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Z. Elizabeth Floyd
Pennington Biomedical Research Center

Jacqueline M. Stephens
Pennington Biomedical Research Center

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Review

Controlling a master switch of adipocyte development and insulin sensitivity: Covalent modifications of PPARγ

Z. Elizabeth Floyd, Jacqueline M. Stephens *

Ubiquitin Biology Laboratory and the Adipocyte Biology Laboratory, Pennington Biomedical Research Center, Louisiana State University Systems, Baton Rouge, LA 70808, USA

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ABSTRACT

Adipocytes are highly specialized cells that play a central role in lipid homeostasis and the maintenance of energy balance. Obesity, an excessive accumulation of adipose tissue, is a major risk factor for the development of Type 2 diabetes mellitus (T2DM), cardiovascular disease, and hypertension. A variety of studies suggest that obesity and T2DM can be linked to a breakdown in the regulatory mechanisms that control the expression and transcriptional activity of PPARγ. PPARγ is a nuclear hormone receptor that functions as a master switch in controlling adipocyte differentiation and development. Also important in controlling glucose homeostasis and insulin sensitivity, PPARγ is a ligand-dependent transcription factor that is the functional receptor for the anti-diabetic thiazolidinediones (TZDs). In the last fifteen years, a variety of covalent modifications of PPARγ activity have been identified and studied. These covalent modifications include phosphorylation, ubiquitylation, O-GlcNAcylation and SUMOylation. Covalent modifications of PPARγ represent key regulatory mechanisms that control both PPARγ protein stability and transcriptional activity. A variety of PPARγ transgenic models, including mice heterozygous for PPARγ, have demonstrated the importance of PPARγ expression in glucose homeostasis and insulin resistance. In the following review, we have highlighted the regulation of PPARγ by covalent modifications, the interplay between these interactions and how these post-translational modifications impact metabolic disease states.

1. Introduction

1.1. Role of PPARγ in disease

Our understanding of adipose tissue biology and the role of adipocytes in obesity-related diseases such as type 2 diabetes (T2DM) has benefited greatly from the discovery that the lipid-activated peroxisome proliferator-activated receptor gamma (PPARγ, NR1C3) nuclear receptor is essential for adipocyte development [1–3]. Subsequent to its discovery as a critical adipogenic transcription factor, PPARγ was identified as the pharmacological target of the insulin-sensitizing thiazolidinediones (TZD) that have been widely used to treat insulin resistance associated with T2DM [4,5]. Since that time, various genetic studies using animal models of tissue-specific PPARγ deletions have shown that activation of PPARγ in adipose tissue is central to the insulin-sensitizing effects of the TZDs. Mice lacking white adipose tissue [6] are not responsive to TZDs and deletion of PPARγ in adipose tissue causes insulin resistance in adipose and liver, but not in skeletal muscle [7]. Inhibiting PPARγ activity in mature adipocytes leads to insulin resistance associated with decreased expression of key genes required for insulin signaling in adipocytes, reduced uptake of free fatty acids by adipocytes and increased lipolysis [8,9]. Gain-of-function experiments show that adipocyte-specific constitutive activation of PPARγ in mature adipocytes can regulate whole body insulin sensitivity [10] without stimulating adipogenesis. Collectively, these observations indicate that PPARγ activation is as important to lipid and glucose metabolism in fully formed adipocytes as it is for adipocyte development.

1.2. Regulation of PPARγ activity by protein–protein interactions and ligand binding

PPARγ is a ligand-activated transcription factor that is expressed in adipocytes as PPARγ2 and PPARγ1; the two forms differing by a thirty amino acid N-terminal extension found in PPARγ2 (refer to Fig. 1). While PPARγ1 is expressed in multiple tissues [11], PPARγ2 is expressed primarily in adipocytes [12]. PPARγ2 is the more adipogenic PPARγ isoform *in vitro* [13] and it is also the isoform transcriptionally regulated by nutrition [11]. PPARγ has an overall domain structure typical of nuclear hormone receptors. This includes an N-terminal activation function-1 (AF-1) domain that functions as a ligand-independent activation domain and confers specificity of target gene activation [14]. PPARγ also contains a DNA binding domain followed by a hinge domain and a large ligand binding domain that contains a short region at the C-terminus called the activation

* Corresponding author at: Louisiana State University, Pennington Biomedical Research Center, Baton Rouge, LA 70808, USA. Tel.: +1 225 763 2648; fax: +1 225 578 2597.
E-mail address: jsteph1@lsu.edu (J.M. Stephens).

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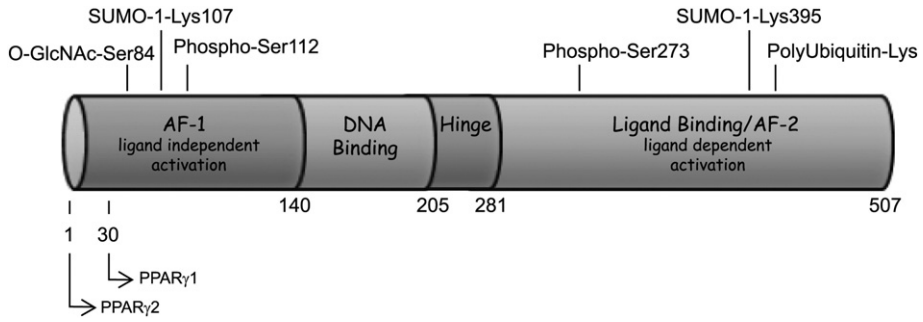

Fig. 1. Schematic of PPARγ2 covalent modifications.

function 2 (AF-2) domain that is responsible for ligand-dependent activation [12] (refer to Fig. 1). PPARγ transcriptional activity is regulated at multiple levels. PPARγ forms a heterodimeric complex with RXRα that is mediated by the PPARγ ligand binding domain. Interaction with RXRα is required for PPARγ to bind the PPARγ responsive element of its target gene promoters. Once bound to DNA, PPARγ activation can be modulated by a large number of coregulators classified as corepressors and coactivators whose association with PPARγ is determined by ligand binding to PPARγ. Corepressor proteins that regulate PPARγ activity include the nuclear receptor corepressor NCoR-1 and its homolog SMRT (silencing mediator for retinoid and thyroid receptors) [15,16] and the receptor interacting protein 140 (RIP 140) [17]. In recent studies of transgenic mice lacking NCoR in adipocytes, it was shown that deletion of this corepressor leads to increased adipogenesis, reduced inflammation, and enhanced systemic insulin sensitivity [18]. These observations are reminiscent of a TZD treatment and support other studies that show transcriptionally active PPARγ promotes insulin sensitivity. In the absence of ligand, PPARγ can be bound to target gene promoters while associated with the nuclear corepressors that recruit chromatin-modifying enzymes to regulate access to the DNA as has been shown in the glycerol kinase promoter [19]. Of note, the most active cis-regulatory elements bound by PPARγ differ between mouse and man [20]. Also, studies in adipocytes and macrophages have demonstrated that PPARγ DNA binding is predominantly cell type specific [21]. Upon ligand binding, the corepressor proteins are replaced by coactivating proteins that promote PPARγ transcriptional activity. A major category of the coactivators is the p160 family of proteins that includes the cAMP response element binding protein (CBP)/p300 and Steroid Receptor Coactivators (SRC)-1,-2,-3, which recruit histone modifiers to the chromatin structure (reviewed in Ref. [22]). A second category of coactivators includes subunits of the mediator complex such as the PPAR-binding protein (PBP)/thyroid hormone receptor-associated protein (TRAP) 220/ vitamin D receptor-associated protein (DRIP) 205 [23–25]. These coactivators interact with the general transcriptional machinery to control assembly of the transcription preinitiator complex [23]. TRAP220/DRIP205, originally cloned as a coactivator of the vitamin D receptor [24], interacts directly with PPARγ [25]. An additional coactivator, PRIP (peroxisome proliferators-activated receptor gamma interacting protein) serves to link TRAP220/DRIP205 bound PPARγ to the CBP/p300 coactivator [26]. These coregulators orchestrate the selective binding of PPARγ to an enormous array of gene promoters.

Our understanding of the genes regulated by PPARγ binding has greatly expanded from the set of genes initially identified as direct targets of this nuclear receptor. Earlier studies established that PPARγ controls the expression of genes that are required for lipid synthesis, transport, and storage [1,27–29] in adipocytes. Recent studies that take advantage of chromatin immunoprecipitation and genome-wide sequencing technology demonstrate the PPARγ/RXRα heterodimer binds to more than 5000 sites, directly activating the expression of a substantial number of genes involved in lipid and glucose metabolism during adipogenesis and in mature adipocytes [30–32]. In a majority of the binding sites, C/EBPα is also present [30,32], in agreement with the accumulated evidence that PPARγ and C/EBPα activities coordinate to form a fully functional insulin sensitive adipocyte. The overlap between PPARγ and C/EBPα binding sites persists in mature adipocytes [32].

1.3. Regulation of PPARγ by covalent modifications

As indicated above, PPARγ can be associated with a large number of proteins. These important protein–protein interactions are noncovalent, but these interactions control PPARγ activity and can be significantly regulated by covalent modifications of PPARγ. PPARγ is known to be modified by phosphorylation, SUMOylation, O-GlcNAcylation, and ubiquitylation (refer to Fig. 1). The multiple covalent modifications of PPARγ increase the levels of control of both PPARγ expression and transcriptional activity that contribute to its ability to modulate adipocyte development and insulin sensitivity.

1.3.1. Phosphorylation of PPARγ

The most well described of these modifications is phosphorylation of PPARγ. Growth factor activation of the mitogen activated protein kinases (MAPK) results in phosphorylation of PPARγ2 in the N-terminal AF-1 domain at serine^{112} (Ser^{82} in PPARγ1) [33–37], the single MAPK consensus recognition site in PPARγ. The first studies on PPARγ phosphorylation were prompted by observations of the MAPK-inducing growth factors including epidermal growth factor (EGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) inhibited adipogenesis. Mutation of serine^{112} to alanine abolishes both the growth factor stimulated phosphorylation of PPARγ at serine^{112} and the inhibitory effect of PDGF and EGF on PPARγ transcriptional activity [35]. *In vitro* studies have demonstrated that the non-phosphorylated Ser^{112}Ala mutant form of PPARγ is more active than wild-type PPARγ in the presence of ligand [33,36,37]. The MAPK-mediated phosphorylation of PPARγ at serine^{112} in the AF-1 domain functions to inhibit PPARγ activity by regulating ligand binding in the C-terminal ligand binding domain of PPARγ [38]. The physiological relevance of PPARγ phosphorylation at serine^{112} was demonstrated in mice homozygous for the Ser^{112}Ala mutation of PPARγ2. Although diet-induced obesity is associated with insulin resistance, elimination of the phosphorylation site protected the Ser^{112}Ala mice from diet-induced insulin resistance even though the mice were obese [39]. Preservation of insulin sensitivity in the obese Ser^{112}Ala mice correlated with higher expression of PPARγ target genes and increased serum levels of adiponectin, an insulin-sensitizing factor secreted exclusively by adipocytes. An additional PPARγ phosphorylation site at serine^{273} in the PPARγ ligand-binding domain that is regulated by Cdk5 [40,41] may have important implications for the treatment of T2DM. Cdk5-dependent phosphorylation is known to play a role in neurobiology and neurodegenerative diseases [42,43], but this kinase is also expressed in non-neuronal tissue, including the insulin secreting pancreatic beta cells [44], and adipocytes [40]. Cdk5 activity

can be regulated by TNF-α [45], a pro-inflammatory cytokine that is up-regulated in adipose tissue in conditions of obesity and insulin resistance [46]. The Cdk5-mediated phosphorylation of PPARγ in adipose tissue has been linked to obesity [40]. Importantly, treatment with thiazolidinediones, the insulin sensitizing drugs that act as high affinity PPARγ ligands, increases PPARγ transcriptional activity and decreases cdk5-dependent phosphorylation of PPARγ2 at serine^{273}. However, Cdk5 phosphorylation at serine^{273} of PPARγ2 does not regulate PPARγ transcriptional activity in general, but does alter the expression of metabolically important target genes in adipocytes such as adiponectin. Moreover, PPARγ ligands that are less potent agonists than the thiazolidinediones, but have insulin-sensitizing effects, also inhibit Cdk5-mediated phosphorylation in PPARγ2. Transgenic mice lacking the corepressor NCoR in adipocytes exhibit improvements in adipogenesis, reduced inflammation, and enhanced systemic insulin sensitivity and a notable decrease in CDK5-mediated PPARγ ser^{273} phosphorylation [18]. These studies suggest that NCoR can function as an adaptor protein to enhance the ability of CDK5 to associate with and phosphorylate PPARγ. Collectively, these studies suggest that phosphorylation of PPARγ2 at serine^{273} by Cdk5 is a key determinant of whole body insulin sensitivity and that new classes of anti-diabetic drugs may emerge that are based on regulating Cdk5-mediated phosphorylation of the PPARγ ligand binding domain. One compound that fits these criteria has recently been described [41].

In addition to modulating PPARγ transcriptional activity, MAPK signaling also affects PPARγ by regulating the subcellular location of PPARγ. These data demonstrate an interaction of MEK1 with the C-terminal AF2 domain of PPARγ, yet this interaction does not directly alter phosphorylation of PPARγ [47,48]. However, there is evidence that cytoplasmic–nuclear shuttling of PPARγ may be regulated by phosphorylation of PPARγ in the AF-1 domain at serines 46 and 51 of PPARγ2 (Ser 16 and Ser 21 of PPARγ1) [49]. In this instance, phosphorylation of PPARγ is mediated by casein kinase II (CK-II) and leads to cytoplasmic localization of PPARγ and decreased PPARγ transcriptional activity. These studies were largely performed in HEK 293 cells and in adipocytes the overall majority of PPARγ is found in the nucleus. Hence, the physiological relevance of these observations is still unclear.

1.3.2. SUMOylation of PPARγ

PPARγ is reversibly modified by SUMO-1 (small ubiquitin-related modifier) at lysine^{107} (lysine^{77} in PPARγ1) and lysine^{395} (lysine^{365} in PPARγ1) [50–53]. First identified in 1995 [54] as a suppressor of a centromere-associated protein, SUMO-1 is an approximately 10 kD protein that regulates protein activity, stability, or cellular location. Although found in the cytoplasm and nucleus, SUMO-1 is best described as regulating a wide range of nuclear functions, including replication, transcription, DNA repair, and cytoplasmic–nuclear protein transport [55]. SUMOylation of PPARγ in the N-terminal AF-1 domain (lysine^{107}) strongly represses PPARγ transcriptional activity, suggesting that SUMO-1 modification of PPARγ at lysine^{107} accounts for the repressive effect of amino acids 100–138 [56]. The potential for "crosstalk" between SUMO-1 modification at lysine^{107} and phosphorylation at serine^{112} within this region is supported by at least one study showing that SUMOylation of PPARγ is impaired in the phosphorylation deficient form of PPARγ2 (Ser^{112}Ala) [52], consistent with the repressive effect of serine^{112} phosphorylation.

SUMOylation-mediated repression of PPARγ transcriptional activity occurs even though only a small percentage of PPARγ protein is modified by SUMO-1 at steady-state. This disproportionate effect of SUMOylation on PPARγ activity is consistent with the effect of SUMOylation on other transcription factors and may be due to SUMO-dependent recruitment of "downstream" cofactors that regulate PPARγ activity. SUMOylation of PPARγ in the AF-1 domain occurs within a sequence specific context found in many nuclear receptors that is termed a "synergy control" motif [57,58]. At promoters containing multiple response elements, complex protein–protein interactions with transcription factors give rise to synergistic interactions that control gene expression patterns [59,60]. The repressive activity of the synergy control motif is determined by SUMO-1 (or SUMO-2) modification of a lysine within the synergy control region and the SUMO-1 modification provides a surface for interactions with transcriptional repressors such as p300 [61] and the histone deacetylases [62]. Evidence of the effect of SUMOylation on the PPARγ AF-1 domain synergy control motif awaits additional experiments to examine the interactions of PPARγ when modified at lysine^{107} by SUMO-1. However, ligand-dependent SUMOylation of PPARγ1 at lysine^{365} (lysine^{395} in PPARγ2) has been shown to promote the interaction of PPARγ with nuclear receptor corepressor (NCoR)-histone deacetylase-3 (HDAC3) complexes in macrophages [53]. This SUMO-1 dependent interaction prevents removal of the NCoR-HDAC3 complexes by proteasomal degradation and maintains repression of PPARγ activity. The physiological relevance of PPARγ SUMOylation has been observed in FGF21 null mice that have alterations in PPARγ signaling. Mice lacking FGF21 are lipodystropic and have less body fat and decreased expression of PPARγ target genes [63]. These transgenic mice also have increased PPARγ SUMOylation and FGF21 is capable of inhibiting PPAR SUMOylation at lysine^{107}. Collectively, these observations suggest that hormones including FGF21 modulate PPARγ transcriptional activity by regulating SUMOylation at lysine^{107} and contribute to whole body insulin sensitivity. To date, no studies have examined the SUMOylation of the PPARγ2 ligand-binding domain in adipocytes. Hopefully, future studies will determine if this modification occurs in adipocytes and if there is any "cross-talk" between SUMO-1 modification at lysine^{395} and Cdk5-dependent phosphorylation at serine^{273}.

1.3.3. Ubiquitylation of PPARγ

SUMO-1 is structurally related to ubiquitin, an 8.5 kD polypeptide that regulates myriad cellular functions ranging from receptor-mediated endocytosis to regulation of transcription when covalently attached to targeted proteins [64]. Proteins modified by multiple ubiquitin polypeptides can be targeted to a number of possible fates, including proteasomal degradation, the best described endpoint for a polyubiquitylated protein. Covalent modification of proteins by ubiquitin occurs *via* a highly regulated cascade of enzymes that catalyze the activation of ubiquitin followed by the transfer of ubiquitin to the targeted protein. The process of activating and transferring ubiquitin to a substrate is repeated multiple times to form the polyubiquitin chains that are recognized by the proteasome.

PPARγ proteins have a short half-life in adipocytes [65] and the turnover rate of PPARγ proteins is regulated by the ubiquitin–proteasome system under ligand-independent and ligand-dependent conditions [66,67]. Ligand binding increases ubiquitin modification of PPARγ and proteasomal degradation of PPARγ and *in vitro* assays indicate that PPARγ is modified by ubiquitin in the ligand binding domain, although the exact site of ubiquitylation has not been identified [66,68]. Although ubiquitylation occurs in the absence of the AF2 domain, the AF2 domain is required for maximal ubiquitin modification of the PPARγ ligand binding domain [68]. This suggests ubiquitylation of PPARγ may precede coactivator binding of the AF-2 domain and that ubiquitin modification of PPARγ functions as an integral part of PPARγ transcriptional activation. The link between ubiquitin modification of PPARγ and activation of PPARγ is further supported by the observations that inhibiting proteasome activity increases PPARγ activity and ubiquitylation in general is required for PPARγ activity in adipocytes [50,68].

The simplest interpretation of the studies on PPARγ ubiquitylation is that ubiquitin modification of PPARγ regulates PPARγ activity by controlling the abundance of PPARγ proteins. Regulation of PPARγ protein steady state levels by promoting ubiquitin modification of PPARγ may represent an important aspect of the action of the insulin

sensitizing thiazolidinediones. Genetic studies using mouse models of insulin resistance show decreased PPARγ gene expression is as effective as thiazolidinedione activation of PPARγ in improving insulin sensitivity [69–71]. The decreased PPARγ gene expression is associated with lower steady state levels of PPARγ protein in adipose tissue [72], lending substantial support to the possibility that modification of PPARγ by the ubiquitin–proteasome in adipocytes is an important regulation of systemic insulin sensitivity that can be exploited in the treatment of type 2 diabetes.

1.3.4. O-GlcNAcylation of PPARγ

A type of glycosylation that occurs on both cytosol and the nuclear proteins is the β-O-linked N-acetylglucosamine (O-GlcNAc) post-translational modification. Recent mutational analysis and mass spectrometry studies have revealed that the threonine^{54} in the AF-1 domain of PPARγ1 (threonine^{84} in PPARγ2) is a major O-GlcNAc site [73]. The transcriptional activity of wild type PPARγ was inhibited in the presence of an inhibitor of this modification, whereas the threonine mutant (Thr^{54}Ala) was unresponsive to inhibitor treatment. Although these studies were conducted *in vitro* in cultured 3T3-L1 adipocytes, the results clearly show that O-GlcNAcylation of PPARγ functions to reduce PPARγ transcriptional activity.

1.3.5. Potential interplay of PPARγ post-translational modifications

The phosphorylation and SUMOylation sites in the AF-1 domain of PPARγ are contained within a highly conserved motif (ψKxExxSP, where ψ is a hydrophobic residue; in PPARγ the sequence is IK`^{107} VEPAS^{112}P) that is considered to be a phosphorylation-dependent SUMOylation motif. Many of the proteins containing this motif are transcription factors, including the nuclear receptors [74]. In PPARγ, phosphorylation and SUMOylation in the AF-1 domain function to repress transcriptional activity. Although phosphorylation within the "phospho-SUMO switch" in other nuclear receptors acts to block SUMOylation of the AF-1 domain [75], the evidence that SUMOylation is impaired in the phosphorylation-deficient form of PPARγ [52] supports the possibility that phosphorylation of serine^{112} regulates SUMOylation of lysine 107 to repress PPARγ activity. In addition, phosphorylation of serine^{112} may affect ubiquitylation of PPARγ since inhibition of MAPK signaling alters the stability of PPARγ and the level of PPARγ ubiquitylation [66,67]. This may also be the case for SUMOylation. While SUMOylation of lysine^{107} can occur in the absence of ubiquitylation, inhibiting SUMO-1 modification of lysine^{107} increases proteasomal degradation of PPARγ [50], suggesting SUMOylation regulates ubiquitin modification of the PPARγ ligand-binding domain. Stabilization of PPARγ protein *via* SUMO-1 modification is consistent with studies that indicate SUMOylation and ubiquitylation have opposing functions, but the interplay between SUMOylation and ubiquitylation in PPARγ may be more complex and involve cooperative as well as antagonistic functions [76].

Phosphorylation, SUMOylation, and ubiquitylation work in concert with acetylation in many transcription factors, including several of the nuclear receptors [77]. PPARγ contains twenty potential acetylation sites [78], although no sites in PPARγ have been definitively identified as modified by acetylation. However, the possibility of acetylation accompanying phosphorylation, SUMOylation, and ubiquitylation in regulating PPARγ activity is intriguing given that four of the potential sites for acetylation overlap or are in close proximity to SUMOylation and phosphorylation sites in the AF-1 (at lysines 107 and 119 of PPARγ2) and ligand binding domain (at lysine 268, 272 and 382). Although speculative, it seems highly likely that acetylation will participate with other covalent modifications in regulating the transcriptional activity of PPARγ.

1.4. The role of PPARγ post-translational modifications in metabolic disease

It appears that all of the PPARγ covalent modifications identified to date including phosphorylation, SUMOylation, O-GlcNAcylation and ubiquitylation largely function to decrease PPARγ transcriptional activity and/or expression levels (see Fig. 2). For over a decade, we have known that PPARγ has been implicated in the regulation of systemic insulin sensitivity. This was first demonstrated when PPARγ was shown to be the functional receptor for the synthetic antidiabetic thiazolidinediones (5). Moreover, direct evidence from genetic studies in humans has revealed that mutations in the ligand-binding domain of PPARγ are associated with severe insulin resistance and T2DM [79]. To date, none of the naturally occurring genetic mutations in humans has been shown to occur at the exact residues in PPARγ where covalent modifications are known to occur. Nonetheless, these post-translational modifications have important

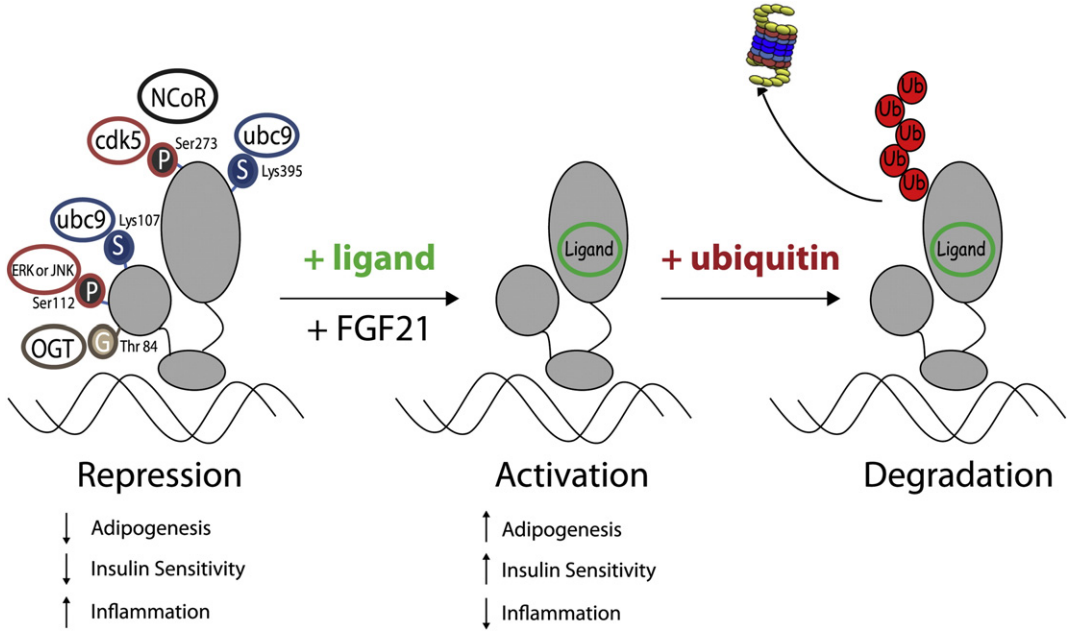

Fig. 2. Covalent modifications of PPARγ are associated with transcriptional repression.

Table 1
Covalent Modifications of PPARγ2.

Modification of PPARγ2	Site	Function
Phosphorylation	Serine 112	General decrease in transcriptional activity
	Serine 273	Decrease in activation of specific target genes
SUMOylation	Lysine 107	Inhibits transcriptional activity
	Lysine 395	Prevents NCoR removal to maintain repression of activity
Ubiquitylation	Ligand binding domain	Targets PPARγ for degradation to reduce protein expression
O-GlcNAcylation	Threonine 84	Inhibits ligand-independent transcriptional activity

effects on PPARγ activity and expression levels. However, a well documented missense mutation in the PPARγ2 genes results in the conversion of proline to glutamine at position 115 and some studies indicate this polymorphism is associated with obesity [80,81]. Ectopic expression of this mutant PPARγ results in decreased serine phosphorylation and increased PPARγ transcriptional activity [80]. Collectively, these studies revealed that the Pro115Gln mutation in PPAR2 enhanced the differentiation of adipocytes and this phenotype was likely a result of decreased serine phosphorylation.

As reviewed above and summarized in Table 1, there are a large number of studies demonstrating how covalent modification of PPARγ plays a role in modulating its activity and expression levels. In the last year, two new PPARγ covalent modifications have been discovered including O-GlcNAcylation and CDK5 mediated serine phosphorylation. Also, FGF21 has recently been identified as a potent modulator of PPARγ SUMOylation [63]. It will be interesting to learn whether other insulin sensitizers act by modulating PPARγ covalent modifications. Transgenic models of PPARγ modulation have shown that the activity and expression of PPARγ play a role in both adipocyte development and insulin sensitivity. Yet, few studies on covalent modification of PPARγ have been performed in the whole animal setting. To date, the best evidence to support that covalent modifications of PPARγ may contribute to metabolic disease states comes from the Lazar laboratory. In these studies, mice containing a PPARγ2 gene that cannot be phosphorylated on serine^{112} developed obesity following high fat feeding but were partially protected from diet induced insulin resistance [34]. Hopefully, future studies will reveal equally important roles for other post translational modification of PPARγ under physiological and/or pathological conditions. The ability to modulate PPARγ activity and improve insulin sensitivity by specifically inhibiting a covalent modification of PPARγ may represent a viable therapeutic option in the treatment of insulin resistance and T2DM.

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