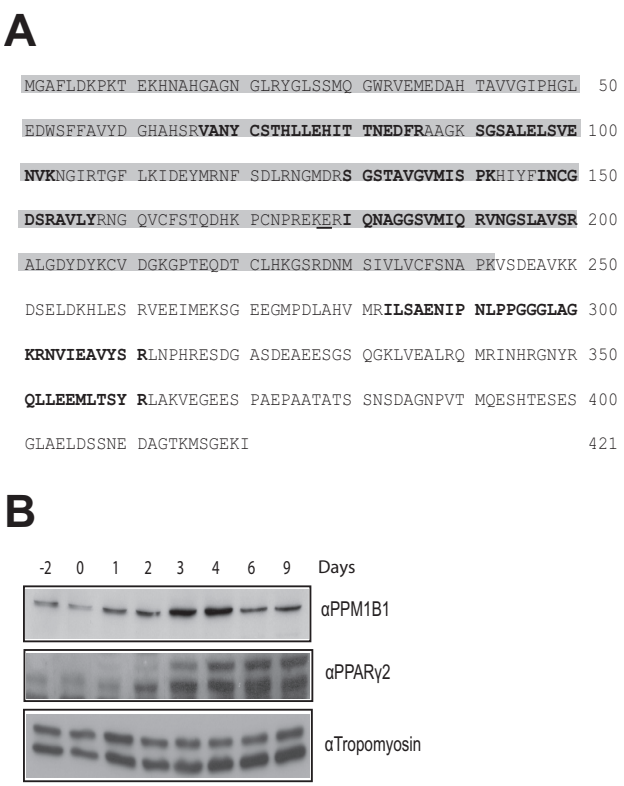

Figure 1. The phosphatase PPM1B is a novel PPARγ interacting protein. A. Amino acid sequence of PPM1B isoform 1. Indicated in grey is the catalytic domain conserved between different PPM1B isoforms. The peptides identified by mass spectromic analyisis are shown in bold. The R179G mutation that was generated for a catalytic inactive PPM1B protein is underlined. 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.

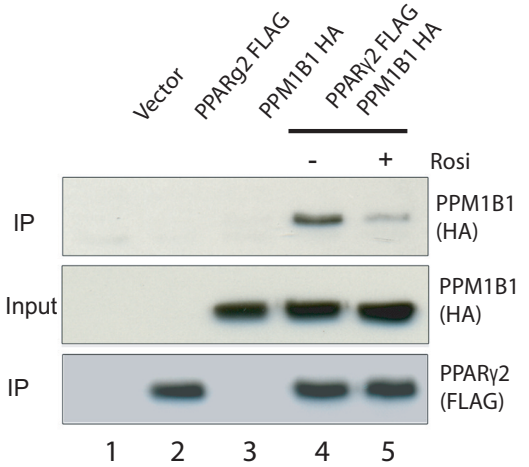

Figure 2.. PPARγ2 and PPM1B1 interact in intact cells. FLAG-PPARγ2 and HA-PPM1B1 were overexpressed in HEK 293T cells in the presence (+)or absence (-) of 2μM Rosiglitazone. Cells were lysed in RIPA buffer and PPARγ2 was precipitated using anti-FLAG beads. Immunorecative proteioens were detected on Western blots by anti-HA and anti-FLAG antibodies.

Next, we examined the protein expression of PPM1B1 during differentiation of 3T3-L1 cells into mature adipocytes. Expression levels of PPM1B isoform 1 increased up to day four, while PPARγ expression was detectable from day three onwards (Fig. 1B). These findings indicate that both PPARγ and PPM1B1 are expressed in mature 3T3-L1 adipocytes.
To confirm the interaction between PPARγ2 and PPM1B in living cells, HA-tagged PPM1B was overexpressed in U2OS cells together with FLAG-tagged PPARγ2 in presence or absence of synthetic ligand rosiglitazone. As is shown in figure 2, HA-tagged PPM1B coimmunopreciptated with FLAG-tagged PPARγ2. The presence of rosiglitazone decreased the interaction between PPM1B and PPARγ2. As a control, immunoprecipitations were performed on lysates of cells expressing either PPM1B or PPARγ2 alone, or neither protein. No coimmunoprecipitations were detected in these lysates (Figure 2).

Next we examined whether PPM1B can dephosphorylate PPARγ. First, FLAG-PPARγ2 and 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 PPARγ2 was readily detected either in absence or presence of 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; (36)). This mutant displayed reduced dephosphorylation abilty 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

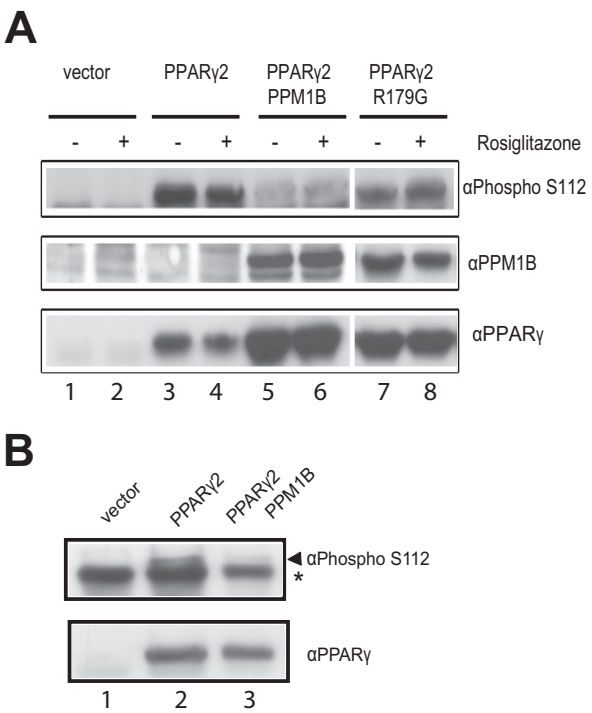

Figure 3. PPM1B functions as a direct phosphatase for PPARγ2, dephosphorylating serine 112 within the AF1 region. A. In HEK 293T cells PPM1B1 and FLAG PPARγ2 were overexpressed in presence or absence of 2μM Rosiglitazone. PPARγ2 was immuno-precipitated in RIPA buffer using FLAG beads. Dephosphorylation of PPARγ2 serine 112 was detected with Western blot labeling using phospho serine 112 specific antibodies. B. In vitro dephosphorylation was assesed using PPARγ2 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 (*).

vitro assays were performed. FLAG-PPARγ2 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.

Finally, we examined whether PPM1B affected PPARγ activity using luciferase reporter assays. As is shown in Figure 4A, cotransfection of PPARγ together with PPM1B increased PPARγ activity approximately 2-fold, either in the absence or the presence of the synthetic ligand rosiglitazone. Importantly, a 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. The only phosphorylation site known for PPARγ is a serine residue at position 112 within the AF1 region. Mutating serine 112 into alanine resulted in increased activity as described earlier (10;11;37;38). PPM1B was not able to further increase transcriptional activity of this PPARγ2 protein harbouring a mutation in the N-terminal AF1 region. Reporter assays in which the N-terminal AF1 region or the LBD were fused to a heterologous

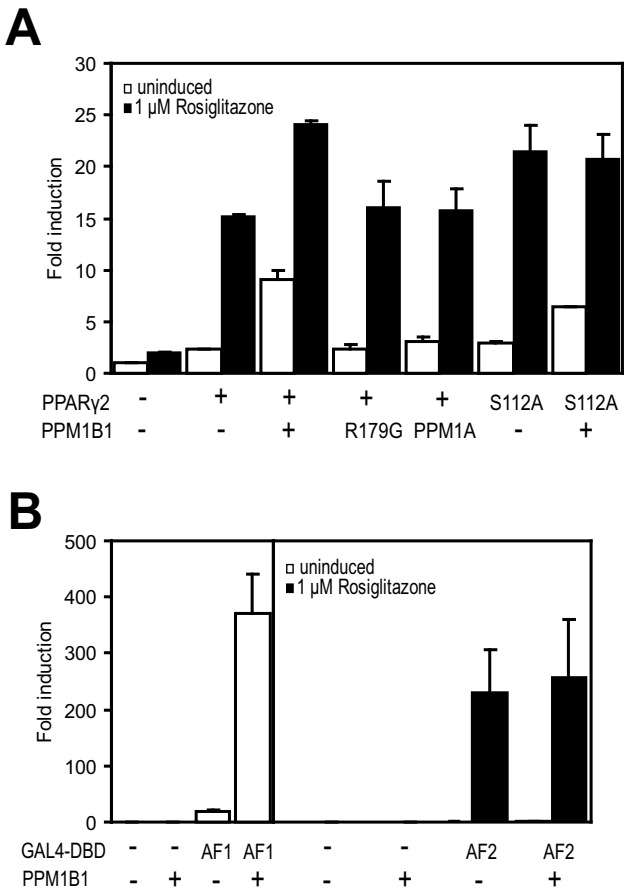

Figure 4. PPM1B is a specific activator of PPARγ2. A. U2OS cells were transfected with expression vectors encoding PPARγ2, PPARγ2 S112A (phosphorylation site mutant) PPM1B1, PPM1B R179G (catalytic inactive mutant), PPM1A and 3xPPRE-tk-Luc reporter. Activation of the luciferase reporter in absence or 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. B. 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.

DNA binding domain (Gal4DBD) indicated that PPM1B specifically targeted the AF1 region, and not the AF2 region (Figure 4B). From these findings we conclude that PPM1B-mediated PPARγ2 activation involves the serine 112 residue. Furthermore PPM1B mediated PPARγ2 activation is specific for PPM1B and requires its catalytic activity.

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 transcriptional activity. As such, PPM1B is the first nuclear receptor 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. At least five different splice variants have been found in humans and mice, all identical in the catalytic domain but differing in the N- and C-terminal domains, which are thought to be involved in localisation and substrate specificity (39;40). While PPM1B isoform 1 is expressed ubiquitously, expression of other PPM1B isoforms is tissue specific (40-43). Furthermore PPM1B phophatases have significant nuclear localisation and one of the PPM family members (PP2Cγ) has been identified as an important component of the spliceosome (23;44;45). Recently disruption of PPM1B in mice was shown to lead to early pre-implantation lethality (26). 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 (26). Our findings add PPARγ to a short list of PPM1B substrates, including the pro-apoptotic protein Bad, the cyclin dependent kinases cdk2 and cdk6 and the IKKβ kinase (28;46;47).

As the first post-translational modification described for PPARγ2, phosphorylation of serine 112 (serine 82 in PPARγ1) has been shown to reduce ligand binding via interdomain communication, resulting in reduced transcriptional activity (6;15;38). However, an laternative mechanism for phosphorylation-mediated inhibition of PPARγ activity has recently emerged. Several research groups have reported phosphorylation to enhance PPARγ SUMOylation (small ubiquitin-like modifier). Interestingly, it was found that ψKxExxSP motifs, which are present in many different proteins and first shown in MEF2 (48), functionally couple phosphorylation to sumoylation (49). PPARγ2 contains two functional sumoylation sites, lysine 107 in the AF1 region and lysine 395 in the AF2 region (50;51). Lysine 107 was shown to be the sumo acceptor site in a functional ψKxExxSP motif, and mutation of either S112 or K107 both decreased sumoylation while increasing transcriptional activity on PPAR responsive reporter assays (16;17). Although lysine 107 sumoylation has been reported to decrease the stability of the PPARγ protein (52), the exact molecular mechanism behind sumoylation dependent repression of PPARγ remains obscure and possibly involves specific binding of a repressor protein. One of the candidates could be the repressor protein TAZ. As a transcriptional modulator of mesenchymal stem cell differentiation, TAZ was shown to specifically repress PPARγ activity while stimulating the osteogenic transcription factor Runx2, thereby inducing osteogenesis in favour of adipogenesis (53). It will be of interest to investigate whether TAZ, or another repressor protein, plays a role in the physiological effects of serine 112 phosphorylation. Fat content and weight of knock-in mice expressing PPARγ S112A were found to be comparable to wild-type animals although the average size of their adipocytes was smaller (38).

Furthermore, increased glucose uptake was detected and indeed the PPARγ S112A mice have become insensitive towards diet-induced insulin resistance.
Although much is still unclear about PPARγ modifications, the modifying enzymes could offer the possibility of therapeutic modulation and provide new ways of improving insulin sensitivity. The phosphatase PPM1B described here might potentially be one of many novel targets for therapeutic modulation to improve treatment of type 2 diabetes. Although not much is known about upstream signalling cascades, PPM1B catalytic activity was shown to be activated by unsaturated fatty acids isolated from human lipoproteins (54). Systematic analysis of a variety of different free fatty acids revealed several of the chemical features of compounds that can activate PPM1B phosphatases. These include a chain length of 15 C-atoms and a free negatively charged group. Interestingly, one of the compounds that fullfills these requirements is nitro-oleic acid, which was recently found to function as a ligand for PPARγ2 (55). Treatment of nitro-oleic acid stimulates adipogenesis of 3T3L1 cells and significantly increases glucose uptake in mature adipocytes. This might indicate that nitro-oleic acid could function as “double ligand” inducing both PPARγ2 and its phosphatase PPM1B. More research is needed to explore the possibility of PPM1B as a future target for the development of novel insulin sensitizing drugs.

Footnotes

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Abbreviations used are: AF, activation function; DBD, DNA binding domain; HA, haemaglutinin; LBD, ligand binding domain; PP, protein phosphatase; PPAR, peroxisome proliferator activated receptor; PPRE, PPAR response element PTM, posttranslational modification.

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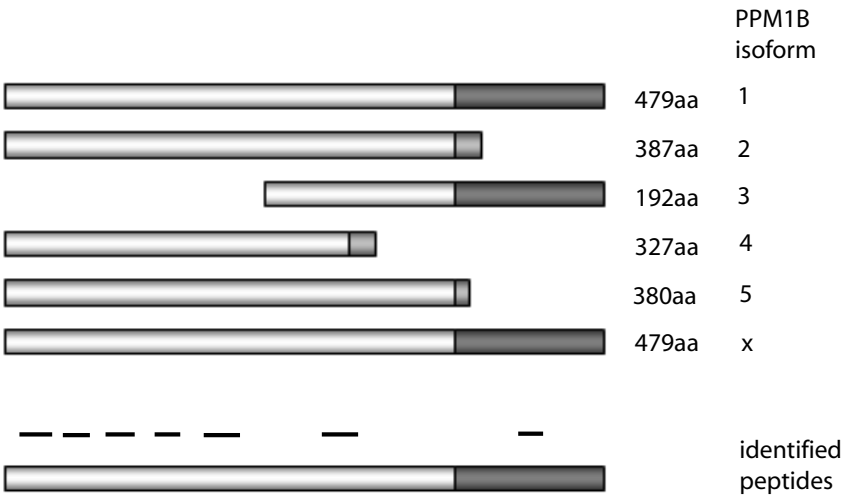
PPM1B
isoform
479aa
1
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3
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Chapter 6

A novel screening method identifies USP7 as a PPARγ2 regulator

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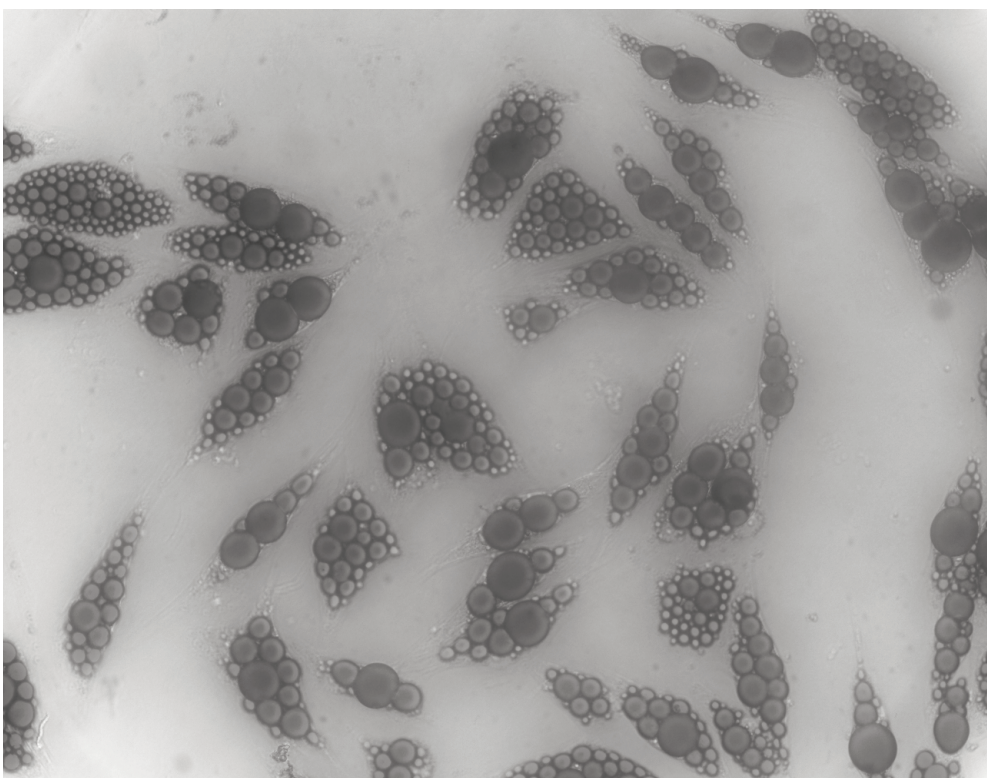

Manuscript in preparation

ABSTRACT

The nuclear hormone receptor Peroxisome Proliferator Activated Receptor γ (PPARγ) plays essential roles in metabolism, differentiation and immunity. Various PPARγ knock-out models and patients carrying PPARγ mutations underscore the importance of PPARγ as a key regulator of adipocyte differentiation, maintenance and function. To control energy homeostasis, tight regulation of PPARγ activity is required. Different post-transcriptional modifications have been identified that control PPARγ activity, including phosphorylation, SUMOylation and ubiquitination. Interestingly, it was shown that inhibition of the proteasome blocks ligand-dependent degradation of the PPARγ protein, indicating a role for ubiquitnation in PPARγ mediated transcription. However, the role of deubiquitination in PPARγ signalling and fat cell differentiation has not been investigated. Since the discovery of siRNA as a way to specifically downregulate gene expression, a broad spectrum of siRNA libraries have been developed that are now widely used to discover novel protein interactions and unravel signalling cascades. However, the use of siRNA libraries in PPAR research is often hampered by the lack of effective screening methods. Here we describe a novel screening assay based on microRNA expression under the control of a PPARγ responsive RNA polymerase II promoter. This method of screening enabled the identification of the deubiquitinating enzyme USP7 as a novel regulator in PPARγ signalling. As such, USP7 is also the first ubiquitin protease described for any of the PPAR proteins.

Introduction

The nuclear hormone receptor Peroxisome Proliferator Activated Receptor γ (PPARγ) plays essential roles in lipid and glucose homeostasis (1-3). Alternative splicing and differential promoter usage results in two isoforms, PPARγ1 and PPARγ2. PPARγ2 has a 30 residue extension at the N-terminus and is primarily expressed in adipocytes while PPARγ1 is more ubiquitously expressed. PPARγ plays a key role in the regulation of glucose and lipid metabolism in adipocytes by regulating their differentiation, maintenance and function (4;5). Compelling genetic evidence for this view comes from human FPLD3 patients, harbouring heterozygous mutations in the PPARG gene, as they are characterized by aberrant fat distribution and metabolic disturbances, including insulin resistance and dyslipidemia (6;7). In addition, heterozygous deletion of the PPARG gene in mice results in a failure to develop adipose tissue (8-10).

PPARγ is a member of the nuclear receptor superfamily of transcription factors. Upon binding of ligand these proteins undergo a conformational change, initiating a cascade of protein interactions and modifications that finally results in the induction of specific target genes (11;12). Interestingly, thiazolidinediones (TZDs), drugs that ameliorate insuline resistance in humans, are able to initiate such a sequence of events since they are ligands for PPARγ (13). Besides ligand binding, PPARγ activity can also be regulated through post-transcriptional modifications like phosphorylation (14) and sumoylation (15-17). Phosphorylation of serine 112 and SUMOylation of lysine 107 (PPARγ2 numbering) both reduce the activity of PPARγ, while SUMOylation of lysine 395 is involved in transrepression of NFκB activity by PPARγ (16). The importance of such post-translational modifications in vivo is underscored by in vivo studies showing that homozygous PPARγ2 S112A knock-in mice remain sensitive to insulin despite diet-induced obesity (18). Furthermore a P113Q mutation in the human PPARG gene, which causes diminished phosphorylation of the adjacent serine 112, has been associated with both adiposity and subsequent risk of type 2 diabetes (19). These data point to the importance of post-transcriptional modifications of the PPARγ protein in regulating energy homeostasis.

Besides phosphorylation and SUMOylation, ubiquitination has also been described as an important mechanism to regulate PPARγ function. Poly-ubiquitination is a common way to target proteins for proteasomal degradation forming an important regulatory mechanisms in virtually any cellular process (20). Recently the connection between ubiquitination and transcriptional regulation has become more clear (21;22). It was shown for nuclear receptors, including PPARα (23), that a functional proteasome pathway is required for transcriptional activity. The underlying mechanism is thought to reside in the cyclic recruitment of transcription factors and coactivators. Following gene activation, proteasomal degradation is an essential step in enabling the sequential recruitment of the different transcription complexes towards the promoter region (24). Ubiquitination has also been studied in the context of PPARγ transcriptional activity. Hauser et al. reported that inhibition of the proteasome blocked ligand-dependent degradation of PPARγ (25). Furthermore, it was reported that interferon-γ treatment of adipocytes decreased PPARγ levels in a proteasome-dependent mechanism (26). These data point to the importance of the ubiquitin-proteasome system

in determining PPARγ function and turn-over. However, the role of deubiquitination in adipogenesis and PPARγ signalling in particular remains obscure. Deubiquitinating enzymes have only recently emerged as important players in various signalling cascades, fulfilling key functions in different processes like tumor supression, cell cycling and cell metabolism (27-29). The deubiquitinating enzymes are part of the superfamily of proteases and can be divided into a large group of cysteine proteases and a smaller group of metalloproteases. In case of the cysteine proteases, a conserved cysteine residue resides within the active site and is crucial for enzymatic activity (30). The metalloproteases on the other hand, use a metal ion, mostly Zn^{2+}, within the catalytic cleft to release the ubiquitin moiety from the bound substrate (31).
Since the discovery of siRNA as a way to specificaly down regulate gene expression, a broad spectrum of siRNA libraries have been developed that are now widely used to discover novel protein interactions and unravel signalling cascades (32). For example, Tang et al. employed a small 96 well-scale siRNA library screen to identify protein kinases involved in inhibition of insulin-induced glucose uptake in fully differentiated 3T3-L1 adipocytes (33). From this screen the integrin-linked protein kinase MAP4K4 was identified as a negative regulator of adipogenesis supressing expression of the adipogenic transcription factors C/EBPα, C/EBPβ and PPARγ. The use of siRNA libraries in high throughput screening assays for the identification of novel PPARγ regulators is however hampered by the lack of adequate screening strategies. Here we describe a novel screening method based on microRNA expression by a PPARγ responsive RNA polymerase II promoter. Targeting of the expressed miRNA towards a resistance marker makes stimulation of PPARγ2 transcriptional activity lethal to mammalian cells when put under selective pressure (see also Figure 1). Specific knock-down of PPARγ regulators under these conditions results in a rescue of the induced lethality phenotype since the expression of the miRNA targeting the resistance marker is now impaired. This system potentially allows the identification of genes affecting PPARγ activity in a high-troughput setting and enables the use of different siRNA libraries in PPAR research.

MATERIAL AND METHODS

Materials

Rosiglitazone was purchased from Alexis. FugeneÒ6 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; ant-FLAG M2 HRP (A8592), anti-HA (H9658), Sigma-Aldrich; anti-rabbit-HRP (111035144) and anti-mouse-HRP (115035146), Jackson Immunoresearch Laboratories Inc.

Plasmids

The pPrime miRNA expression CMV miRNA FF2 and FF3 vectors were a kind gift from the Elledge lab and used for subsequent cloning (34). Target sequence with a mismatch at the first base for FF2; cCCGCCTGAAGTCTCTGATTAA and for FF3; aGCTCCCGCTGAATTGGAATCC. A BglII-HindIII fragment encompassing the 3xPPRE-TK promoter was digested from the 3xPPRE-TK-luciferase reporter and subcloned into pLNCX ΔClaI. The miRNA FF2 cassette was digested from pPrime using ClaI, NotI and subcloned behind the 3xPPRE-TK promoter to generate the pLNCX 3xPPRE FF2miRNA retroviral vector. A SV40 polyA signal oligo was ligated at the 3' end of the FF2 cassette, using a ClaI site. All recombinant DNA work was performed according to standard procedures (35). All mutations were generated by Quickchange mutagenesis (Stratagene) and verified by sequencing.

Cell culture, transient transfections and reporter assays

The human osteosarcoma cell line U2OS, MCF-7 breast carcinoma cell line and the Phoenix amphotropic packaging cell line (7) were maintained in DMEM Glutamax (Dulbecco) containing 10% foetal calf serum (Gibco Life Technologies), 100 mg/ml penicillin and 100 mg/ml streptomycin (Gibco Life Technologies). Reporter assays were performed in 24-well plates with 1 µg 3xPPRE-tk-Luc reporter construct, 2 ng PPAR expression construct, 2 ng pCMV-Renilla (Promega). Reporter assays as well as retroviral transductions were performed as described before (7;36). Electroporation of U2OS cells were performed with the Biorad Genepulser Xcell using 2µg and 100ul cell suspension in electroporation buffer (2mM Hepes pH 7.2, 15mM K_2PO_4/KH_2PO_4, 250mM Manitol and 1mM $MgCl_2$ pH 7.2) per electroporation, with two times 8 pulses at 140V, 1.5 msec burst duration at intervals of 1.5 s.

Western blot analysis

FLAG-PPARγ2 was overexpressed in U2OS cells together with USP7 wild-type or the C223S catalytic mutant. The next day cells were incubated for 4-8 hours with 1ug/ml doxicyclin prior to lysis in 2X Laemmli sample buffer. Samples were analysed by Western blotting. Blots were probed with various primary antibodies and immunoreactive complexes were visualised by enhanced chemiluminescence as described (7).

RESULTS

PPARγ2 driven miRNA expression results in loss of resistance

While vector-based siRNA techniques generally employ RNA polymerase III-driven expression (37), Stegmeier et al. recently reported efficient knock-down of gene expression from RNA polymerase II-driven vectors. (34). This was achieved by embedding the short hairpin RNA (shRNA) expression cassette in a miRNA environment. Amongst others, this sytem was tested using two pPRIME (potent RNA interference using microRNA expression) vectors expressing different miRNAs directed against firefly luciferase mRNA, named FF2 and FF3. Based on these vectors, we developed a novel screening method depicted here as "miRNA based siRNA screening" (Figure 1).

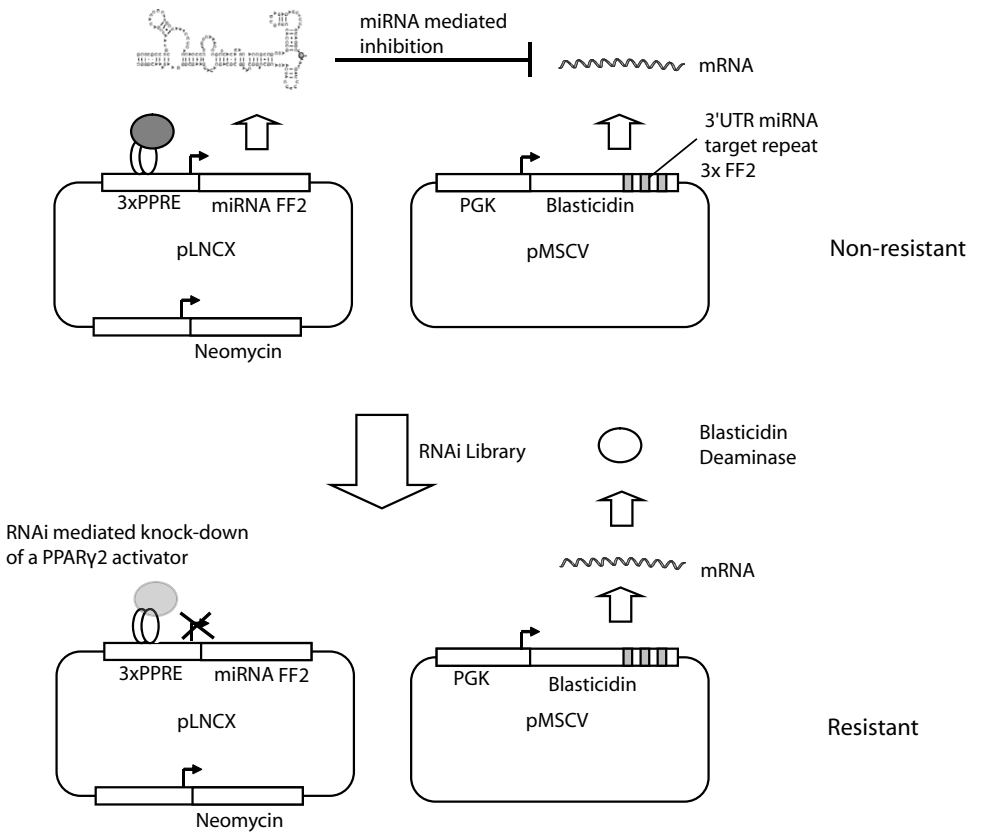

Figure 1. Schematic model of miRNA mediated siRNA screening. The upper panel illustrates the situation of cells with active PPARγ2-mediated gene expression. PPARγ drives miRNA FF2 expression, resulting in repression of the Blasticidin expression cassette via the target repeat FF2 sequences present in the 3' UTR. The lower panel depicts the situation of cells with an siRNA mediated knock-down of a PPARγ2 activating protein. Expression of the FF2 miRNA is diminished and Blasticidin deaminase expression is no longer repressed, resulting in Blasticidin resistance.

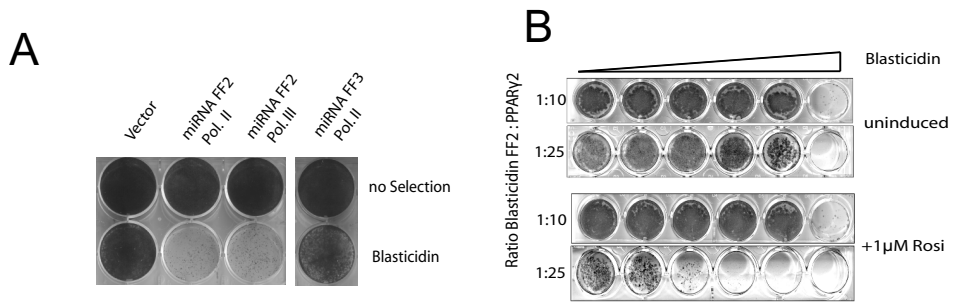

Figure 2. A RNA polymerase II and RNA polymerase III driven miRNA FF2 expression results in loss of Blasticidine resistance. U2OS cells expressing the Blasticidin 3xFF2 expression cassette were transduced with miRNA FF2 expressing virus and selected (50µg/ml Blasticidin) for 1 week. As a control either empty virus or not FF2 specific miRNA FF3 virus was used, the latter expressing miRNA not targetting the modified 3'UTR of the Blasticidin mRNA. B. U2OS cells stably transduced with 3xPPRE miRNA FF2 virus were retransduced with Blasticidin 3xFF2 virus and PPARγ2 virus at a ration of 1:10 or 1:25 respectively. Cells transduced at a ratio of 1:25 show loss of resistance when incubated in 1µM Rosiglitazone and increasing amounts of Blasticidin.

We first tested whether miRNA FF2, originally directed against luciferase gene mRNA, can be directed towards a heterologous transcript by the addition of 3 FF2 target sequences to the 3' UTR of the Blasticidin resistance expression cassette. For this, U2OS cells stably expressing the Blasticidin-3xFF2 cassette, driven by a CMV promoter, were isolated using retroviral transduction and selection on neomycin and Blasticidin. In order to test whether constitutive miRNA FF2 expression resulted in loss of resistance, these cells were subsequently transduced with a miRNA FF2 expressing retrovirus, under control of either the RNA polymerase II-driven CMV promoter or the RNA polymerase III-driven U6 promoter. As shown in figure 2A, cells expressing miRNA FF2 driven by either RNA polymerase II or RNA polymerase III showed a significant loss of Blasticidin resistance. As a control, empty virus or non-specific miRNA FF3 expressing virus were used, and neither resulted in significant loss of Blasticidin resistance. From these experiments we conclude that this system allows efficient expression of miRNAs RNA polymerase II and III promoters. Furthermore, the FF2 targeting sequences, originally found in the firefly luciferase gene, can be transferred to a heterologous gene (Blasticidin), resulting in significant loss of expression of this gene when exposed to the miRNA-based FF2 vector.

PPARγ2 dependent miRNA expression

Next we investigated whether the constitutively active CMV promoter, driving miRNA FF2 expression, could be replaced by a PPARγ2 responsive promoter (see also figure 1). We first isolated U2OS cells stably transduced with a 3xPPRE miRNA FF2 virus. The resulting U2OS 3xPPRE miRNA FF2 stable cell line underwent a second round of retroviral transduction to express Blasticidin deaminase and PPARγ2. When these two virusses were used at a ration of 1:10, activation of PPARγ by rosiglitazone had no effect on the Blasticidin resitance (Figure 2B). However, when PPARγ expression was increased by shifting the ratio 3xPPRE FF2-Blasticidin: PPARγ2 to 1:25, a significant loss of resistance for Blasticidin was observed in the presence of 1µM rosiglitazone. These experiments indicate that miRNA-FF2 expression from the

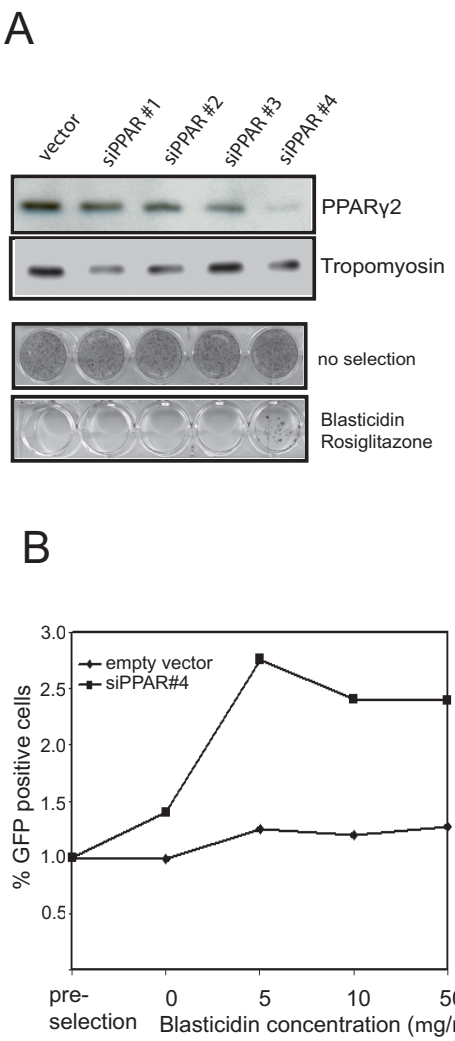

Figure 3.A siRNA-mediated knock down of PPARγ rescues miRNA FF2 mediated loss of Blasticidin resistance. Different PPARγ specific siRNA vectors were generated and tested for their ability to rescue the loss of Blasticidin resistence. The functional siRNA vector #4 rescues the miRNA FF2 induced loss of resistance. B. The U2OS 3xPPRE miRNA FF2 cells were partially rescued with PPAR#4 siRNA expressing GFP virus. The percentage of GFP positive cells was determined using FACS analysis after 1 week of Blasticidin selection at various concentrations

3xPPRE-miRNA-FF2 vector can be induced by rosiglitazone in cells expressing sufficient amounts of PPARγ2, resulting in loss of Blasticidine resistance conferred by the blasticidine-3xFF2 cassette.

In order to verify whether the effect of PPARγ2-driven miRNA expression on loss of resistance is indeed mediated by PPARγ2, we knocked-down PPARγ2 expression by siRNA technology. Different shRNA expression vectors directed against PPARγ were generated and transiently expressed in U2OS 3xPPRE miRNA FF2 cells. As shown in Figure 3A (upper panel), siRNA vector #4 efficiently knocked-down the ex-

pression of PPARγ, while vectors #1-3 were less efficient, as assesed by Western blotting. Next, U2OS 3xPPRE miRNA FF2 cells were transiently transfected with the different PPARγ siRNA expression vectors and incubated in the presence of rosiglitazone. After selection with Blasticidin for 1 week in the presence of rosiglitazone (1μM), colonies were stained using Giemsa staining. As shown in Figure 3A (lower panel), the functional PPARγ siRNA expression vector #4 could partially rescue the loss of Blasticidine resistance observed upon activation of PPARγ by rosiglitazone. The 3 shRNA vectors which gave no efficient knock-down of PPARγ expression (#1, #2 and #3) also failed to rescue Blasticidine resistance (Figure 3A), indicating that the loss of Blasticidine resistance in the presence of rosiglitazone shown in Figure 2B is indeed mediated by PPARγ. To corroborate these findings, FACS analysis was performed on the same cells with different concentrations of Blasticidin. For this we developed a retroviral GFP vector expressing the functional siRNA #4 directed against PPARγ2 described above. U2OS 3xPPRE miRNA FF2 cells were stably transduced and selected at different concentrations of Blasticidin for 1 week. The percentage of GFP positive cells was determined by FACS analysis. The percentage of GFP postive cells after Blasticidin selection increased approximately three fold in case of siRNA induced PPARγ2 knock down while it remained unchanged in empty GFP vector transduced cells (Figure 3B). Taken together, these experiments indicate that the loss of resistance in U2OS 3xPPRE miRNA FF2 induced by rosiglitazone is dependent on PPARγ2 expression.

miRNA based siRNA screening identifies USP7 as a PPARγ2 regulator

To investigate whether the U2OS 3xPPRE miRNA FF2 cell line could be used to identify novel regulators in PPARγ2 signalling, we made use of a siRNA DUB library previously developed by the laboratory of R. Bernards (27). This siRNA library targets 50 deubiquitinating enzymes and contains a set of 4 pSuper siRNA expressions vectors designed to target a single DUB mRNA (27). From this library we used individual siRNA pools directed against 24 different DUB transcripts (see Supplemental Table I). The U2OS 3xPPRE miRNA FF2 cells were selected by Blasticidin in the presence of 1μM rosiglitazone after siRNA vector electroporation. After 3 weeks of culture, colonies were observed when the expression of USP7/HAUSP, USP10, UCH-L3 or UCH-L5 was reduced while reduced expression of the other 20 DUBs did not result in rescue of the PPARγ2 induced loss of resistance phenotype (Figure 4A and data not shown). These findings suggest that 4 different DUBs (USP7/HAUSP, USP10, UCH-L3 and UCH-L5) play a specific role in PPARγ signalling.
To investigate the potential role of one of these DUBs, USP7/HAUSP, in PPARγ2 signalling and to verify the data obtained from the siRNA screen, 2 independent MCF-7 cell lines were generated stably expressing the Tet repressor (38;39) together with a tetracyclin inducible siRNA expression cassette targetting USP7 (28). Treatment of these cells with doxicyclin resulted in reduced protein expression of USP7/HAUSP (Figure 4B). Rosiglitazone-induced activity of the 3xPPRE-TK-Luc reporter was also clearly reduced in the presence of doxicyclin (Figure 4B), suggesting that USP7/HAUSP is required for optimal PPARγ signalling.

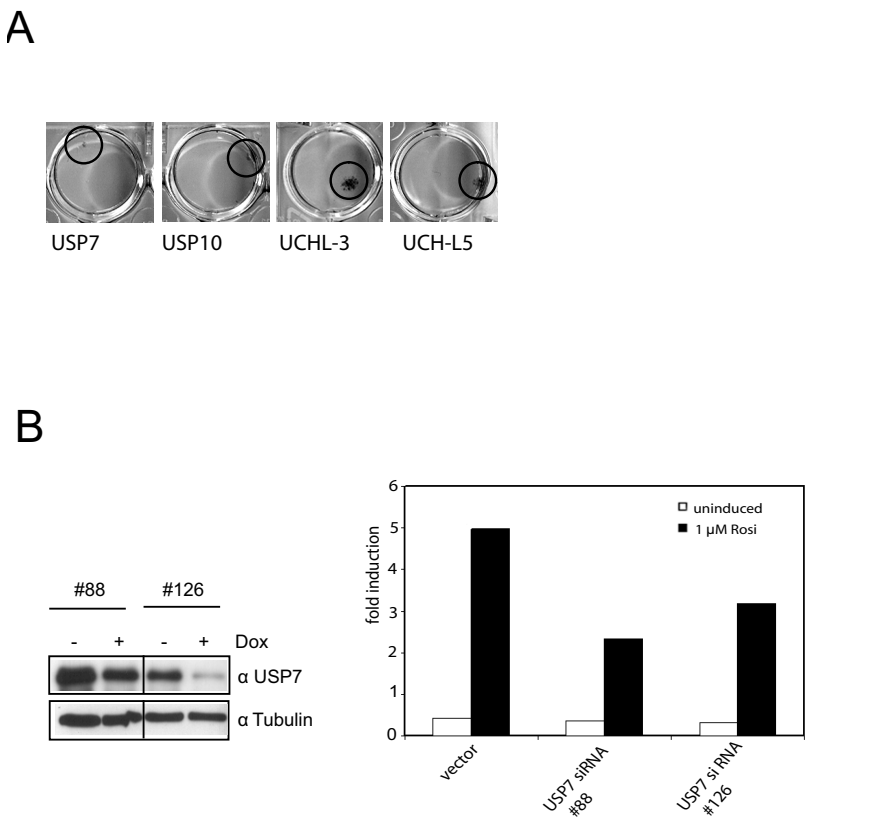

Figure 4. Small siRNA DUB screen perfomed in U2OS 3xPPRE miRNA FF2 cells. A partial siRNA library against 24 different deubiquitinating enzymes was tested for the ability to rescue the miRNA FF2 induced loss of Blasticidin resistance. A total of 4 different DUBs were identified as depicted. B. MCF-7 tetracyclin inducible USP7 knock down cells were induced for 48 hours using 1μg/ml tetracyclin and cotransfected with a 3xPPRE luciferase reporter and a PPARγ2 expression vector. C. USP7 overexpression results in PPARγ destabilistation. U2OS cells were transiently cotransfected with USP7 and PPARγ2 expression vectors. Cells were incubated with cyclohexamide and harvested at the indicated time points. PPARγ2 protein levels were assessed using Western blot analysis and as a positive control p53 was used. As indicated in the left panel, USP7 catalytic activity was checked using a USP active-site specific HA probe recognizing only the catalytically active USP7 conformation.

Since PPARγ is ubiquitinated and degraded by the proteasome upon ligand binding (25), which is thought to be an essential step in nuclear receptor signaling in general, we investigated whether the deubiquitinating enzyme USP7/HAUSP directly affected PPARγ2 protein stability. For this, wild-type USP7 or the C223S catalytic mutant were overexpressed together with PPARγ2 protein in U2OS cells. Part of the obtained lysate was incubated with a active site specific HA-tagged probe, to confirm that the C223S mutant lacks enzymatic activity (40). Two days after transfection cells were treated with cycloheximide, an inhibitor of translation. Cells were harvested at different time points and samples were analysed by Western blotting. Remarkably, co-transfection of USP7 wild-type decreased PPARγ2 protein levels, while the catalytic mutant USP7 C223S did not affect PPARγ2 protein expression. As a control, p53 protein levels were analysed and in this case overexpression of wild type USP7 resulted in higher p53 protein levels, while the C223S mutant was less able to affect p53 expression levels (Figure 4C). These results suggest that USP7/HAUSP regulates PPARγ activity indirectly, and not by counteracting the ubiquitination and subsequent proteosomal degradation of the PPARγ protein itself.

DISCUSSION

To identify novel regulators of PPARγ, we have developed a novel method termed "miRNA based siRNA screening". Our data indicate that miRNA based siRNA screening offers a novel way to identify transcriptional coregulators and could potentially be used as a generic method to identify coregulators of transcription factors.
The miRNA based siRNA screening method enabled us to identify USP7 from a small siRNA library of 24 different deubiquitinating enzymes as a possible regulator of PPARγ2 action. USP7 is a ubiquitously expressed protein belonging to the cysteine proteases of deubiquitinating enzymes. USP7 was first described to interact with a viral protein, ICP0 of the Herpes simplex virus, leading to stimulation of the lytic cycle (41). Recent reports have shown that USP7 also directly interacts and deubiquitinates p53 resulting in p53 stabilisation. Association and stabilisation of the E3 ligase MDM2 by USP7 was also reported and this possibly explains the contradictory reports on USP7 function in p53 signalling (42-44). USP7 is able to stabilise both the E3 ligase MDM2, leading to increased p53 ubiquitination and subsequent proteolytic degradation, as well as deubiquitinate p53, increasing its stability. Most likely the net deubiquitination of both substrates determines the level of functional p53 (45). A similar interaction between a ubiquitin-specific protease and ubiquitin ligase has been identified for Hif-1α signalling in which VHL, an E3 ligase, interacts and ubiquitinates the ubiquitin protease USP33. USP33 itself functions as a deubiquiting enzyme for Hif-1α (46). From studies in Drosophila it was shown that USP7 catalyses the deubiquitination of the histone protein H2B contributing to epigenetic silencing of homeotic genes. Furthermore this study indicated that USP7 associates with guanosine 5'-monophosphate synthase (GMPS) . It was found that GMPS enables the USP7 mediated removal of mono-ubiquitin which was not observed for USP7 alone (47). USP7 has also been identified as a deubiquitinating enzyme of the transcription factor FOXO (Forkhead box O). Upon oxidative stress FOXO is mono-ubiquitinated. Interestingly, USP7 mediated FOXO deubiquitination does not influence protein half-life but decreases its transcriptional activity by inhibiting nuclear translocation (28). Our results on the effects of USP7/HAUSP on PPARγ protein levels and activity support a model analogous to the role of USP7/HAUSP in p53 signalling. In this model USP7/HAUSP does not directly deubiquitinate PPARγ, but rather deubiquitinates and stabilizes its E3 ligase, hence the decreased PPARγ levels observed upon overexpression of USP7/HAUSP (Figure 4C). siRNA-mediated knock-down of USP7/HAUSP expression would then result in reduced levels of the PPARγ E3 ligase, and thereby PPARγ activity, as ubiquitination and degradation are viewed as essential steps in the transciption process among the PPAR proteins (25;48-50). Further research is required to identify the PPARγ E3 ligase and thereby elucidate the exact role of USP7 in PPARγ2 mediated gene activation.
Another deubiquitinating enzyme identified from the DUB library we screened was USP10. This DUB was described as a regulator of the androgen receptor (AR) and identified as part of a DNA-bound AR complex purified from nuclear extracts. USP10 was reported to stimulate AR transcriptional activity and a direct interaction was shown using GST pull down assays further confirming a coactivator function (51). The role of USP10 together with the carboxy-terminal hydrolases UCHL3 and UCHL5

in PPARγ signalling remains to be further established, but the functional link between USP10 and AR suggests that USP10 may be a more general regulator in nuclear receptor signalling.
In conclusion, USP7 to our knowledge, is the first deubiquitinating enzyme found to regulate the activity of any of the PPAR family members. So far little is known about the function of deubiquitination in the regulation of PPAR signalling. More research is required to unravel the function of USP7 and other members of the ubiquitin-proteases in the regulation of the different PPAR members. Considering the important function of PPARs in regulating lipid and glucose homeostasis, the identification of novel PPAR regulators could have important clinical implications.

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Chapter 7

General Discussion

Using both immunoprecipitation coupled to mass spectromic analysis and miRNA based siRNA screening we have identified several novel PPARγ2 interactors. Mass spectromic analysis has revealed the histone acetyltransferase Tip60 discussed in chapter 4 as well as the serine/threonine phosphatase PPM1B discussed in chapter 5 as novel PPARγ regulating enzymes. In addition, mass spectromic analysis revealed the methyltransferase PRMT5 and its partner MEP50 as well as the serine/threonine kinase STK38 as putative PPARγ2 regulators (unpublished data). Furthermore as discussed in chapter 6, a novel miRNA based siRNA screening method was developed. Using this method we identified the ubiquitin proteases USP7, USP10, UCHL-3 and UCHL-5 as potential regulators of PPARγ signalling. So far this is the first indication that ubiquitin proteases function in PPARγ-mediated gene activation.

PPARγ mutations

The discovery that thiazolidinediones function as ligands for PPARγ initiated screening for mutations in the PPARG gene of patients suffering from severe insulin resistance. These investigations indeed led to the identification of several genetic variants of the PPARG gene. The most common genetic variant of PPARγ2 is a polymorphism replacing alanine for proline at codon 12 (Pro12Ala). Although only modestly affecting insulin sensitivity, this common polymorphism causes a significant risk in the development of insulin resistance (1). A rare PPARγ Pro113Gln mutation found in the AF1 region on the other hand is linked to severe obesity and a high risk of developing type 2 diabetes (2). Most PPARγ mutations however have been identified in the DBD or AF2 domain and these can affect PPARγ function at different levels, as described in chapter 3. Interestingly, patients carrying mutations in the PPARγ DBD or AF2 domain suffer from familial partial lipodystrophy (FLP), in contrast to the patients described earlier harboring the Pro113Gln mutation in the AF1 domain. Since the P113Q missense mutation causes loss of the repressive serine 112 phosphorylation, this might indicate the important function of AF1 repression in determining fat distribution in the peripheral limb depots (3;4). Much is still unclear about the role of PPARγ in fat depot distribution and further research is therefore required to elucidate the underlying mechanisms.

Tip60 a PPARγ FAT or HAT?

In chapter 4 we have described Tip60 as a novel acetyltransferase in PPARγ activation. However, the exact molecular mechanism by which Tip60 activates PPARγ remains to be established. Since Tip60 mutants affected in the acetyltransferase activity

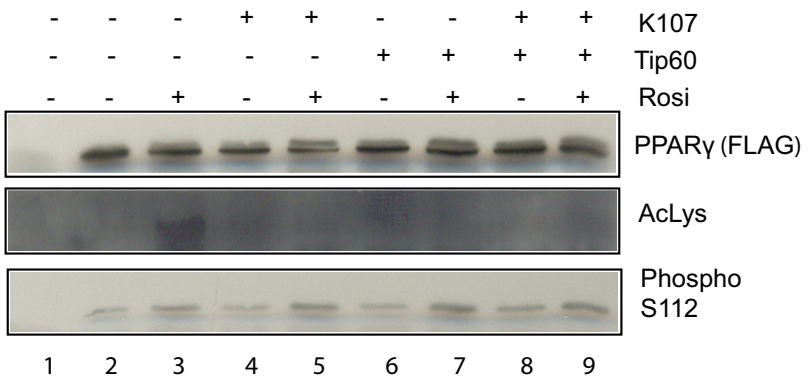


Figure 1. PPARγ acetylation at lysine 107. Either FLAG tagged PPARγ wild-type or K107A mutant PPARγ was transiently overexpressed in 293T cells and immunoprecipitated using FLAG beads. Acetylation of PPARγ was assessed by Western blot analysis using anti-acetyl lysine antibodies.

were less able to activate PPARγ, a Tip60-mediated acetylation event is likely to be involved. Tip60 is both known as a histone acetyltransferase (HAT) as well as a factor acetyltransferase (FAT) and several non-histone substrates have been described, including the transcription factor p53 (5). Tip60 was also reported as an acetyltransferase for the oncogene and transcription factor PLAGL2, which is involved in the development of various malignancies . Three repressive sumoylation sites were identified in PLAGL2. The repressive effect of sumoylation can partially be explained by effects on nuclear localization, as was concluded from data collected from PLAGL2 sumoylation mutants (6). Similar to PPARγ2, Tip60 functions in increasing the transactivation function of PLAGL2. Interestingly, it was suggested that Tip60-mediated PLAGL2 transactivation is achieved by competitive acetylation of the PLAGL2 sumoylation sites, thereby counteracting SUMO-mediated repression of PLAGL2 activity. This led us to investigate whether a similar mechanism was present for Tip60-mediated PPARγ activation. So far, acetylation has not been reported for any of the PPAR members, but, although technically challenging, PPARγ acetylation can be detected in the presence of rosiglitazone by anti-acetyl lysine antibodies (Figure 1, lane 3). Furthermore, mutation of the sumoylation acceptor lysine K107 present in the AF-1 domain abolished PPARγ2 acetylation (Figure 1, lane 5). Mutation of the sumoylation site by changing E109 to alanine did not affect PPARγ2 acetylation (data not shown). This would suggest a mechanism similar to what was found for PLAGL2 in which sumoylation is inhibited via competitive acetylation. However, co-expression of Tip60 together with wild-type PPARγ2 inhibited rather than stimulated PPARγ acetylation (Figure 1, lane 7). In addition, reporter assays showed that mutation of either the PPARγ2 sumoylation consensus site (E109A), the sumoylation acceptor lysine itself (K107A) or the phosphorylation site (S112A, P113Q) all affected coactivation by Tip60 in a similar fashion, indicating that this effect on Tip60 mediated-PPARγ activation is not specific for lysine 107 (Figure 2). Therefore, competitive acetylation of the sumoylation site seems unlikely to be mediated by Tip60. So although Tip60 acetyltransferase activity is required for Tip60 mediated PPARγ activation, this activity is more likely directed against other lysine residues and does not involve PPARγ lysine 107.

Exactly how Tip60 co-expression results in decreased PPARγ2 acetylation remains to be established.

Interestingly PPARγ2 acetylation at lysine 107 seems linked to PPARγ2 S112 phosphorylation since acetylation was only detected for the slower migrating band, which represents the phosphorylated form of PPARγ2 as was also confirmed using phospho-serine S112 specific antibodies. Acetylation of lysine 107 following phosphorylation of serine 112 is remarkable since PPARγ phosphorylation has been reported to stimulate repressive sumoylation of lysine 107 (7-9). Therefore, the coupling between phosphorylation and acetylation would point to a novel link between these two different modifications. Interestingly, a link between phosphorylation and acetylation is not uncommon and has been reported for other proteins including RelA (10).

Since acetylation of PPARγ2 is unlikely to be mediated by Tip60, another acetyltransferase must be responsible for modification of lysine 107. Interestingly p300, another well established coactivator for PPARγ2 (11), was found to facilitate acetylation of a conserved sumoylation motif present in the tumor supressor protein HIC1 and oncogenes PLAG1 and PLAGL2 (12;13). These preliminary data point to exciting and possible novel mechanisms in the regulation of PPARγ activity and future experiments should establish the function of PPARγ acetylation.

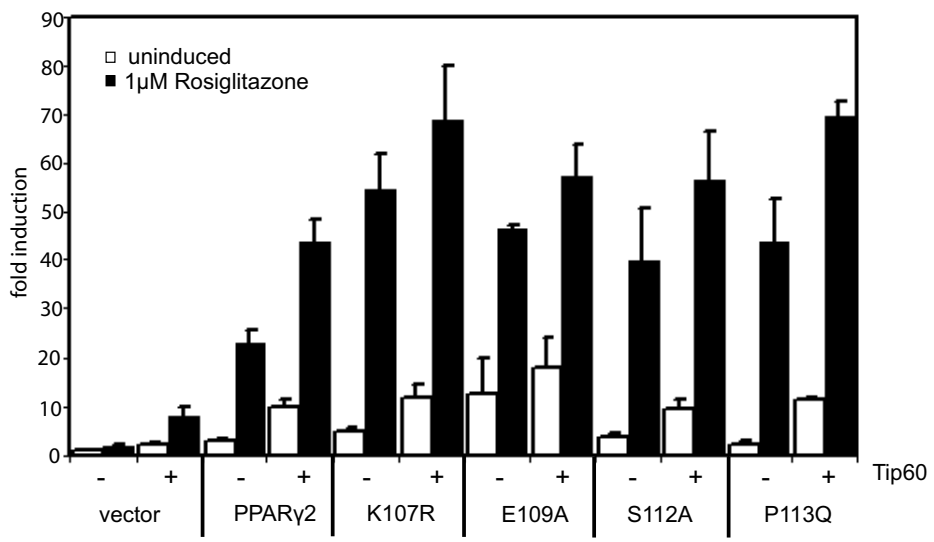


Figure 2. Phosphorylation and sumoylation defficient PPARγ2 mutants are still potentiated by the MYST acetyltransferase Tip60. U2OS cells were transfected with an expression vector encoding PPARγ2 wild type (wt), sumoylation mutants (K107A and E109A) or phosphorylation deficient mutant forms (S112A and P113Q) and a 3XPPRE-TK-Luc reporter. Activation of the luciferase reporter, in the absence or presence of 1 mM rosiglitazone, is expressed as fold induction over that with empty vector in the absence of ligand, after normalisation for Renilla luciferase activity. Results are averages of at least three independent experiments assayed in duplicate ± standard error of the means.

The ING3 complex

Tip60 is a member of the MYST family of acetyltransferases which exsist in large multi-subunit complexes (14). It was found that each MYST member is associated with a specific member of the ING (inhibitor of growth) tumor supressor proteins. ING proteins contain plant homeodomains (PHD), a motif often found in chromatin modifying proteins. All ING members have been implicated in p53 signalling (15). ING3 overexpression, for example, results in decreased numbers of cells in S-phase and induction of apoptosis. It was suggested that ING3 modulates p53 function by thetering chromatin remodelling complexes to p53 responsive genes or by altering the acetylation status of p53 itself (16). The MYST members MOZ and MORF were found to associate exclusively with ING5, while HBO1 both associate with ING4 and ING5. Interestingly, an ING-5 containing HBO1 complex has been implicated in DNA replication and indeed part of the complex was found to interact with the helicase forming MCM proteins (15). The ING4-HBO1 complex on the other hand is not involved in DNA replication, since siRNA mediated knock-down of ING4 did not affect replication, but instead led to accumulation of cells in G2/M phase (15). These findings imply that ING proteins play an important role in determining MYST protein function (15).
ING3 was found as part of the Tip60 HAT complex, in which it is required for acetylation of chromatin substrates (17). We therefore tested whether subunits of the Tip60 complex also affected PPARγ mediated gene expression. As shown in Figure 3, coexpression of ING3 in PPARγ responsive reporter assays indeed increased PPARγ transcriptional activity. However, Western blot analysis indicated that ING3 coexpression significantly increased PPARγ levels, which probably explains the increase in PPARγ transcriptional activity. Exactly how ING3 modulates PPARγ protein levels remains to be established but may involve increased protein stability or increased activity of the PPARγ promoter. These findings do however show that ING family members are likely to be involved in the regulation of PPARγ function.

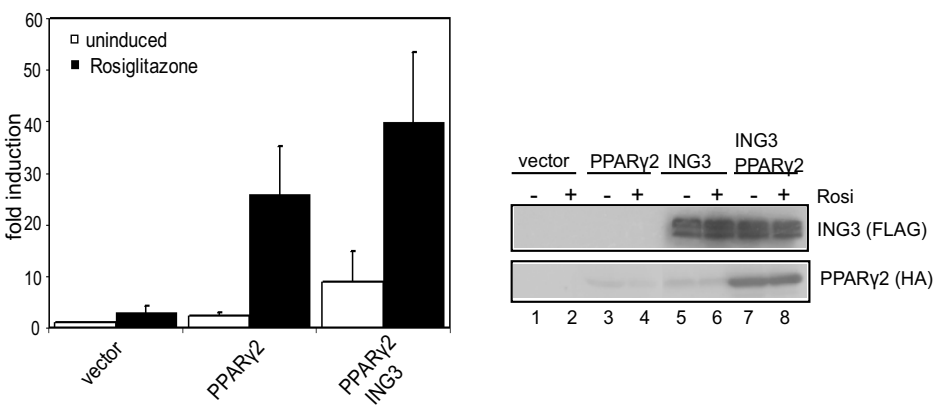


Figure 3. ING3 potentiates PPARγ mediated gene expression and affects PPARγ protein levels. U2OS cells were transfected with an expression vector encoding PPARγ2 wild type (wt) together with ING3. Results of the reporter assay are presented as described in figure 2. Expression of PPARγ2 and ING3 proteins in U2OS cells, as assessed by Western blot analysis using anti-HA or anti-FLAG antibodies.

Nitro-oleic acid, a novel insulin sensitizer?

With the finding that PPM1B is a phosphatase of PPARγ2 (chapter 5) the question arose whether unsaturated fatty acids, as potent stimulators of PPM1B phosphatase activity, could also affect PPARγ activity. As mentioned in chapter 5, analysis of various unsaturated fatty acids has revealed several of the chemical features of compounds that can activate PPM1B phosphatases. These include a chain length of 15 C-atoms and a free negatively charged group. Although nitro-oleic acid has not been described as a PPM1B stimulating compound, it fulfills the afforementioned requirements. Interestingly, nitro-oleic acid was also found to function as a ligand for PPARγ2 and significantly increased glucose uptake in mature adipocytes (18). As one of the most abundant nitroalkene fatty acid derivatives, nitro-oleic acid is present at concentrations of around 0.5μM in serum of healthy induviduals (19). Our preliminary data have indicated a possible involvement of nitro-oleic acid in activating the ligand independent domain of PPARγ (unpublished data). Using a GAL-4 reporter assay we were able to show strong increases of transactivation function when GAL-4 DBD PPARγ-AF1 was coexpressed together with PPM1B (see supplementary data, chapter 5). Addition of rosiglitazone or a nitro-oleic acid derivative, oleic acid, did not show any significant increases in activity. Addition of 1μM of nitro-oleic acid, however, further increased AF-1 transactivation approximately two-fold (unpublished data). Mechanistically nitro-oleic acid could therefore function as a dual agonist, activating both PPARγ2 via the ligand dependent AF-2 domain and via stimulation of PPM1B phosphatase activity resulting in dephosphorylation of PPARγ AF1. Further research is required to establish whether the PPM1B phosphatase forms a putative novel drug target in the treatment of T2DM.

miRNA based siRNA screening

In chapter 6 we described a novel siRNA screening method which may be used for several other applications (Figure 4). For example by replacing the PPRE-TK cassette, which is located in front of the miRNA sequence, with other TF binding sites this method may be used for many other transcription factors. Another possibility could be the use of a Gal4 responsive promoter, which will then offer the possibility of using Gal4-DBD fusion proteins and screen for activators of particular transactivating regions present within transcriptional regulators. A third option is the use of natural promoters which can be cloned in front of the miRNA cassette allowing the identification of activators for a specific promoter of interest. As a final possibility tissue-specific knock-down of genes could be achieved in vivo by generating transgenic mice harbouring tissue specific promoters in front of a specific the miRNA cassette. For now, all these options remain purely hypothetical, but hold the promise of improving our knowledge in this exciting field of gene regulation.

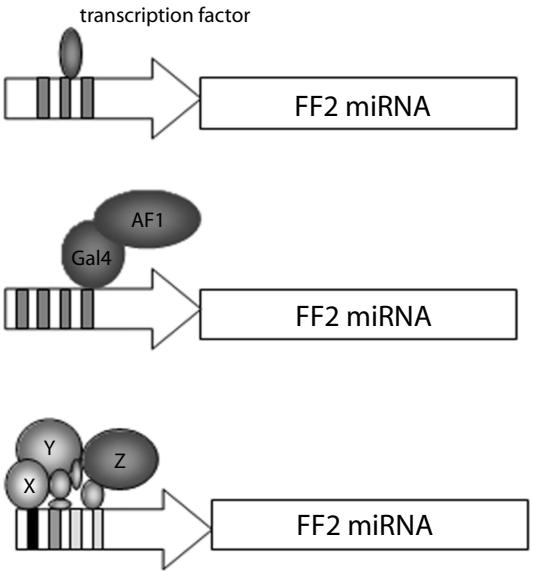
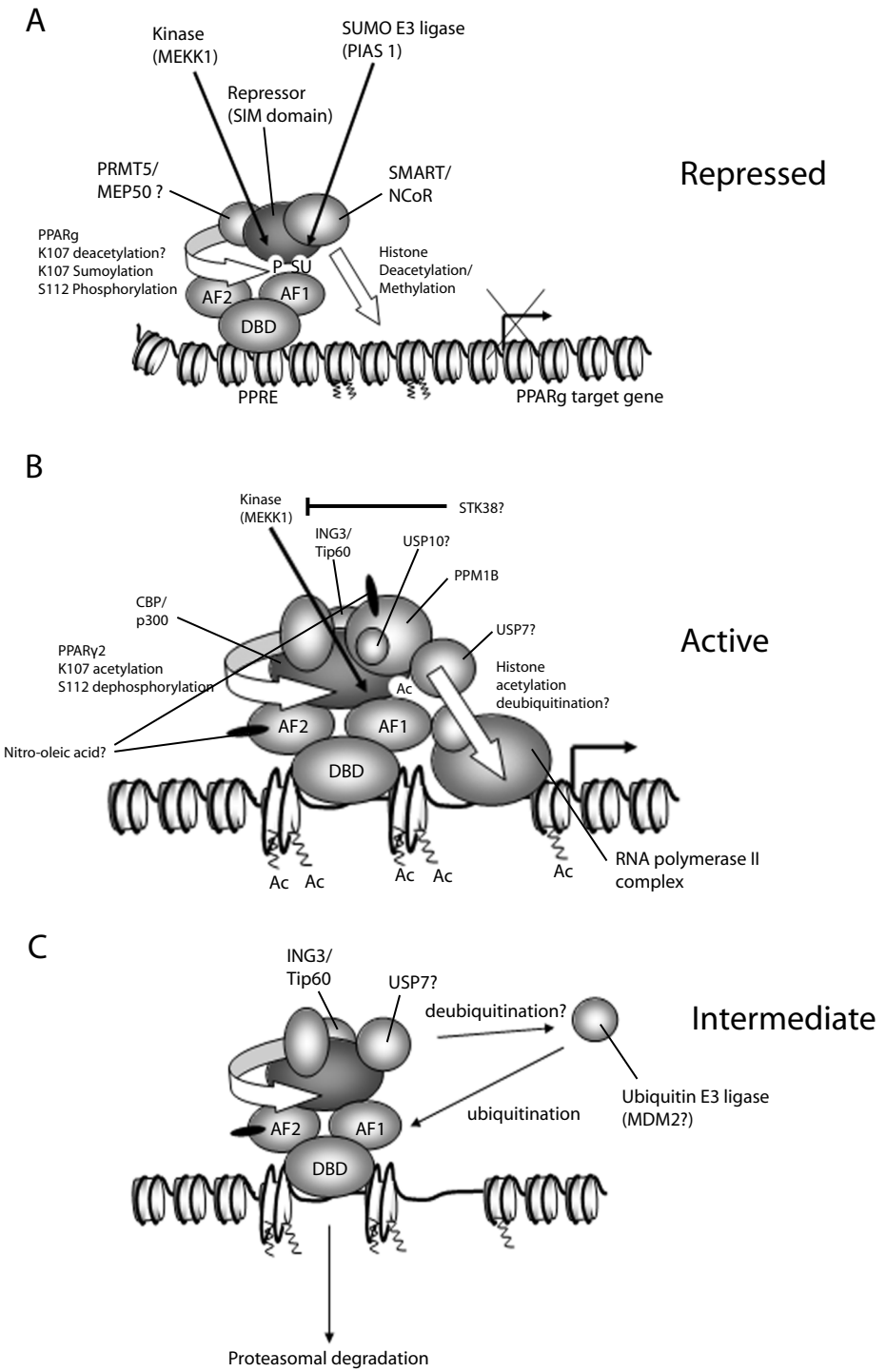


Figure 4. Several other aplications of miRNA based siRNA screening (see text for details).

PPARγ2 regulated gene expression, a hypothetical model

As depicted in Figure 5 a simplified model of PPARγ mediated gene expression can be extracted from the data obtained from mass spectrometry analysis and miRNA based siRNA screening (chapter 4,5 and 6). In a repressed state, PPARγ is phosphorylated at serine 112 which can be mediated by kinases like ERK1 or MEKK1/2(20;21). PPARγ phosphorylation at serine 112 stimulates lysine 107 sumoylation mediated by SUMO E3 ligases like PIAS1 (7;9). These modifications will inhibit ligand binding by via interdomain communication but probably also initiate binding of a putative repressor protein containing a so called SUMO interacting domain (SIM) or WW domain (22;23). Absense of PPARγ ligand also allows the recruitment of the repressor proteins SMRT and NCoR both associating with the AF-2 domain. The methyltransferase PRMT5 and its binding partner MEP50, both identified by mass spectrometry analysis (MS) in our laboratory (unpublished data) have been linked to gene repression through di-methylation of histones H3 and H4 may be involved in PPARγ-mediated transcription in this way (24).
Upon ligand binding conformational changes take place, allowing the formation of a protein docking site around helix 12 present within the ligand binding domain of PPARγ. As discussed above, certain unsaturated fatty acids, like nitro-oleic acid, could function as twofold agonists, activating both PPARγ and PPM1B, a phosphatase found to dephosphorylate PPARγ at serine 112 (also discussed in chapter 5). Furthermore, the serine/threonine kinase STK38 which was also identified by MS

Figure 5. A simplified model of PPARγ mediated gene expression can be extracted from the data obtained from mass spectrometry analysis and miRNA based siRNA screening (see text for details). →


A
Kinase
(MEKK1)
SUMO E3 ligase
(PIAS 1)
Repressor
(SIM domain)
PRMT5/
MEP50 ?
SMART/
NCoR
Repressed
PPARg
K107 deacetylation?
K107 Sumoylation
S112 Phosphorylation
P
SU
Histone
Deacetylation/
Methylation
AF2
AF1
DBD
PPRE
PPARg target gene
B
Kinase
(MEKK1)
STK38?
ING3/
Tip60
USP10?
PPM1B
CBP/
p300
USP7?
Active
PPARγ2
K107 acetylation
S112 dephosphorylation
Ac
Histone
acetylation
deubiquitination?
Nitro-oleic acid?
AF2
AF1
DBD
Ac
Ac
Ac
Ac
Ac
RNA polymerase II
complex
C
ING3/
Tip60
USP7?
deubiquitination?
Intermediate
Ubiquitin E3 ligase
(MDM2?)
ubiquitination
AF2
AF1
DBD
Proteasomal degradation

analysis could indirectly be involved in dephosphorylation of PPARγ by inhibiting the PPARγ kinase MEKK1 (25). The PPARγ repressing proteins are now released and several histone modifying proteins are recruited. Besides general nuclear receptor coactivators like SRC-1 and CBP, also several more specific activating proteins are now thethered towards the PPARγ transcription complex. These may include Tip60, USP10 and USP7. PPARγ itself may at this stage be acetylated at lysine 107 (see also Figure 1), possibly mediated by the acetyltransferases CBP or p300. Lysine 107 acetylation counteracts repressive 107 sumoylation, a mechanism also described for the oncogenes PLAG1 and PLAGL2 (see also chapter 2). Whether Tip60 and PPM1B which both are AF1 interacting proteins, will be recruited at this stage of ligand mediated PPARγ activation remains a matter of debate. It should be noted that so far there are no indications pointing to the presence of PPM1B and Tip60 at endogenous promoters in absence of PPARγ ligand. Furthermore the function of both USP7 and USP10 remains highly speculative: USP10 was recently reported as a coactivator of the androgen receptor but no mechanistic data are available yet. USP7-mediated PPARγ activation could involve deubiquitination of H2B, previously described in mediating gene activation (26). Also, USP7 could initially be involved in stabilizing PPARγ by direct deubiquitination. However, at some point PPARγ should clear the way to allow sequential recruitment of different transcription complexes. This may be achieved by redirecting USP7 deubiquitinating activity towards a ubiquitin E3 ligase, a mechanism previously described for the ubiquitin E3 ligase MDM2 and its substrate p53 (27-29). Deubiquitination of the E3 ligase would result in its stabilisation and hence increased activity. The increased ubiquitin E3 activity could then be directed towards PPARγ and possibly other coactivating proteins, like Tip60, and subsequently target these proteins for proteasomal degradation, thereby clearing the promoter region for subsequent protein complexes.

Final remarks

People are unlikely to cut back on food intake and start exercising more in the near future. This implies the number of obesity-induced type 2 diabetic patients will rise even further in the coming years. Although TZDs may offer many benefits to type 2 diabetic patients, PPARγ ligands often do not achieve a large enough response to eliminate the use of insulin or insulinotrophic drugs and show severe side effects like fluid retention, increased adiposity and possibly CVD risk (30). Therefore further research is required to learn more about the molecular mechanisms involved in the regulation of PPARγ mediated gene activation as these could offer novel ways of treating obesity-induced type 2 diabetes mellitus.

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Nieuwe mechanismen in door PPARγ gemedieerde vetcel differentiatie

Nederlandse samenvatting voor leken

Eten is een van de meest essentiele bezigheden van al het leven en het grootste deel van de tijd is een organisme dan ook kwijt aan het vergaren van voedsel. Bij een groot deel van de mensheid is dit echter sinds ongeveer de laatste honderd jaar anders. In de westerse maatschappij is het door de industrialisatie mogelijk geworden nog maar een fractie van de tijd te besteden aan voedsel vergaring. Hoewel dit natuurlijk grote voordelen biedt we hebben immers tijd om andere dingen te doen schuilt hierin ook een groot gevaar. Voor een groeiend aantal mensen in voornamelijk de westerse landen vormt het gemak van de grote voedsel beschikbaarheid namelijk een gezondheidsprobleem. Door de combinatie van te weinig bewegen en meer eten dan nodig is wordt een groeiend aantal mensen te dik (obesitas).

Obesitas en type 2 diabetes

Obesitas vormt vooral de laatste twee decennia een steeds grotere gezondheidsbedreiging in de westerse wereld, waaraan een nog steeds groter groeiend aantal mensen leidt. Dit is zorgwekkend omdat obesitas aan de oorsprong ligt van een groot aantal ziekten zoals hoge bloeddruk, nierfalen, hartkwalen en kanker. Aan de grondslag van veel van deze ziekten ligt de verhoogte kans op de ontwikkeling van type 2 diabetes. Vroeger kwam type 2 diabetes alleen voor bij ouderen (“ouderdoms suiker”). Tegenwoordig wordt door het groeiende aantal mensen met obesitas type 2 diabetes steeds vaker ook bij jongere mensen geconstateerd. Ongeveer 80% van alle mensen met type 2 diabetes zijn obese.
Diabetes is een chronische ziekte met complicaties die voornamelijk worden veroorzaakt door een verslechterende doorbloeding. Dit zorgt ervoor dat patienten een verminderde doorbloeding van de benen hebben en daardoor een verhoogde kans op het verliezen van een been of voet. Verder hebben diabetes patienten meer kans op nier en hart problemen.
Glucose (suiker) vormt de brandstof voor veel organen en processen in het menselijk lichaam, zo gebruiken de hersenen ongeveer 100 gram suiker per dag. De concentratie glucose in het bloed wordt constant gehouden door het hormoon insuline. Bij een verhoogde glucose (suiker) concentratie in het bloed, als gevolg van voedsel inname, reageert het lichaam met de aanmaak van insuline. Bij een lage concentratie glucose in het bloed door vasten of meer bewegen, zorgt vooral de lever voor de aanmaak en afgifte van glucose. Op deze manier blijft de concentratie glucose in het bloed ongeveer gelijk gedurende verschillende activiteiten. Insuline is een signaal stof die wordt aangemaakt door zogenaamde β cellen in de alvleesklier. De verhoging van insuline wordt herkend door de insuline gevoelige organen: vet-

weefsel, spieren maar met name de lever. Deze organen worden door de insuline aangezet de glucose op te nemen en op te slaan in de vorm van triglycerides, in het vetweefsel, of glycogeen (aan elkaar gekoppelde suiker moleculen), in de lever en spieren.
Type 2 diabetes karakteriseert zich niet door een tekort aan insuline in het bloed zoals bij de veel minder voorkomende type 1 diabetes. Bij type 2 diabetes maakt het lichaam nog steeds insuline maar reageert het lichaam niet of in onvoldoende mate op een verhoging van de insuline concentratie (insuline resistentie). Hierdoor is de glucose concentratie in het bloed van diabetes patienten verhoogt en kan deze ook erg schommelen. Verhoging van de glucose concentratie in het bloed is schadelijk voor het lichaam.

Type 2 diabetes en vetcellen

Waarom ontwikkelen mensen met obesitas, type 2 diabetes? Dat is een vraag waar de wetenschap zich al een groot aantal jaar mee bezighoud en waar nog geen duidelijk antwoord op is te geven. Wel is het duidelijk geworden uit onderzoek dat vetcellen (adipocyten) niet alleen functioneren in de opslag van vet maar ook functioneert als endocrien orgaan. Dit betekent dat vetweefsel signaal stoffen afgeeft, ook wel hormonen genoemd en in het geval van vetweefsel, adipocytokines. Deze adipocytokines functioneren op een andere plaats in het lichaam als een signaal. Zo functioneert het misschien wel meest bekende adipocytokine: leptine, als een signaal naar de hersenen dat we nu genoeg hebben gegeten. Muizen waarbij leptine genetisch is uitgeschakeld stoppen dan ook veel minder snel met eten en worden veel te dik.

Vetcellen en PPARγ

In 1995 is duidelijker geworden wat een belangrijke groep van medicijnen, de thiazolidinedionen, die worden gebruikt bij de behandeling van type 2 diabetes precies doen in het lichaam. Over het algemeen kun je zeggen dat medicijnen functioneren door processen in het lichaam te beinvloeden, dit kan op heel veel verschillende manieren. Van de meeste medicijnen is niet bekend wat het werkingsmechanisme is, dus welk proces precies wordt verandert en is er alleen door uitproberen (empirisch) door de eeuwen heen gevonden dat sommige stoffen helpen bij de genezing van een bepaalde ziekte. Pas de laatste decennia wordt langzaam duidelijk wat medicijnen precies doen in het lichaam. De thiazolidinedionen zijn medicijnen die het lichaam gevoeliger maken voor insuline (“insulin sensitizers”) omdat ze de insuline resistentie verlagen. Thiazolidinedionen doen dit door een eiwit (“machientje” in cellen) genaamd PPARγ te activeren. PPARγ is een eiwit dat wordt geactiveert door een ligand en thiazolidinedionen functioneren als niet natuurlijke (synthetisch) liganden. PPARγ is een transcriptiefactor wat betekent dat het een eiwit is dat genen “aan” en “uit” kan zetten. Een gen is gemaakt van DNA en is simpel gezegd het instructie boekje van een eiwit waaruit een cel op kan maken hoe het eiwit eruit moet zien, welke vorm, wel of niet oplosbaar in water en heel belangrijk wat het moet doen. Een cel kun je zien als een zak met sap met daarin duizenden eiwitten die weer duizenden chemische reacties in de cel vloeiend laten verlopen. Zoals elke cel een functie heeft in het li-

chaam zo heeft elke cel een ander pakket aan eiwitten nodig, een spiercel heeft niets aan eiwiten die betrokken zijn bij het waarnemen van licht of het aanmaken van gal. Zo zal een spiercel dus vooral genen aanzetten (lees instructies lezen) van eiwitten die een cel kunnen laten samentrekken. Elke cel in ons lichaam heeft een volledige uitgave van alle bestaande instructieboekjes in tweevoud. Er zijn naar schatting 30.000 verschillende genen en het goed aflezen van deze genen is dan ook een gecompliceerd proces. Zoals gezegd is PPARγ een van de eiwitten in een cel die bij het aflezen van genen betrokken is. Het PPARγ eiwit is op te delen in drie gedeelten, een deel dat helpt bij het activeren van genen zonder ligand (AF1) een gedeelte dat DNA bindt (DBD) en een gedeelte dat alleen helpt bij het activeren van genen wanneer er ligand gebonden is (AF2).
Activatie van PPARγ leidt er toe dat meer cellen in vetcellen veranderen (differentieren). Dit lijkt tegenstrijdig; mensen met diabetes als gevolg van te dik zijn, worden nog dikker door de medicijnen die ze krijgen. Toch is te verklaren waarom deze medicijnen werken. Mensen met obesitas hebben meer vetten in hun bloed, ook wordt vet opgeslagen in organen waar het niet thuis hoort zoals in de lever en het hart. Doordat onder invloed van thiazolidindionen meer vetcellen worden gevormd krijgt het lichaam de kans vet uit de organen waar het niet hoort weer veilig op te slaan in vetweefsel. Verder is het zo dat door activatie van PPARγ het eiwitten pakket dat normaal voorkomt in vetcellen veranderd. Zo komen er meer eiwitten die er voor zorgen dat vet uit je bloed wordt opgeslagen in een speciaal ingericht gedeelte van de vetcel. Kortom het lichaam is minder uit balans en kan weer beter reageren op insuline.

PPARγ en companen

Bij het aan en uit zetten van genen zijn veel verschillende eiwitten betrokken die alle een andere functie vervullen. Voor PPARγ is dit ook zo en een aantal van deze ''companen'' zijn ook geidentificeerd. Uit onderzoek met muizen is gebleken dat deze eiwitten van groot belang zijn voor een goed functioneren van PPARγ. Zo zijn muizen die genetisch zijn veranderd waardoor ze een van de eiwitten die PPARγ gebruikt om genen aan te zetten mist heel erg mager en ook insuline resistent. Dit is een belangrijke aanwijzing geweest om hier meer onderzoek aan te doen en de projecten die hier zijn beschreven zijn er op gericht om beter te begrijpen welke eiwitten PPARγ gebruikt om genen aan en uit te zetten en welke functie deze eiwitten daarin dan precies hebben.
In hoofdstuk 1 is er gekeken naar wat er gebeurt met het functioneren van PPARγ wanneer er een fout in een van de twee het instructie boekjes (gen mutatie) voor PPARγ zelf staat. Deze fout zorgt ervoor dat PPARγ minder goed functioneerd op een groot aantal vlakken. Zo kan deze “mutant'' minder of bijna niet geactiveerd worden door thiazolidinedionen. Verder kan PPARγ door deze verandering minder goed aan DNA binden en dus genen activeren. Patienten met deze genetische verandering in de genen van hun cellen zijn dan ook (gedeeltelijk) lipodystrofisch wat betekent dat er minder vetweefsel gevormd wordt. In hoofdstuk 2 wordt een nieuw eiwit beschreven genaamd Tip60, dat PPARγ helpt in het activeren van genen. Tip60 bind en activeert het gedeelte van PPARγ dat niet geactiveerd hoeft te worden door ligand. Door het Tip60 eiwit niveau te verlagen in cellen hebben we gevonden dat

Tip60 essentieel is voor de differentiatie van vetcellen. In Hoofstuk 3 staat een ander eiwit beschreven genaamd PPM1B dat ook functioneert als een activator van PPARγ. We hebben gevonden dat PPM1B PPARγ activeert door een modificatie (vlaggetje) weg te halen in dit geval een fosfor groep. Hierdoor wordt PPARγ niet langer geremt in zijn activiteit en is door PPM1B dus activer geworden. PPM1B is hiermee het eerste “fosfatase” voor de groep van nucleaire receptoren verder is interessant dat PPM1B ook kan worden geactiveerd door liganden net als PPARγ. In hoofdstuk 4 wordt een nieuwe techniek (‘’miRNA based siRNA screening’’) beschreven die is bedacht en opgezet om nieuwe eiwitten te vinden die PPARγ activeert. Uit de eerste experimenten met deze methode blijkt dat dit systeem inderdaad gebruikt kan worden voor het identificeren van nieuwe factoren in PPARγ gemedieerde gen activatie. Zo is met deze ‘’screeningsmethode’’ het eiwit USP7 gevonden als regulator van PPARγ activiteit. De toekomst zal uitwijzen of deze techniek ook voor andere transcriptie factoren dan PPARγ gebruikt kan worden.

Conclusies

In dit proefschrift is onderzoek beschreven over PPARγ en de eiwitten die deze transcriptie factor reguleren. Uit onderzoek aan een mutatie in het ligand afhankelijke domein van PPARγ is gebleken dat deze verandering het functioneren van PPARγ op meerder niveaus beinvloed Zo zijn onder andere DNA binding en activator of repressor binding verstoord. Pogingen nieuwe regulatoren van PPARγ te identificeren hebben onder andere de acetyltransferase Tip60 opgelevert. Tip60 is een nieuw gevonden activator van PPARγ die essentieel is voor de vorming van vetcellen. Ook een ander nog niet eerder geidentificeerd eiwit PPM1B is een activator van PPARγ. PPM1B functioneert als een phosphatase van PPARγ en naar alle waarschijnlijkheid delen beide eiwitten in sommige gevallen hetzelfde ligand. Een nieuwe screeningsmethode ‘’miRNA based siRNA screening’’ biedt voor het eerst de mogelijkheid grote siRNA banken te screenen voor nieuwe activatoren van PPARγ. Mogelijk biedt deze screeningsmethode ook mogelijkheden voor het screenen van andere transcriptie factoren. Kortom regulatoren van PPARγ zijn minstens zo interessant en belangrijk als PPARγ zelf. Verder biedt het identificeren en achterhalen van de functie van regulatoren van PPARγ mogelijk nieuwe aangrijpingspunten in het ontwikkelen van nieuwe medicijnen voor de behandeling van type 2 diabetes. Dit is van groot belang aangezien type 2 diabetes een van de grootste bedreigingen vormt van de huidige westerse samenleving.

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Olivier

“Thus grew the tale of Wonderland:
Thus slowly, one by one,
Its quaint events were hammered out
And now the tale is done,
And home we steer, a merry crew,
*Beneath the setting su*n.“

Lewis Carroll, 1865

Curriculum vitae

Olivier van Beekum werd geboren op 1 mei 1975 te Delft. Na het behalen van het VWO diploma op het st. Bonifatius college te Utrecht in 1995, begon hij datzelfde jaar met de studie Biologie aan de Universiteit Utrecht. Tijdens zijn studie werden stages gevolgd bij de afdeling moleculaire plantenfysiologie (Prof. Dr. S.C.M. Smeekens) onder begeleiding van Dr. H. Schluepmann en bij de afdeling Virologie (Prof. Dr. P.J.M. Rottier) onder begeleiding van Gert-Jan Godeke. In augustus 2001 werd het doctoraal examen Biologie behaald. Van oktober 2001 tot augustus 2003 werkte hij als assistent in opleiding op de afdeling Genregulatie (Prof. Dr. C.P. Verrijzer). Vanaf september 2003 is hij opnieuw als assistent in opleiding gestart op de afdeling Metabole en endocriene ziekten (Prof. Dr. R. Berger) onder begeleiding van Dr. E. Kalkhoven. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. In april 2008 zal hij beginnen als postdoctoraal onderzoeker op de afdeling farmacologie en anatomie (Prof. Dr. R.A. Adan) van het Rudolf Magnus instituut te Utrecht. Hij zal hier onderzoek gaan doen aan de cellulaire mechanismen in de hersenen die ten grondslag liggen aan voedingsgedrag.

List of abbreviations

AF1	Activation function 1
AF2	Activation function 2
DBD	DNA binding domain
ERK	Extracellular signal-regulated kinase
FA	Fatty acid
K107	Lysine 107 of human PPARγ2
LBD	Ligand binding domain
NR	Nuclear receptor
OA	Oleic acid
PPAR	Peroxisome proliferator-activated receptor
PPM	Protein phosphatase Mg^{2+} /Mn^{2+} dependent
PPRE	PPAR responsive element
PTP	Protein tyrosine phosphatase
RXR	Retinoid X receptor
S112	Serine 112 of human PPARγ2
TZD	Thiazolidinedione
SIM	SUMO interacting motif
WW	Conserved Trp residues within consensus sequence
SUMO	small ubiquitin-like modification
Tip60	Tat interacting protein 60 kD