Chapter 21
PPAR Modulation Through Posttranslational Modification Control

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Abstract The peroxisome proliferator-activated receptors (PPAR) are transcription factors modulated by ligands and members of the nuclear receptor superfamily. There are three different human PPAR isotypes: PPARα, PPARδ/β, and PPARγ, which regulate the transcription of their target genes involved with energy metabolism, inflammatory process, and cellular differentiation in different human tissues. Because of these activities, PPARs are considered important targets for drugs to treat metabolic diseases, including diabetes, dyslipidemia, and obesity. Besides ligand modulation, PPARs activities can be modulated by posttranslational modifications (PTM), such as phosphorylation, SUMOylation, ubiquitination, acetylation, and O-GlcNAcylation. The understanding of PTMs modulation of PPARs function could contribute for the development of metabolic diseases treatment with more specificity and fewer side effects. Therefore, in this chapter, we present an overview

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of PTMs that modulate the activity of each PPAR isotype and strategies to modulate these PTMs and thus regulate PPARs action.

Keywords Post translational modification · PPAR modulation · PPAR Phosphorylation · PPAR Acetylation · PPAR Sumoylation · PPAR Ubiquitination

21.1 Introduction

Peroxisome proliferator-activated receptor (PPAR) is a transcription factor included in the nuclear receptor (NR) superfamily, within are included the receptors for steroid hormones, thyroid hormone, lipophilic vitamins, and cholesterol metabolites [10, 179]. All of them have central roles as regulators of energy metabolism, tissue development, and cell differentiation, and most of them binds ligands and modulates gene expression in response to them.

The PPAR structure is highly conserved, a characteristic shared with the other members of the NR superfamily [274], and is composed of six functional regions, named from A to F (Fig. 21.1a). In the N-terminal portion, the A/B region is responsible for transcriptional activity and harbors the activation function 1 (AF-1), a

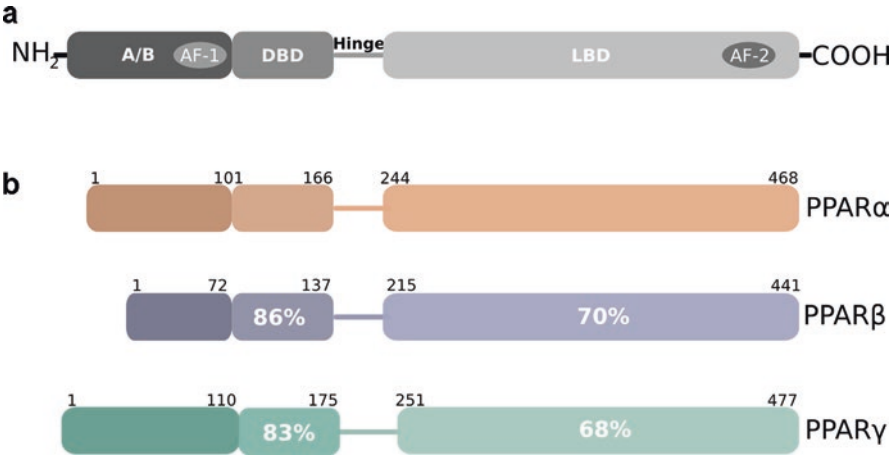


Fig. 21.1 PPARs primary structure. (**a**) The general domain structure of nuclear receptors encompasses four domains. The A/B domain at N-terminal, which contains the ligand-independent activation function 1 (AF-1); the DNA-binding domain (DBD); the hinge region; and the ligand-binding domain (LBD), that contains the ligand-dependent activation function 2 (AF-2). (**b**) The three isoforms of PPARs present different domain lengths. PPARα in orange, PPARβ in purple, and PPARγ in green. The numbers inside each domain correspond to the amino acid sequence identity of human PPARβ and PPARγ relative to PPARα

constitutive activation function independent of ligand binding, which is modulated by PTMs. The C region, also called the DNA-binding domain (DBD), is the most conserved among NRs and consists of two zinc-finger motifs involved in DNA recognition and protein-protein interaction [10, 179]. The DBD recognizes the promotor region of target the genes for peroxisome proliferator response elements (PPREs), formed by six nucleotide sequences with one nucleotide spacer (Direct Repeat 1, AGGTCAnAGGTCA) [179, 274]. The D region is the hinge region, a flexible structure that connects DBD with the ligand-binding domain (LBD), region E/F, in the C-terminal portion of PPAR. This last domain is an essential region responsible for dimerization, where the ligand binding pocket (LBP) and the activation function 2 (AF-2) are present [10, 248].

Although less conserved than the DBD, LBD structure is well conserved compared to all the NR members and is composed of 12 α-helices and 1 β-sheet harboring the LBP [18, 333]. The variation in the LBP residues contributes to the PPARs distinct physiological roles and ligand selectivity among PPAR subtypes [177, 248, 333].

21.1.1 PPAR Isotypes

PPARs are found in three subtypes: PPARα (nuclear receptor subfamily 1, group C, member 1, NR1C1, encoded by the PPARA gene), PPARδ/β (NR1C2, encoded by the PPARD gene), and PPARγ (NR1C3, encoded by the PPARG gene) (Fig. 21.1b) [3, 248]. These three different isotypes mediate the physiological actions of a large variety of fatty acids (FAs) and FA-derived molecules. Despite overlapping roles, each subtype has a distinct role and owns their expression profiles in different tissues, sensitivities to agonists, and regulation of target genes. They play essential roles in energy metabolism; however, they differ in a spectrum of their activity [48, 220].

PPARα The primary function of PPARα is to regulate the expression of genes related to FA oxidation, an activity that is linked to its presence in different tissues [153, 197]. PPARα is highly expressed in high energy requiring tissues, like kidney, liver, brown adipose tissue (BAT), heart, and skeletal muscle, tissues with high levels of mitochondrial and peroxisomal FA catabolism [153, 197, 370]. This isotype is implicated in the lipid regulation through the lipid metabolism control, and its activity is connected to the nutritional (fed and fasted) states [56, 152]. Moreover, this receptor activity is related to inflammation, mainly by limiting inflammatory responses by inhibiting transcription of vascular cell adhesion molecule-1 (VCAM-1), which is essential for leukocyte adhesion and entry into the vessel wall. PPARα also inhibits the secretion of pro-inflammatory cytokines and the nuclear factor kappa B (NF-κβ) signaling pathway [170, 360]. It is important to highlight the liver's PPARα role, where a selective deletion of the receptor was sufficient to promote hepatic steatosis, impairing whole-body FA homeostasis [223].

PPARδ/β This isotype has a broader expression pattern, being found in high levels in tissues related to FA metabolism as the skeletal muscle, adipose tissue, heart, and in gastrointestinal tract, kidney, and skin [14, 220, 232, 310]. Besides roles on FA metabolism, PPARδ/β is involved in suppressing macrophage-derived inflammation, reducing the expression of inflammatory mediators and adhesion molecules [54, 196]. Many studies have already revealed the important role of this receptor on the transcriptional regulation of mitochondrial biogenesis in skeletal muscle, mainly due to the regulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) expression [131, 282]. In addition, it was reported that PPARδ/β is also a key regulator of antioxidant defense and mitochondrial biogenesis in adult heart [337]. In summary, PPARδ/β not only regulates plasma lipid levels through FA oxidation (FAO) in several tissues but also modulates glucose handling in muscle and liver and mitochondrial biogenesis in skeletal muscle and heart [319].

PPARγ The third isotype, PPARγ, is found in two different protein isoforms, PPARγ1 and PPARγ2, which differ from each other by amino acid extension: PPARγ1 lacks the first 30 amino acids due to alternative splicing in mouse (28 amino acids in human) [48, 319]. PPARγ1 is expressed in a wide variety of cells, including the gut, adipose tissues, immune, and brain cells, while PPARγ2 is highly expressed in white adipose tissues (WAT) and BAT [48, 319]. PPARγ is considered a master regulator of adipogenesis and lipid storage, controlling FA uptake and lipogenesis, especially in WAT and BAT [48, 202, 325]. This NR also has an indispensable role in insulin sensitivity and lipid metabolism by forming different transcription complexes with distinct cofactors depending on the physiological condition to regulate a specific set of genes. PPARγ anti-diabetic effects are significantly linked with its anti-inflammatory ones, acting as a suppressor of cytokine release by macrophages and monocytes [209], also inhibiting endothelial cell migration and controlling immune cells differentiation and function [149]. Besides, this NR also acts in controlling the balance between browning of white fat and bone marrow adipogenesis and bone formation mainly by posttranslational modifications, which guides its transcriptional activity to osteogenesis or adipogenesis [202].

21.1.2 Classic Modulation and Activation

To start its activity, all PPAR isotypes form obligate heterodimers with the Retinoic X Receptor (RXR), and a ligand binding induces a conformational change in the receptor, promoting the closure of the LBP entrance by helix 12 repositioning (Fig. 21.2a) [179, 370]. Such change leads to the dissociation of corepressors complexes and the recruitment of coactivators, as CREB binding protein (CBP), steroid receptor coactivator (SRC-1), and PGC1α, promoting the transcription of target genes by binding to the specific PPRE in each promoter region [172] (Fig. 21.2b).

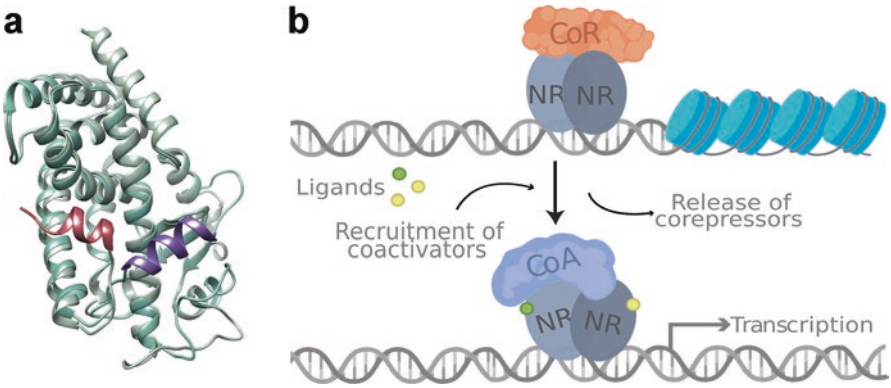


Fig. 21.2 Classical mechanism of action of nuclear receptors. (**a**) Aligned structures of PPARγ-LBD in its agonist (PDB ID: 2prg) and antagonist (PDB ID: 6c5t) conformation; the H12 is highlighted in red for agonist and in purple for the antagonist structure. (**b**) In absence of ligands, the receptors are coupled to corepressor proteins (CoR) that repress transcription. In the presence of ligands, the receptor undergoes a conformational change, main in H12, that leads to the release of corepressors and the recruitment of coactivators (CoA) that activates the transcription of the target gene. (Created with BioRender.com)

In this scenario, coactivators interact with the PPAR-LBD through the LXXLL motif (L, leucine; X, any amino acid), recruiting chromatin modifiers, which act acetylating the nucleosome histones and improving the access of the polymerase machinery to transcript genes [179, 211] (Fig. 21.2b). In contrast, in the absence of a ligand, PPAR-RXR forms a complex with corepressors, as nuclear receptor corepressor 1 (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), blocking transcription of the target genes and keeping it in basal levels [172] (Fig. 21.2b). This mechanism occurs as the corepressors interact with PPAR through the LXXXIXXXL/I motif and are capable of maintaining the chromatin closed by deacetylating it, inhibiting the transcription of the PPAR gene targets [136, 169].

Beyond the mechanism described above, i.e., the classic activation of PPAR/RXR, other action mechanisms have been described for NRs. One example is the formation of atypical heterodimers, which are still not well characterized for PPARs and are limited to certain types of cells or strict physiological conditions, but which can have substantial effects on gene expression [63]. The formation of atypical heterodimers is an example of crosstalk and can be formed either by direct or indirect interaction. Regarding the direct interaction, it involves physical contact between each NR, and one, both, or none of them bind the DNA, involving the participation of other transcription factors [63]. In the case of PPARα, a report of direct

interaction with liver X receptor alpha (LXRα) was shown, in which the atypical heterodimer binds to two directly adjacent hexameric sequences in overlapping PPARα and LXRα response elements, resulting in antagonizing the interaction of PPARα:RXRα or RXRα:LXRα with the murine cytochrome P450 family 7 subfamily A member 1 (*Cyp7a1*) gene promoter [94]. PPARα was also reported to directly interact with glucocorticoid receptor alpha (GRα) by cellular immunoprecipitation and in vitro assays, interfering in GRα gene regulation [30, 269].

In indirect crosstalk, the NR pair has no physical interaction and can affect each other's activity on chromatin by competing for overlapping DNA binding sites, by redistributing common protein partners of the transcriptional machinery, by up- or downregulation of shared coregulators, or by acting as a pioneering factor, facilitating chromatin loosening, and allowing binding of another nuclear receptor [63]. One example of indirect crosstalk is the interaction between PPARα and ERR subfamily members, in which they regulate overlapping pathways [5, 139, 246], and there are some reports of ERRα upregulating PPARα [5, 58, 139, 246]. The relationship of PPARα and GRα can also be described in some cases as indirect interaction, in which reports of sharing control in various steps of the intermediate metabolism and inflammatory pathways signal transduction cascades [184, 264, 302] and of GRα regulating PPARα expression [184, 302].

With all this information in mind, it is clear that the knowledge about modulation of NR is increasing, which is extremely positive on the development of new ligands, which may have a versatile approach by targeting dual receptors and various disorders at the same time [63].

21.1.3 Posttranslational Modulation

Another mechanism of PPAR regulation is mediated by posttranslational modifications (PTMs). The PTM is a covalent attachment of chemical groups to certain amino acids side chains that can lead to a broad spectrum of consequences on the properties of target proteins by modulating their functions [116, 322]. Therefore, PTMs are important regulators of practically every aspect of protein biology, including protein stability, cellular localization, enzyme function, and cofactor interaction [116, 322]. Some examples of PTMs that modulates PPAR are phosphorylation, SUMOylation, ubiquitination, acetylation, and O-GlcNAcylation [8, 34, 332]. In this chapter, we aim to give an overview of research on PTMs present in the PPAR isotypes (PPARα, PPARδ/β, and PPARγ) and their functional roles (Fig. 21.3a, b). Moreover, here we present several mechanisms of how to modulate PTMs and thus regulate PPAR action.

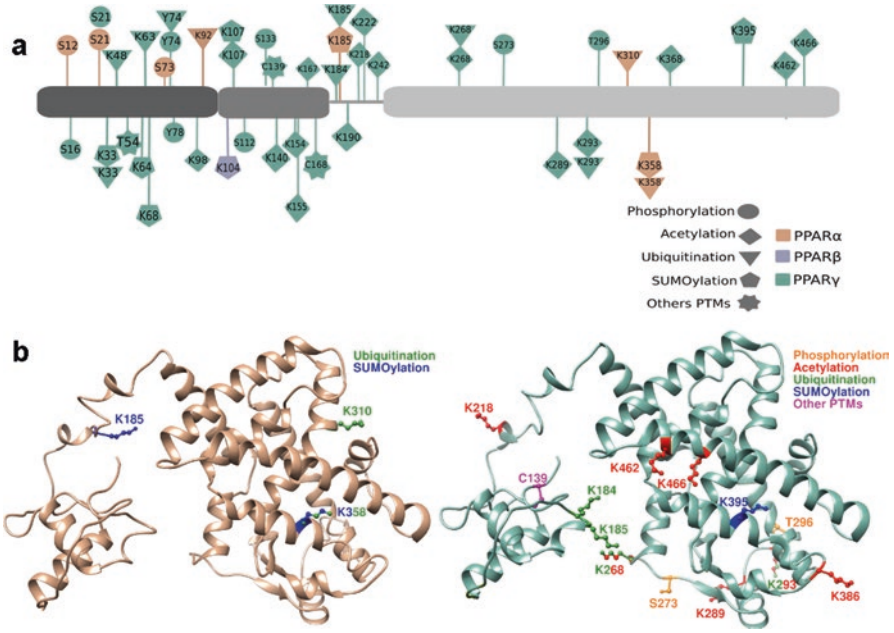
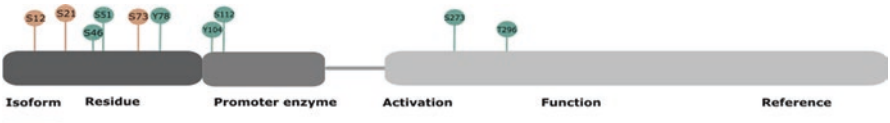


Fig. 21.3 Representation of reported PPARs PTMs. (**a**) Primary structure of PPARs with the identified PTMS in three PPAR isoforms. PTMs without residue specification, such as polyubiquitination, are not showed. (**b**) Structures of PPARα in orange (modeled from PDB ID: 3e00) and PPARγ (PDB ID: 3e00) in green. Residues involved in PTMs had their side chains highlighted in orange for phosphorylation, red for acetylation, green for ubiquitination, blue for SUMOylation, and pink for other PTMs. The structure of the A/B domain at N-terminal is not presented here because this region is intrinsically disordered

21.2 PPARs and Their Posttranslational Modifications (PTM)

21.2.1 Phosphorylation

Protein phosphorylation is the most frequent PTM, a reversible mechanism that occurs through the action of protein kinases, such as cAMP-dependent protein kinase (PKA) and cyclin-dependent kinase 5 (CDK5), which add a phosphate group (PO_4) to the polar group of serine, threonine, and tyrosine. Phosphorylation adds a negative charge to the residue, increasing its size, causing conformational changes that may affect the protein functions [11]. In the case of PPARs α and γ, phosphorylation alters the mechanisms of ligand, DNA, and cofactors binding, affecting their action on insulin sensitivity, inflammation, cancer, and osteogenesis, among others [35, 265, 300, 350] (Fig. 21.4). Up to now, no phosphorylation sites were identified at PPARδ/β.



Isoform	Residue	Promoter enzyme	Activation	Function	Reference
PPARα	S12 and S21	p38 MAPKs or CDKs	↑	Increases coactivation by PGC1 Insulin-induced transactivation Dissociation of corepressors	Barger et al. 2001 Juge-Aubry et al. 1999 Molzer et al. 2016
	S73	GSK3β	↓	Induces hepatic steatosis Increases plasma glucose Increases insulin levels Decreases glycogen storage	Hinds et al. 2016; 2017
PPARγ	S46 and S51*	Casein-kinase II	↓	Cytoplasmatic localization	von Knethen et al. 2010
	Y104*	EGFR kinase	–	Increases cell proliferation and survival Degradation signaling	Xu et al. 2016 Shi et al. 2016
	Y78	c-Src	↑	Anti-inflammatory Insulin sensitivity	Choi et al. 2015
	S112	MAPK, CDK7, CDK9 and ERK	↓	Decreases adiponectin levels Increases FFA levels Insulin resistance Decreases osteoblastic effects Decreases adipocyte differentiation	Hu et al. 1996 Adams et al. 1997 Camp and Tafuri 1997 Shao et al. 1998 Rangwala et ak. 2013 Compe et al. 2005 Iankova et al. 2006 helenius et al. 2009 Pang et al. 2014 Shu et al. 2016 Ge et al. 2018
	S133	ERK or MERK	–	–	Banks et al. 2014
	S273	CDk5 and ERK	↑	Insulin resistance Decreases osteoblastic effects Decreses adipocyte differentiation Glucose intolerance High fasting glucose and insulin levels Decreases diponectin	Choi et al. 2010 Stechschulte et al. 2016 Banks et al. 2014
	T296	CDK5	–	–	Banks et al. 2014

Fig. 21.4 PPARs phosphorylations. (Top) Primary structure with representative phosphorylation in PPARs. (Bottom) Table summarizing identified PPARs phosphorylation sites and their effects. Residues in PPARγ are numbered after γ2 isoform. There is an asterisk for PTM only described for PPARγ1 isoform. Effect on PPAR activation is described as upward arrow for activation and downward arrow for repression

PPARα Three phosphorylation sites were identified in AF-1 of PPARα: S12/S21[17, 147] and S73 [127].

Phosphorylation of S12/S21 in PPARα is targeted by mitogen-activated protein kinases (MAPKs) [17, 147] or by cyclin-dependent kinase 7 (CDK7) of transcription factor II H (DNA-binding domain IIH) complex [55]. These phosphorylations could be stimulated by insulin treatment in human hepatocytes [147]. Functionally, S12/S21 phosphorylations correlate to increased PPARα basal activation, independent of ligand, in rat cardiac myocytes and in human hepatocytes in the presence of insulin, possibly due to decreased corepressor interaction with NCoR or increased interaction with the coactivator PGC1α [17, 147]. In the presence of PPARα ligand, oleic acid, the phosphorylation-promoted activation is even further increased [17, 147]. Phosphorylation-defective mutants (S12A/S21A) are not responsive to p38 MAPK in vitro, in the presence or absence of ligands, confirming that these are the phosphorylation sites for this enzyme [17].

Inhibition of phosphorylation by phosphorylation-defective mutant (S12A and S21A) or by MAPK inhibitor PD98059 decreased the ligand-dependent or insulin-dependent PPARα activation [147], corroborating the hypothesis that the

phosphorylation state modulates PPARα activity. Trichothiodystrophy (TTD) mice, a mouse model, carrying a mutation in the CDK7-containing TFIIH complex, showed lower levels of S12/S21 phosphorylation [55]. This decreased phosphorylation resulted in lower PPARα ligand-induced activity in TTD fibroblast cells, with downregulation of cytochrome P450 4A1 (*Cyp4a1*) and peroxisomal acyl-CoAthioesterase (*Pacoth*) expression, and decreased PPARα recruitment on the CYP4A1 promoter in TTD liver cells [55]. Phosphomimetics (S12E/S21E) showed increased ligand-dependent activation in both native and TDD fibroblasts [55].

Another phosphorylation that regulates PPARα function is at S73 [127], which seems to have an opposite effect of S12/S21 phosphorylations. This PTM is mediated by Glycogen synthase kinase 3β (GSK3β) [127] and not p38 MAPK [17]. In Cos7 green kidney monkey cells, the PPARα activator WY-14643 increased GSK3β concentrations, decreasing PPARα activation, indicating that this phosphorylation decreases the receptor activity [127]. Moreover, phosphorylation-defective mutant (S73A) increased basal activation, and phosphorylation mimetic mutation (S73D) decreased activation in the presence and absence of ligand, corroborating the initial observations [127]. Co-expression of PPARα and GSK3 dramatically increased the ubiquitination of PPARα in Cos7 cells. This ubiquitination was smaller with the phosphorylation-defective mutant, indicating that the receptor's reduced activity after phosphorylation might be due to increased ubiquitination and protein degradation [127].

Liver-specific Biliverdin reductase A (BVRA) knockout (KO) mice, which does not reduce Biliverdin to Bilirubin, had shown increased GSK3β activity and S73 phosphorylation of PPARα, leading to hepatic steatosis, increased plasma glucose and insulin levels, and decreased glycogen storage [127]. In LBVRA-KO, it was also observed a reduction in PPARα activity indicated by a decrease in the expression of several of its target genes in the liver (fibroblast growth factor 21 - *Fgf21*, carnitine palmitoyltransferase I - *Cpt1a*, and fatty acid translocase - *Cd36*). In contrast, a mouse model of human Gilbert's syndrome, a genetic condition that results in moderate hyperbilirubinemia, showed lower S273 phosphorylation levels, along with higher levels of PPARα protein [128]. These animals also showed an improved glucose tolerance, a protective effect against hepatic steatosis, and against insulin resistance, alongside with increased expression of PPARα target genes and increased resistance to metabolic effects of a high-fat diet (HFD) [128]. These two animal models contribute to the hypothesis that phosphorylation modulates PPARα activity in the liver. Bilirubin was reported to also act as a PPARα agonist, increasing receptor activity in high concentration (>50 μM) and upregulating the *Cd36, Cpt1a,* and *Fgf21* in adipocytes and pyruvate dehydrogenase kinase 4 (*Pdk4),* angiopoietin like 4 *(Angptl4)*, and *Fgf21* in liver cells [300]. Furthermore, bilirubin's effect on lowering glucose and reducing body fat percentage was absent in PPARα KO mice [300]. Treatment with bilirubin seems to agree with the hyperbilirubinemia mouse model, where bilirubin's presence favors PPARα activation. More experiments are necessary to confirm if bilirubin is involved in increasing or allowing S73 phosphorylation.

PPARγ Phosphorylations at PPARγ have been reported since 1996, stimulated by insulin and 12–0-tetradecanoylphorbol-13-acetate (TPA) treatment [135, 361] . The first phosphorylation site identified in PPARγ was S112 (or S84 in PPARγ1) [135] which is located at AF-1 domain and is modulated by MAPKs and CDK7 action [122, 135], decreasing PPARγ activity. Specifically, PPARγ1 S84 is phosphorylated in vitro by the MAPKs extracellular signal-regulated kinases 2 (ERK2) and Jun NH2-terminal kinase (JNK), also decreasing the receptor activity [2, 37], without altering its DNA binding activity [37].

Experiments with CDK7 knockdown and PPARγ2 phosphorylation-defective mutants S112A showed that inhibition of phosphorylation leads to increased receptor activity in the presence and absence of rosiglitazone, a PPARγ strong agonist and member of thiazolidinediones (TZDs) family, increasing adipocyte differentiation in vitro [122, 135, 287]. Phosphomimetic mutant S112D showed decreased activity in rosiglitazone's presence, reduced interaction with the coactivator SRC-1, and increased proteolysis [287]. Inhibition of MAP kinase and ERK kinase/ERK (MEK/ERK), reducing S112 phosphorylation, also decreased PPARγ degradation, indicating that phosphorylation at this residue may favor protein stability [83]. Interestingly, CDK9 was also reported to phosphorylate residue S112; however, CDK9-mediated phosphorylation increased PPARγ activity in the presence or absence of rosiglitazone [141]. Pharmaceutical inhibition of CDK9, with DRB, impaired adipocyte differentiation, indicating that CDK9 activity by phosphorylating PPARγ has an opposite effect as MAPK and CDK7-mediate phosphorylation [141].

Concerning the S112 phosphorylation effects (same as PPARγ1 S84), several studies using genetic S112A mutant mice aimed to elucidate this phosphorylation's roles in vivo. Blockage of this phosphorylation with S112A mouse preserved insulin sensitivity on HFD-induced obesity, retrieving smaller fat cells, increased serum adiponectin, and reduced free fatty acid (FFA) levels without increasing body weight [265]. The S112A mice also showed a reduction in bone formation, with decreased osteoblastic activity and increased expression of adipocyte markers: CCAAT/enhancer-binding protein alpha (*Cebpa*), fatty acid-binding protein 4 (*Fabp4*, also called aP2), *Pparg*, and adiponectin (*Adipoq*), revealing its importance on controlling bone mass and marrow adiposity, also affecting energy metabolism [95]. Phosphorylated S112 PPARγ directly interacts with a circadian clock protein, called period circadian protein homolog 2 (PER2), which represses the NR transcriptional activity by blocking its recruiting to target promoters [105]. On the other hand, phosphorylation-defective mutant S112A reduced PER2 binding to PPARγ, and Per2−/− mice cells showed in vitro increased activation of adipogenic genes and brown adipogenic markers [105].

The PPARγ1 S84 phosphorylation was upregulated in a diethylnitrosamine (DEN) mouse model of hepatocellular carcinoma (HCC) and in human liver tumors, respectively. Inhibition of this phosphorylation through phosphorylation-defective mutant S84A or kinase pharmaceutical inhibition (by MEK inhibitor PD0325901) decreased proliferation of human tumoral and normal liver cells [293]. The presence

of this mutation also downregulated genes related to glycolysis and pro-proliferation genes, indicating that S84 phosphorylation may have a role in promoting glycolysis and cell proliferation in hepatocellular carcinoma [293]. Moreover, S84 phosphorylation was reported in another tumor cell line to increase cell proliferation of human fibrosarcoma cells [243]. The PPARγ1/2 (S84 and S112), as well as PPARα (S12/S21), is significantly less phosphorylated in the adipose tissues and liver from the TTD mice model, carrying a mutation in the CDK7-TFIIH complex [55]. Contrary to previous S84 phosphorylation and S112 phosphorylation studies, this decreased phosphorylation in TTD mice was accompanied by decreased PPARγ1/2 ligand-induced activity in TTD fibroblast cells, whereas phosphomimetic mutants (S84E and S112E) showed increased ligand-dependent activation in both native and TDD fibroblasts [55].

The importance of S112 phosphorylation blockage was observed in W1P1 deficient mice [187]. W1P1 is a serine/threonine phosphatase belonging to the protein phosphatase Mg2+/Mn2+ (PPM) family, which plays a critical role in adipogenesis and fat accumulation. W1P1-deficient mice showed impaired body weight growth, decreased fat mass, triglycerides, and leptin levels on circulation. These phosphatase's pro-adipogenic roles were shown to be due to its interaction with PPARγ and dephosphorylation of S112, in vitro and in vivo [187].

Another phosphorylation site, the S273 (or S245 in PPARγ1), was first reported in 2010, and it is one of the most studied posttranslational modifications of PPARγ [45]. This residue is located at the LBD domain of PPARγ and is preferentially phosphorylated by the activated form of CDK5 [45]. However, the MEK/ERK signaling pathway can also be involved in this modification, in which ERK kinase promotes S273 phosphorylation [16]. S273 phosphorylation did not change the basal activity of PPARγ, but its inhibition, for the mutant phosphorylation-defective S273A, increased basal and ligand-dependent activity of this receptor [66]. Moreover, this phosphorylation does not affect DNA binding [45], being the reduced activity explained by increased corepressor recruitment, as shown by the phosphorylation-defective mutant S273A, which presented decreased affinity for the corepressors SMRT and NCoR [66]. In fact, NCoR seems to have a role as an adaptor protein that enhances the ability of CDK5 to associate with and phosphorylate PPARγ.

The phosphorylation of S273 is increased in obesity and has been associated with insulin resistance, occurring mainly in adipose tissues [45]. Studies in vitro confirmed that S273 phosphorylation by CDK5 is related to a scenario of obesity-induced by the tumor necrosis factor alpha (TNFα), mainly due to CDK5 activation through released pro-inflammatory cytokines [45]. It was also shown that reduction of S273 phosphorylation is correlated with pro-osteoclastic activity in vitro, increasing bone turnover through Wnt/β-catenin signaling pathway [301].

In vivo, mice on HFD showed an increased level of S273 phosphorylation, accompanied by insulin resistance and glucose intolerance [45]. S273 phosphorylation was also reported to deregulate genes involved with insulin resistance in vivo, such as *Adipoq*, leptin, and complement factor D (*Cfd*, Adipsin), among others [16, 45].

Phospho-defective (S273A) homozygous PPARγ$^{A/A}$ mice showed no differences in body weight compared to wild type in chow and HFD [108]. Regarding glucose metabolism, on HFD PPARγ$^{A/A}$, mice were as glucose intolerant as wild type; however, they were less insulin resistant. Hyperinsulinemic-euglycemic clamp experiments confirmed an improvement on insulin sensitivity due to an increase in glucose uptake. RNA-seq in epididymal WAT (eWAT) of PPARγA/A mice revealed a downregulation of growth differentiation factor 3 (*Gdf3*), a secreted protein member of transforming growth factor β (TGFβ) family, which was found upregulated in wild-type mice under HFD in both eWAT and inguinal WAT (iWAT), as well as skeletal muscle. Overexpression of GFD3 in vitro decreased glucose uptake in the presence of insulin and in vivo impaired glucose and insulin tolerance tests [108], suggesting the importance of S273 phosphorylation for insulin resistance by the influence of GDF3 factor.

In another mice model, NCoR-KO mice, it was reported a decreased S273 phosphorylation, and PPARγ was found in a constitutive active state, with upregulation of its target genes (*Fabp4*, *Cd36*, solute carrier family 2 member 4 (*Slc2a4*, former *Glut4*, Periplin, long-chain acyl-CoA synthetase 1 - *Acsl1*) in the adipose tissue [189]. These NCoR-KO mice showed enhanced insulin sensitivity, indicating that modulation of S273 phosphorylation has an essential role in insulin resistance and that PPARγ activation, independent of the phosphorylation state, has an adipogenic role [189]. NCoR importance on CDK5-mediated phosphorylation may be revealed during ligand binding to PPARγ. The association of an agonist or non-agonist at the NR LBD may induce conformational changes that dismiss NCoR from the transcriptional complex, decreasing S273 phosphorylation [189]. In addition, it was verified that some PPARγ ligands could block S273 phosphorylation in vitro, promoting an improvement in glucose tolerance and improving insulin sensitivity, as it will be further discussed later in this chapter.

Moreover, in 2020 it was found a phosphatase of protein phosphatase Mg2+- or Mn2+-dependent (PPM) family, called protein phosphatase 1A (PPM1A), that is capable of dephosphorylating S273, restoring the expression of most genes dysregulated by S273 phosphorylation, as adiponectin and CFD [156]. This activity occurs due to the physical interaction of this protein with PPARγ in a phosphorylation-independent manner. PPM1A is positively associated with insulin sensitivity since in vitro assays showed that its expression is decreased when adipocytes are treated with TNFα. In agreement, it was shown that HFD-fed animals have lower expression of this phosphatase, indicating its negative association with S273 phosphorylation and its potential role as a target for obesity and metabolic disorders [156].

PPARγ1 Y74 is another site already described for phosphorylation by epidermal growth factor receptor (EGFR) kinase. This PTM is related to inhibition of the receptor, since Y74 phosphorylation leads to PPARγ1 degradation by murine double minute 2 (MDM2), a ubiquitin ligase system, which recognizes the phosphorylation, destabilizes the receptor, and signalizes for ubiquitin complex PPARγ degradation pathway [350]. This modification occurs more frequently in colonic cancer tissues, being related to its progression and metastasis since the inhibition of

this phosphorylation using a Y74A mutant decreased tumor-associated gene expression (c-MYC proto-oncogene, Ciclo-oxygenase-2 - *COX2*, and interleukin-6 - *IL-6*) and inhibited cell proliferation, colony formation, and antiapoptotic gene expression in human cell culture. These results revealed the importance of Y74 phosphorylation on cell survival and proliferation due to the activation of the EGFR/NF-κβ signaling pathway [350]. Moreover, another study showed that pioglitazone, a known TZD, can block Y74 phosphorylation and consequently inhibit cancer cell chemoresistance by increasing PPARγ protein stability [289].

PPARγ Y78 (Y48 on PPARγ1) was reported as other PPARγ phosphorylation site, being phosphorylated by the proto-oncogene tyrosine-protein kinase (c-SRC) and dephosphorylated by protein tyrosine phosphatase (PTP-1B) [49]. Tyrosine kinase Abelson murine leukemia viral oncogene (c-ABL) was also reported to promote Y78 phosphorylation, once its physical association with PPARγ2 resulted in the receptor phosphorylation on two tyrosine residues (Y78 and Y102) [154]. This phosphorylation promotes the activation of PPARγ, increasing its transcriptional activity, being involved with the suppression of pro-inflammatory cytokines and chemokines expression in adipocytes, also reducing macrophage migration [49]. Pharmacological inhibition of c-SRC kinase raised insulin resistance on obese mice, increasing fasting insulin levels without altering body weight, suggesting that Y78 phosphorylation might have positive effects on controlling insulin and obesity. PPARγ phosphorylation-defective mutants (Y78F) resulted in an increased expression of chemokines and cytokines involved in inflammation in vitro [49].

The other two PPARγ1 phosphorylations, on S16 and S21, are located at AF-1 and are related to the ligand-independent transcriptional activity being the target of casein-kinase II (CK-II) activity under control conditions and promoting a decrease on PPARγ activity. These effects were confirmed using phosphomimetics (S16E/S21E) and phosphorylation-defective mutants (S14A/S21A), demonstrating that CK-II-dependent phosphorylation of PPARγ1 at S16 and S21 provokes its cytosolic localization, impairing this receptor shuttle for the nucleus of the cells, reducing its transcriptional activity in vitro. However, the physiological relevance of these modifications remains unclear [331]. Finally, two other PPARγ sites were identified as targets for phosphorylation: T296 by CDK5 and S133 by MEK/ERK, but their physiological effects are still unknown [16].

21.2.2 Acetylation

Protein acetylation encompasses a transfer of an acetyl group (CH3CO) onto protein lysine residues. However, acetylations on serine, threonine, and histidine residues were also reported, and the acetyl addition can change the protein hydrophobicity, solubility, and surface properties, leading to alterations in the protein physiological effects [50]. Regarding PPARs, this PTM occurs only in PPARγ (Fig. 21.5) and was firstly identified in 2010, being more frequent on lysine residues and promoted by the action of histone acetyltransferases, as CBP and p300 [110,

Isoform	Residue	Promoter enzyme	Function	Reference
PPARγ	K98	CBP	No study about its effects	Qiang et al. 2012
	K107	CBP	No study about its effects	Qiang et al. 2012
	K170*	–	No study about its effects	Tian et al. 2014
	K184 and 185*	–	Lipogenic diferrentiation	Tian et al. 2014
	K197*	–	No study about its effects	Tian et al. 2014
	K218	CBP	No study about its effects	Qiang et al. 2012
	K220*	–	No study about its effects	Tian et al. 2014
	K252*	–	No study about its effects	Tian et al. 2014
	K272*	–	No study about its effects	Tian et al. 2014
	K268 and K293	CBP	Favors lipid storage Insulin resistance	Qiang et al. 2012
	K289	–	No study about its effects	Qiang et al. 2012
	K386	–	No study about its effects	Tian et al. 2014
	K462	–	No study about its effects	Jianget al. 2014
	K466	–	No study about its effects	Jiang et al. 2014
	K184 and 185*	SIRT1	Decrease lipogenic activity	Tian et al. 2014
	K268 and 293	SIRT1	Insulin sesivitivity Increase "browning" gene expression Protec from obesity	Qiang et al. 2012

Fig. 21.5 PPARs acetylations. (Top) primary structure with representative acetylations in PPARs. (Bottom) Table summarizing identified PPARs acetylation sites and their effects. Residues are numbered after PPARγ2 isoform. There is an asterisk for PTM only described for y1 isoform. Effect on PPAR activation is described as upward arrow for activation and downward arrow for repression. In black is described acetylation studies and in red the deacetylation studies

260]. Moreover, this PTM importance was revealed by studies focusing on PPARγ deacetylation, reporting beneficial metabolic effects, as browning and insulin sensitization [34].

PPARγ This PPAR isotype was reported to suffer acetylation by acetyltransferase CBP [260] and p300 [110] and to be deacetylated by NAD-dependent deacetylase sirtuin-1 (SIRT1) [110, 260] and histone deacetylase 3 (HDAC3) [146]. PPARγ acetylation levels were increased in differentiated adipocytes, and the acetylated state was shown to promote activation of the receptor. Inhibition of deacetylation by knockdown or pharmaceutical inhibition of the deacetylase HDAC3 leads to an increased expression of the target genes *Fabp4* and *Adipoq*, increased adipocyte differentiation, and insulin-induced glucose uptake in 3T3 cells [146]. In vivo, HDAC3 inhibitor significantly reduced glucose levels and enhanced insulin sensitivity [146]

In 2004, a report of SIRT1 repressing PPARγ activity described that the deacetylase promoted fat mobilization in white adipocytes by repressing PPARγ, reducing its activity, and reducing fat and triglycerides content during 3T3-L1 differentiation [250]. Despite of this clues, the confirmation that SIRT1 deacetylates PPARγ emerged later [110, 260]. Deletion of Sirt1 from adipocytes led mice to exacerbated insulin resistance, glucose intolerance, and inflammation on short-term HFD feeding. However, these mice fed chronic HFD showed reduced inflammation, improved glucose tolerance, and enhanced insulin sensitivity, relative to wild-type mice [210]. PPARγ acetylation levels increased through HFD in both groups, indicating that this PTM has a role in the metabolic syndrome phenotype [210].

Nine acetylation residues were identified on PPARγ2 by mass spectrometry: lysines 98, 107, 218, 268, 289, 293, 386, 462, and 466 [146, 260, 314]. Native acetylation levels of PPARγ are very low (1%) [314], and the residues were only identified by mass spectroscopy after CBP treatment for acetylation enrichment [260] or PPARγ overexpression in 293 cells [146]. It was the case of K268 and K293 residues, in the helix 2-helix 2′ region of the ligand-binding pocket, which are highly acetylated in obese tissue and were identified after acetylation enrichment with CBP in 293 cells [260]. PPARγ acetylated in both residues interacts with corepressor NCoR in human cells, favors cell proliferation in 3 T3 fibroblasts, and favors lipid storage in adipocytes in vitro and in vivo [260].

K268 and K293 were reported to be deacetylated by SIRT1 [260]. Treatment with rosiglitazone or resveratrol (RSV, SIRT1 activator) also promoted the deacetylation of K268 and K293 by SIRT1 [260]. Deacetylations mimetics promoted expression of "browning" genes (*Ucp1, Cidea, Elovl3, Cox7a1, Pgc1a*) and increased mitochondrial activity in adipocytes under differentiation, whereas acetylation mimetic (K293Q) delayed adipocyte differentiation, failed to induce "brown" genes, and favored expression of "white" genes in adipocytes [260].

In rosiglitazone's presence, the browning effect of deacetylated PPARγ could be explained by its interaction with the brown adipogenic activator PR domain containing 16 (PRDM16). This interaction with the PRDM16 occurs mainly by deacetylated K293 [260]. PPARγ overexpression and Sirt1 gene deletion in mice liver upregulated lipid metabolism pathways as biosynthesis of unsaturated FA, FA metabolism, and FAO in a micro-array screening [314]. On the other hand, SIRT1 gain-of-function in mice promotes "browning" of WAT by deacetylating PPARγ at K268 and K293 [260]. Corroborating the previous findings, another report showed that mice with constitutive deacetylation mutation (K268R/K293R, 2KR) are protected from obesity and its associated comorbidities, through increased energy expenditure and augmented brown remodeling of WAT [171]. These results combined indicate that control of the PPARγ acetylation state could serve as a metabolic switch to regulate lipid metabolism and thermogenesis, where the acetylated receptor increased lipogenesis and the deacetylated receptor favors "browning" of WAT and thermogenesis. With this in mind, selective modulation of PPARγ K268/K293 could have therapeutic importance in obesity and type II diabetes (T2D).

Among the nine acetylation residues identified by mass spectroscopy analysis, K107, a strongly acetylated residue, did not have its acetylation affected by rosiglitazone, indicating that SIRT1 does not deacetylate this residue [260]. This residue was also reported to suffer SUMOylation [84], which indicates potential crosstalk among these two PTM, as will be discussed later in the chapter. Regarding K98, K107, K218, K289, K386, K462, and K466, we did not find reports better characterizing these acetylations in PPARγ2.

Nine other lysine residues were later identified as targets of acetylation in PPARγ1: K140, K154, K167, K188, K190, K222, K238, and K242 in human HEK293 cells [314], of which K188 and K238 correspond to the same site observed in PPARγ2 (respectively, K218 and K268 sites) [260]. K154, one of the lysyl targets identified by mass spectroscopy, is, together with K155, part of a conserved lysine motif (RIHKK) present in PPARγ1 [314]. This motif is present in other evolutionarily related NRs, and it is located just carboxyl-terminal to the zinc finger DBD [314]. Lysines present in this motif were reported to suffer acetylation in estrogen receptor alpha (ERα), androgen receptor (AR), progesterone receptor (PR), and glucocorticoid receptor (GR) [62, 88, 163, 336], strongly suggesting that PPARγ1 K155 could also suffer acetylation. An enzymatic deacetylation assay confirmed K155 deacetylation by SIRT1, and K154/K155 acetylations were confirmed by labeling assay on human HEK293 cells [314].

PPARγ1 acetylation mimetic mutant (K154R/K155R) was very similar to native PPARγ on lipogenic differentiation verified by Oil Red Staining and mRNA expression of multiple lipogenic genes in microarray analysis in ERbB2-positive breast cancer cells [314]. Moreover, acetylation-defective mutants of PPARγ1 showed decreased lipogenic differentiation, protein expression of FAPB4, and mRNA expression of lipogenic genes in a human lineage of breast cancer cells [314]. Besides this, both residues are deacetylated by SIRT1 through enzymatic assay, being the deacetylation inhibited in the presence of nicotinamide (NAM, a SIRT1 inhibitor) [314].

21.2.3 SUMOylation

SUMO (small ubiquitin-related modifier) proteins are <10-kD polypeptides that are bound covalently to the ε-amino group of lysine residues. This process involves a cascade of enzymatic steps that requires an E1 activating enzyme, an E2 conjugating enzyme, and an E3 SUMO ligase [82, 96]. SUMOylation can affect molecular interactions by adding or disguise of surface interactions. In consequence, it can alter the activity, localization, and stability of target proteins. SUMOylation of transcription factors such as NRs frequently is related to inhibition of transcription [96]. On PPARs, SUMOylation predominantly induces negative regulation of target genes (Fig. 21.6).

Isoform	Residue	SUMO	Promoter enzyme	Activation	Function	Reference
PPARα	K185	SUMO-1	PIAS1	↓	Increases NCoR interaction Decreases L-fabp and pkd4 expression	Pourcet et al. 2010
	K358	SUMO-1	–	↓	Increases corepressor interaction	Leuenberger, Pradervand and Wahli 2009
PPARβ	K104	–	SENP2	↑	Increases FAO gene expression	Koo et al. 2015
PPARγ	–	–	PIAS1	–	Decreases chemokines expression	Lu et al. 2013
	K63*	SUMO-2	–	↓	–	Diezko and Suske 2013
	K94*	SUMO-2	–	↓	–	Diezko and Suske 2013
	K98*	SUMO-2	–	↓	–	Diezko and Suske 2013
	K107	SUMO-1/2	PIASxβ	↓	Increases CoR interaction Anti-inflamatory Insulin resistance Adipogenesis	Chung et al. 2011
	K395*	–	PIAS1	↓	Increases CoR interaction Anti-inflamatory	Pascual et al. 2005
	K107	–	SENP2	↑	Increases DNA binding Increses cd36 and pabp3 expression	Chung et al. 2011

Fig. 21.6 PPARs SUMOylations. (Top) Primary structure with representative SUMOylation in PPARs. (Bottom) Table summarizing identified PPARs SUMOylation sites and their effects. Residues are numbered after PPARγ2 isoform. There is an asterisk for PTM only described for y1 isoform. Effect on PPAR activation is described as upward arrow for activation and downward arrow for repression. In black is described SUMOylation studies and in red the deSUMOylation studies

PPARα SUMOylation on PPARα is linked to increased repressive activity by improving corepressor recruitment. Two lysine residues have been reported undergoing this modification: K185 and K358 [185, 257]. In this way, K185 SUMOylation, on the hinge region, downregulates PPARα activity favoring the selective recruitment of the corepressor NCoR [257]. Studies with both COS-7 and HuH-7 cell lines reveals that the presence of proteins related to SUMO enzymatic cascades, such as SUMO-1, SUMO E3, and protein inhibitor of activated STAT1 (PIAS), decreases the transcriptional activity of PPARα and expression of its specific target genes [185]. Cellular assays showed that PPARα ligand GW-7647 blocks this SUMOylation, suggesting that although it does not occur in the receptor's LBD region, it may be ligand-regulated.

The second identified SUMOylation, at K358, leads to a sex-specific and ligand-dependent PPARα repression [185]. K358 SUMOylation in female mice livers

enables PPARα to interact with GA-binding protein (GABP) on the cytochrome P450 family 7 subfamily B member 1 (CYP7B1) promoter via the NR-interacting motif, LKKLL, also recruiting NCoR, HDACs, DNA, and histone methylases, resulting in *Cyp7b1* downregulation [185]. Physiologically, CYP7B1 repression indirectly results in increased testosterone levels and ER activity reduction, which would confer to female mice protection against estrogen-induced toxicity [185]. This effect was reproduced in the male liver using PPARα ligand WY-14643, indicating that the ligand-induced repression was SUMOylation dependent and an agonist-mediated conformational change of the LBD may be necessary for K358 SUMOylation [185].

PPARδ/β This PPAR isotype is the least studied PPAR family member, and the only SUMOylation reported for this isotype is a constitutive one, at K104, which is removed by the SUMO-specific protease 2 (SENP2) [168]. This same protease also acts deSUMOylating PPARγ, and together, these modifications promote the expression of genes involved in FAO, such as carnitinepalmitoyl transferase-1 (*CPT1b*) and *ACSL1* in muscle cells [168].

PPARγ PPARγ conjugation with SUMO proteins commonly results in the negative regulation of its transcriptional activity, either by enhanced transrepression [98, 245] or decreased activation [84, 237, 353]. SUMO-1/2 modification on K107 residue of PPARγ2 (K77 on PPARγ1) is the most studied PPARγ SUMOylation. Through mutational analysis, it was found that inhibited PPARγ K107 SUMOylation can increase the transcriptional activity of the target genes [67, 84, 150, 237, 291, 353]. One possible mechanism that explains this repression is the enhancement of corepressor recruitment by providing a novel interaction site to PPARγ sumoylated [84, 143, 150, 237, 353]. Another possible explanation is that this modification affects PPARγ stability and transcriptional activity, but not its nuclear localization [84].

This repressive state related to corepressor recruitment was found to be important for the anti-inflammatory response. On macrophages, where PPARγ1 acts in the repression of inflammatory responses, K77 (K107 on PPARγ2) SUMOylation triggered by apoptotic cells leads to stabilization of the corepressor NCoR, thereby blocking activation of NF-κβ [143]. In human renal cells, PPARγ ligand-dependent SUMOylation by the PIAS1 inhibits NCoR degradation and NF-κβ activation in lipopolysaccharide (LPS)-stimulated HK-2 cells, also presenting downregulation of chemokines expression [199]. Another SUMOylation site of PIAS1 was identified at residue K365 (K395 on PPARγ2) [245]. In macrophages, this ligand-mediated modification results in repression of the inflammatory response by recruiting PPARγ monomers to NF-κβ and AP1 DNA-binding sites, promoting increased interaction of PPARγ with NCoR and HDAC3, and preventing LPS-induced NCoR degradation [245]. PPARγ agonists also block the activity of the proinflammatory NF-κβ, inhibiting the inflammatory response in macrophages [245].

PPARγ K107 SUMOylation by SUMO-1 can regulate insulin resistance [150], body weight, and adipogenesis [216, 353]. Studies with SUMO-1-null mice demonstrated reduced adipogenesis, resistance to rosiglitazone treatment, decreased weight gain on HFD, and deregulation of PPARγ signaling pathways in adipose tissue [216]. However, SUMOylation-defective (K107R) mutants were able to recover the insulin-sensitizing actions of rosiglitazone without increasing body weight or adiposity [150], presenting increased transactivation [353].

Several studies have reported that the SUMOylation at K107 is regulated by phosphorylation at S112 of PPARγ2. Thus, the lack of phosphorylation at this site promotes K107 SUMOylation, increasing the potency of the SUMOylation repressive effects [291, 353]; however, this correlation is still not clear. Fibroblast growth factor (FGF21), which is a key mediator of the physiologic and pharmacologic actions of PPARγ, was reported to inhibiting the NR SUMOylation at K107 in WAT [71]. The FGF21-KO mice had an increase in K107 SUMOylation, but not in S112 phosphorylation [71]. Additionally, growth differentiation factor 11 (GDF11) can promote the SUMOylation of PPARγ, decreasing its transcription activity in mesenchymal stem cell (MSCs), and in that, this modification occurs without changes in S112 [364]. Interestingly, GDF11 was also reported to induce osteoblastogenesis and to inhibit adipogenesis of MSCs, and these events were supposed to occur via PPARγ modulation through SUMOylation [364].

Moreover, the deSUMOylating at K107 and K104 of PPARγ and β, respectively, were reported to enhance the recruitment of both receptors to the promoter region of their target genes. The SENP2 acts on skeletal muscle, where it selectively increases the expression of some PPARγ target genes (as fatty-acid-binding protein 3 – *Fabp3, Cd36, Cpt1b* and *Acsl1*) [168]. Another SUMO-related mechanism that increases the PPARγ activity is regulated by the E3 ligase PIASxβ/PIAS1 and the SUMO-conjugating enzyme UBC9, which are inhibitors of activated signal transducer and activator of transcription (STAT), leading to the enhancement of the transcriptional activity of PPARγ independent of PPARγ SUMOylation [237].

In addition to the K107, other modification sites can be target by SUMO1 and SUMO2 in PPARγ1: residues K33, K64, and K68 (respectively, K63, K94, and K98 in PPARγ2), and all of them were reported to repress basal and ligand induced PPARγ transactivation when SUMOylated [67]. Besides the K365 (K395 in PPARγ2) SUMOylation in macrophages [245], in adipocytes, this PTM has a role on isoform-specific regulation between PPARγ1 and y2 [12].

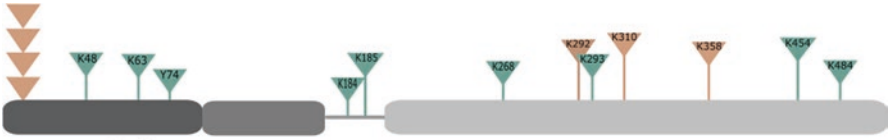
21.2.4 Ubiquitination

Ubiquitination is the covalent coupling of ubiquitin-protein, a 76-amino-acid peptide, to lysine residues in the substrate protein [124, 252]. Through a series of enzymatic processes, ubiquitin can be attached to their substrate proteins as a single

molecule or as polymeric chains in which successive ubiquitin molecules are connected through specific peptide bonds [167, 251].

The ubiquitin-proteasome system (UPS) is an intracellular protein degradation system that regulates the transcriptional activity in different levels [100]; its action goes beyond of the proteolytic role, controlling diverse activities as receptor internalization [312] and ribosome function [297]. The proteasomal degradation of transcription factors is a fundamental step in the fine-tuning regulation of its target genes because this process enables the sequential arrangement of protein complexes at the gene promoter [228].

PPARs ubiquitination regulates the protein content in cells (Fig. 21.7). In most cases, ubiquitination of all isotypes targets them for protein degradation, decreasing receptor activity [97, 102, 157, 158]. Otherwise, treatment with their agonists increases protein stability by inhibiting proteolysis, thereby increasing the receptor



Isoform	Residue	Promoter enzyme	Activation	Function	Reference
PPARα	poliubiquitination	26S proteasome	↓	Degradation	Blanquart et al. 2002
	poliubiquitination A/B domain	MDM21	↓	Degradation	Leuenberger, Pradervand and Wahli 2009
	K292, K310, and K358	MuRF1	–	Inhibits FAO gene expression signalizes cytoplasmatic export	Rodriguez et al. 2015
PPARβ	poliubiquitination	26 S proteasome	–	Degradation	Genini and Catapano 2007
	poliubiquitination	–	–	Degradation	Rieck et al. 2007
PPARγ	poliubiquitination	–	↓	Degradation enhanced by ligand	Hauser et al. 2000
	poliubiquitination	SIAH2	↓	Degradation Adipogenesis	Kilroy et al. 2012
	AF-1 and LBD	–	↑	Degradation	Kilroy et al. 2009
	K48 and K63	NEDD4	–	Decreases stability Promotes adipogenesis	Li et al. 2016
	K63*	Smurf-1	↓	Decreases lipid metabolism gene expression	Zhu et al. 2018
	Y74ph	MDM2	↓	Decreases stability Degradation	Xu et al. 2016
	K184 and K185	MKNR1	↓	Degradation Decreses adipogenesis	Kim et al. 2013
	K268 and K293	CRL4B	–	Decreases stability Decreses adipogenesis	Qiang et al. 2012 Dou et al. 2019
	K454 and K484	pVHL	↓	Degradation Downregulation of ACLY	Noh et al. 2020

Fig. 21.7 PPARs ubiquitination. (Top) Primary structure with representative ubiquitinations in PPARs. (Bottom) Table summarizing identified PPARs ubiquitinations and their effects. When ubiquitination residues were identified, they are numbered after PPARγ2 isoform. There is an asterisk for PTM only described for y1 isoform. Effect on PPAR activation is described as upward arrow for activation and downward arrow for repression

activation [27, 97]. However, some ubiquitin ligases can also increase PPARγ half-life through non-proteolytic ubiquitination, promoting adipogenesis [188].

PPARα Ubiquitination in this isotype was firstly observed in HepG2 cells, in which it was shown that PPARα is degraded by the ubiquitin-proteasome system in a ligand-dependent manner, since the WY-14643 (a PPARα selective agonist) increases the half-life of PPARα, thus protecting the receptor against the ubiquitination [27]. In addition, treatment with MG132, a selective proteasome inhibitor, increases the level of ubiquitinated PPARα and inhibits its degradation [27]. This mechanism allows rapid responses in tightly regulated processes, such PPARα expression in circadian rhythm [183] and acute-phase inflammatory response [20].

MDM2, a ubiquitin ligase, promotes polyubiquitination at the A/B domain of PPARα, regulating its transcriptional activity and promoting its degradation [102]. Furthermore, this process is ligand-dependent, as the increasing concentrations of MDM2, in the presence of WY-14643, leads to decreased PPARα activity.

There is also a report of a mono-ubiquitination of PPARα by the ubiquitin ligase Muscle ring finger-1 (MURF1) in rat cardiomyocytes in vitro [278]. This modification inhibits FAO by inhibiting this isotype activity in cardiomyocytes in a proteasome independent manner, as this single ubiquitination targets PPARα export from the nucleus to the cytoplasm. Residues K292, K310, and K358, located around a newly identified nuclear export signal in the LBD (aa300–308), were identified as putative sites for the mono-ubiquitination [278].

PPARδ/β This isotype can undergo a constitutive polyubiquitination and degradation by 26S proteasome to keep low levels of the receptor in the absence of ligands, despite DNA binding [97]. The presence of PPARδ/β-specific agonists such as L-165041, GW-501516, and prostaglandin (PGI2) completely inhibits PPARδ/β proteolysis, increasing the half-life of the DNA-bound receptor, thus allowing the time for transactivation of target genes. This increase in PPARδ/β half-life can also be achieved in the presence of proteasome inhibitor, such as PS341 [97].

Another level of modulation was revealed by a study that show, in mouse fibroblasts, that the ligand-dependent ubiquitination of PPARδ/β and its subsequent degradation are also influenced by PPARδ/β protein levels [275]. At high PPARδ/β expression levels, the agonist GW-501516 strongly inhibits the receptor ubiquitination and degradation processes, which was not observed at low PPARδ/β levels.

PPARγ The ubiquitination of PPARγ has a role on its stability, as this NR has a short half-life ($t_{1/2}$ = 2 h) [334] and is regulated by ubiquitin proteasome system. The polyubiquitination that marks for degradation usually occurs on AF-2 region [157, 158]. However, the PPARγ activation by ligands, as TZDs, was demonstrated to accelerate the process of ubiquitination and degradation [83, 119]. 3 T3-F442A cells treated with pioglitazone presented increased ubiquitination

levels in a dose-dependent manner and a subsequent decreased in PPARγ2 protein expression [119].

PPARγ degradation by polyubiquitination plays diverse roles in different cell types. In adipocytes, the E3 ligases makorin RING finger protein 1 (MKRN1) [160] and seven in absentia homolog 2 (SIAH2) [157] target PPARγ for proteasomal degradation, determining its physiological effects on adipogenesis. While SIAH2 is required on this process [157], overexpression of MKRN1 inhibits adipocyte differentiation targeting K184 and K185 [160]. It was observed that PPARγ polyubiquitination and degradation by EGFR/MDM2 regulate cancer progression by accumulation of NF-κβ/p65 protein levels and increasing NF-κβ activation [350].

PPARγ polyubiquitination by the ligase complex Von Hippel-Lindau tumor suppressor (pVHL) also leads to the NR proteasomal degradation, being K404 and K434 the potential major ubiquitin acceptor residues in this case [234]. PPARγ degradation via pVHL resulted in the downregulation of ATP citrate lyase protein (ACLY), which is involved on tumor progression and is related to de novo synthesis of lipids, promoting cholesterol synthesis [234].

Despite the proteolytic function of ubiquitination, some ubiquitin ligases can play a role in prolonging PPARγ half-life [188]. In this case, the ubiquitin ligase neural precursor cell-expressed developmentally downregulated 4 (NEDD4) lengthen PPARγ half-life, adding ubiquitin in the hinge (K48 PPARγ2) and in the LBD, stabilizing PPARγ, and promoting adipogenesis in 3 T3-L1 cells, without changing the receptors activity [188]. Another ubiquitin E3 ligase, the tripartite motif protein 23 (TRIM23), has a critical role in the switching from early to late adipogenic function, stabilizing PPARγ protein by atypical polyubiquitin conjugation, that leads to reduced proteasomal degradation [342]. In the liver, the smad ubiquitin regulatory factor 1 (SMURF1) regulates the lipogenic activity of PPARγ attenuating its activity by K63 linked non-proteolytic ubiquitination, leading to hepatocytes protection against nonalcoholic fatty liver disease (NAFLD) [369].

Sites for other covalent modifications were also reported to be sites for ubiquitination. The EGFR-mediated PPARγ Y74 phosphorylation leads to PPARγ ubiquitination and degradation by MDM2 ubiquitin ligase in HEK293 and SW480 [350]. In addition, the targets for acetylation K184/K185 and K268/K293 were reported to suffer ubiquitination, making the protein prone to subsequent proteasomal degradation [69, 160, 260], indicating a possible crosstalk between these PTMs. For example, K184 and K185 are targets for MKRN1 ubiquitin addition, decreasing basal and ligand-dependent activation and targeting PPARγ for protein degradation [160], and K268/K293, for CUL4B-RING E3 ubiquitin ligase (CRL4B), leading to reduced PPARγ stability, as well as adipocyte differentiation (Dou 2019). In this last case, there is one report of aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, acting as the substrate receptor in CRL4B complex [69]. It was observed that after AhR overexpression, PPARγ stability was reduced, as well as adipocyte differentiation. On the other hand, AhR stimulated adipocyte differentiation in 3 T3-L1 cells. These results indicate that AhR could mediate PPARγ activity through posttranslational modifications [69].

21.2.5 Other PTMs in PPARs

21.2.5.1 PPARγ S-Nitrosylation and Nitration

Nitrosative stress occurs with an increase in reactive nitrogen species (RNS) and reactive oxygen species (ROS) formed from oxidative stress. Proteins can suffer two kinds of posttranslational modifications after nitrosative stress: reversible S-nitrosylation of thiol groups and irreversible protein tyrosine nitration [286, 306]. In the case of PPAR, only the isoform γ was shown to be modified by S-nitrosylation and tyrosine nitration (Fig. 21.8).

S-nitrosylation is the reaction of thiols at cysteine residues in the substrate proteins with NO or NO-derived species, resulting in an S-nitrosothiol derivative (RSNO), through a -SNO group formation [125, 306]. This reversible modification is mediated by nitric oxide synthases (NOS), affecting protein activity, protein-protein interactions, and protein location [286, 306]. There are two physiologically relevant denitrosylases to remove NO group from S-nitrosylated Cys thiol side chains: glutathione/S-nitrosoglutathione reductase (GSH/GSNOR) and the thioredoxin/thioredoxin reductase (Trx/TrxR) [125]. PPARγ was first identified to suffer S-nitrosylation in 2003, among S-nitrosylated proteins of activated murine mesangial cells treated with NO donors or appropriate controls [175].

PPARγ Pro-inflammatory macrophage negatively regulated the transcriptional activity of PPARγ in adipocytes by S-nitrosylation of PPARγ1 at the C168 (C198 in isoform γ2), promoted by the release of pro-inflammatory factors like nitric oxide (NO) [358]. This PTM reduced PPARγ ligand-dependent activation in HeLa cells, downregulated PPARγ target genes (*ADIPOQ, FABP4*, and periplin) in 3 T3-L1 adipocytes, and blocked adipogenic differentiation in Rat epididymal preadipocytes and mice 3 T3-L1. This downregulation of PPARγ is due to a decreased binding to the promoters of its target gene, possibly by protein degradation, as 3 T3-L1 cells treated with a NO donor (S-nitrosoglutathione - GSNO) had a decreased level of PPARγ protein. Pretreatment with the proteasome inhibitor MG132 partially prevented the decrease of PPARγ levels, suggesting that the proteasome-dependent degradation might account for the impaired PPARγ stability [358]. These in vitro results agreed with in vivo results showing that obese diabetic db/db mice have severe macrophage infiltration in visceral WAT, while gene expression of NO synthase (iNOS) was increased, and the adiponectin expression was decreased.

A S-nitrosylation in PPARγ2 (C139) was described [38] after observation of the effects of NO in bone-marrow-derived MSCs, precursor cells for adipocytes and osteoblasts [229]. The S-nitrosylation residue (C139) was suggested by the predicted acid-based nitrosylation conservative motif [299] and confirmed through single point mutations [38]. Treatment of HEK-293 T cells with a GSNO decreased PPARγ activity, which was not completely recovered after rosiglitazone treatment. Animal model denitrosylases GSNOR deficient (GSNOR−/−) presented decreased adipogenesis, with smaller adipocytes, lower body weight, and fat mass, with an

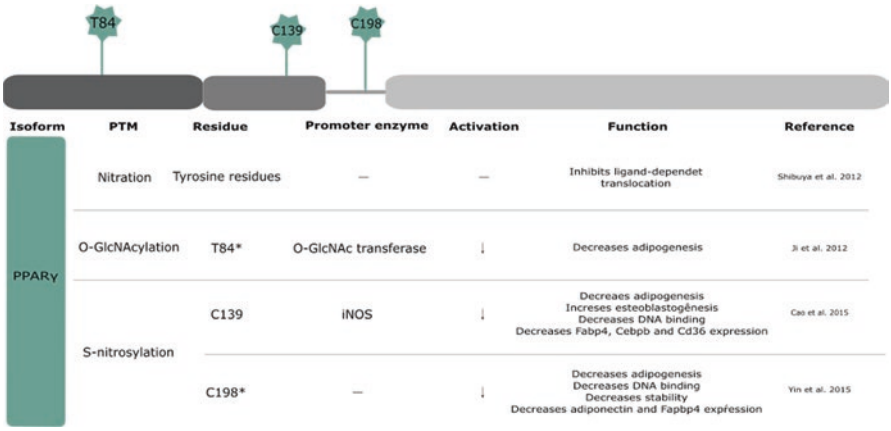
increased proportion of lean mass. Moreover, these animals presented increased osteoblastic differentiation with augmented osteoclastic effects [38]. S-nitrosylation of PPARγ increased 50% in MSCs of GSNOR−/− compared to wild type, suggesting the PPARγ role in the observed effects. In cell culture, GSNOR−/− MSCs showed decreased differentiation and expression of PPARγ target genes involved in adipocyte differentiation (*Cebpb, Fabp4, Cd36*), while adiponectin expression remained the same as wild type. These effects were also observed in wild-type cells treated with GSNOR inhibitor. PPARγ decreased activity observed in reporter gene assays was not followed by a decrease in PPARγ mRNA expression, but it was observed decreased binding affinity by CHIP assays to FABP4 promoter region [38].

Another modification caused by nitrosative stress is the irreversible nitration of tyrosine residues. In this case, the tyrosine amino acid of target proteins reacts with the cytotoxic oxidant peroxynitrite (OONO−), generated from NO and superoxide. This reaction leads to a covalent addition of a nitro group (-NO2) to one of the two equivalent ortho-carbons of the tyrosine residues aromatic ring [286, 306]. This covalent modification affects protein function and structure, including a change in the proteolytic degradation rate and protein activity loss [286]. Nitrosative stress is also present in inflammation; therefore, tyrosine residues' nitration is considered a marker of inflammation [286, 306]. Macrophages are considered key players in inflammation and highly express PPARγ, which also plays a role in the control of inflammation, particularly modulating the production of inflammatory mediators [209]. PPARγ nitration was identified in macrophage-like cell line RAW 264 stimulated by peroxynitrite, LPS, or tumor necrosis factor-K (TNF-K). This nitration inhibits ligand-induced translocation into the nucleus, which might change the PPARγ function [290]; however, the key tyrosine residues that suffer nitration were not identified yet.

21.2.5.2 PPARγ O-GlcNAc

The addition of a single residue of O-linked *N*-acetylglucosamine (O-GlcNAc) is a PTM that occurs in the nuclear and cytosolic compartments of eukaryotic cells [99]. In mammals, this modification is dynamically regulated by two highly conserved enzymes: the glycosyltransferase, named O-linked N-acetylglucosaminyltransferase (GlcNAc transferase, OGT), and the antagonistic enzyme β-*N*-acetylglucosaminidase (O-GlcNAcase, OGA). Analogous to the other PTMs, evidence indicated that O-GlcNAc modification of protein could regulate its activity. O-GlcNAc addition has been mapped to modify serine/threonine (S/T), which are the same sites for phosphorylation addition by kinases, implying that the two modifications might compete for the same site [99].

PPARγ O-GlcNAc modifications were reported in 3 T3-L1 adipocytes [145], by using immunoprecipitation and western blotting techniques (Fig. 21.8). Both PPARγ1 and γ2 were reported to have O-GlcNAc in this cell type, although only PPARγ1O-GlcNAc modification was significantly increased in high glucose condi-



Isoform	PTM	Residue	Promoter enzyme	Activation	Function	Reference
PPARγ	Nitration	Tyrosine residues	–	–	Inhibits ligand-dependet translocation	Shibuya et al. 2012
	O-GlcNAcylation	T84*	O-GlcNAc transferase	↓	Decreases adipogenesis	Ji et al. 2012
	S-nitrosylation	C139	iNOS	↓	Decreaes adipogenesis Increses esteoblastogênesis Decreases DNA binding Decreases Fabp4, Cebpb and Cd36 expression	Cao et al. 2015
		C198*	–	↓	Decreases adipogenesis Decreases DNA binding Decreases stability Decreases adiponectin and Fapbp4 expression	Yin et al. 2015

Fig. 21.8 Others PTMs in PPARs. (Top) Primary structure with representative of other PTMs in PPARs. (Bottom) Table summarizing identified other PTMs (S-nitrosylation, Nitration, O-GlcNAc) sites in PPARs and their effects. Residues are numbered after PPARγ2 isoform. There is an asterisk for PTM only described for y1 isoform. Effect on PPAR activation is described as upward arrow for activation and downward arrow for repression

tions. This modification was not identified for PPARα and PPARδ/β isotypes. Since O-GlcNAc modification for PPARγ2 was not increased under high glucose conditions, this modification was further investigated for the isoform PPARγ1. Protein digestion and single point mutations assays showed that the residue which suffers O-GlcNAc-modification PPARγ1 is T54, present in the AF-1 domain. Through a reporter gene assay in HeLa cells, using T54A mutant protein and OGA inhibitor, it was demonstrated that the presence of O-GlcNAc reduces PPARγ1 activation, which is not rescued by rosiglitazone treatment [145]. Regarding physiological conditions, it was previously described that a general increase in O-GlcNAc is observed in adipocyte differentiation and that inhibition of this PTM decreases adipogenesis [134, 142]. Treatment of 3 T3-L1 adipocytes with OGA inhibitor resulted in more O-GlcNAc modifications of PPARγ1 and reduced transcriptional activity in this cell type, indicating that, although proteins level of PPARγ1 targets in adipocyte differentiation were not changed, the decrease in adipocyte differentiation might be due to the decrease in this receptor activity through O-GlcNAc modification [145].

21.3 Crosstalk of PPARγ PTMs

Reports of crosstalk are usually found between proteins of a signaling cascade; however, PTM crosstalk can occur within a single protein [60, 327]. In general, the PTM crosstalk can be classified into two forms: positive or negative [327]. In positive crosstalk, one PTM can signalize for the addition of a second PTM at the same site or for recognition by a binding protein that carries out a second modification

(e.g., phosphorylation-dependent SUMOylation) [304, 357]. In this second case, the first PTM can induce conformational changes in the protein that enable access for the protein effector of the second PTM. In negative crosstalk, direct competition for modifying a single residue in a protein can occur, or indirectly by masking the recognition site for a second PTM [327]. An example of direct negative crosstalk is the addition of O-GlcNAc mapped to modify serine/threonine (S/T), which are the same sites for phosphorylation modifications by kinases [99, 115, 341]. Furthermore, lysine residues were reported to suffer not only acetylation but SUMOylation and ubiquitination as well, where the latter plays an important role signaling for protein degradation by proteasome pathway and the former is important for regulating cellular processes, including cell cycle, apoptosis, DNA repair, and signal transduction pathways [120].

In the case of PPARγ, direct negative crosstalk was reported in many sites that are a target for different modifications, for example, lysyl residues, which are both targets for acetylation and SUMOylation or ubiquitination. Some positive PTM crosstalks were also reported, such as acetylation, which may induce phosphorylation, phosphorylation-mediated SUMOylation, and ubiquitination. These combinatorial actions of PTMs provide a fine-tuning mechanism in regulating protein function. So far, no PTM crosstalk was identified for the other PPAR isotypes.

21.3.1 Negative Crosstalk at PPARγ K184/K185 and K268/K293 Which Are Shared Residues for Acetylation and Ubiquitination

Examples of direct negative crosstalk that might occur at PPARγ2 K184/K185 are acetylation and ubiquitination. Acetylation in PPARγ1 K154/K155 (which corresponds to PPARγ2 K184/K185) was described, and its acetylation mimetics mutants (K154Q/K155Q) were very similar to wild type in lipogenic differentiation, whereas acetylation-defective (K154R/K155R) mutants decreased lipogenic differentiation, indicating repression of PPARγ1 activity [314]. MKNR1 ubiquitination of these residues in PPARγ2 K184/K185 also reduced basal and ligand-dependent activation, targeting the receptor for proteasome degradation [160]. These results allow us to speculate that the deacetylation of K154/K155 allows for PPARγ1 ubiquitination, targeting for proteasome degradation and reducing protein activity; however, this hypothesis still needs to be addressed experimentally.

Another pair of acetylated residues, PPARγ2 K268/K293, is upregulated in obesity and was reported to favor lipid storage in adipocytes and cell proliferation, in agreement with acetylation mimetics mutants (K268Q/K293Q), increasing expression of insulin-resistant genes on WAT of HFD-obese mice [260]. Deacetylation of K268 and K293 residues led to the browning of WAT and repression of insulin resistance and adipogenic genes [260], in agreement with PPARγ1 K154/K155 deacetylation results of decreased lipogenic differentiation and decreased PPAR

activation with consequent protein degradation [160]. Corroborating the hypothesis of K268/K293 being shared residues for acetylation and ubiquitination, one report of ubiquitin-mediated degradation of AhR transcription factor shows the same lysine residues acting as the substrate and receptor for CRL4B E3 ligase.

Taken together, these reports suggest that PPARγ deacetylation in K184/K185 and K268/K293 pairs both allows ubiquitination and targets for protein degradation, reducing lipogenic differentiation.

21.3.2 Negative Crosstalk at PPARγ K107, Which Is a Shared Residue for SUMOylation and Acetylation

PPARγ2 K107 is a shared residue for SUMOylation and acetylation. This residue's SUMO modification is the most studied PPAR SUMOylation and strongly represses PPARγ, repressing inflammatory genes in macrophages [143]. DeSUMOylating of this residue was shown to increase the receptor activity, increasing expression of some PPARγ target genes, such as *Fabp3* and *Cd36*, both in the absence and presence of rosiglitazone, in rat C2C12 myotubes [51]. K107 was strongly acetylated in a mass spectrometry analysis of 3 T3-L1; however, no studies were done about its physiologic effects [260]. Based on the effects of acetylation in other PPARγ residues (K154/K155, K268/K293), acetylated mimetics mutants of PPARγ had effects very similar to native, whereas deacetylation of these residues was shown to decrease lipogenic differentiation, and to promote expression of "browning" genes and adiponectin in WAT [260, 314]. Extrapolating to K107 deacetylation, its effect could also be of decreased lipogenic differentiation, which would agree with SUMOylation effects of PPARγ repression, suggesting that when K107 is deacetylated, it could be SUMOylated. Although it is clear that this same residue can suffer two types of PTM, the molecular basis of this alternance is not determined yet. It will be of interest to determine if there is a reciprocal regulation of acetylation and SUMOylation at this site during browning or adipogenesis.

21.3.3 Positive Crosstalk of PPARγ1 T74 and PPARα S73 Phosphorylation-Dependent Ubiquitination

PPARγ1 T74 and PPARα S73 were reported to be target of phosphorylation-dependent ubiquitination, signaling for protein degradation [102, 350]. Y74 phosphorylation occurs more frequently in colonic cancer tissues, where PPARγ phosphorylation leads to ubiquitination by MDM2, signaling for protein degradation. Y74 phosphorylation seems important to colonic cancer cell survival and proliferation since Y74A mutants decreased tumor-associated gene expression (c-MYC,

COX-2, and IL-6), inhibited cell proliferation, colony formation, and anti-apoptotic gene expression [350].

In the case of PPARα S73 phosphorylation, a BVRA-KO mouse model addressed this phosphorylation's effects in the liver. In the BVRA-KO mice, upregulation of S73 phosphorylation was observed together with hepatic steatosis and decreased expression of PPARα target genes in the liver (*Fgf21, Cpt1a, Cd26*), suggesting that the phosphorylation decreased PPARα activity [127]. Lower levels of ubiquitination observed with S73A PPARα indicates that the reduced activity of the receptor after phosphorylation might be due to increased ubiquitination and protein degradation [127].

21.3.4 Positive Crosstalk of PPARγ K268/K293 Acetylations and S273 Phosphorylation

In PPARγ, K268/K293 acetylation might be an example of positive crosstalk, whereas the presence of a modification induces the occurrence of a second one. S273 phosphorylation was reported to correlate with K268 and K293 acetylation because this phosphorylation was increased in the presence of K293 acetylation mimetic mutant (K293Q) [260]. Both modifications were reported independently to have overlapping effects in adipogenesis and browning: deacetylation mimetics mutants (K268R/K293R) and S273 phosphorylation-inhibition by rosiglitazone increased expression of adipokine and "brown genes" in WAT [45, 236, 260]. However, phospho-defective mutation (S273A) alone did not promote upregulation of "brown genes", whereas the combined deacetylation and dephosphorylation mimetic (S273A/K268R/K293R) had a similar result as the deacetylation mimetic (K268R/K293R) [260]. This upregulation of "brown genes" in both deacetylation mimetics was more pronounced together with rosiglitazone treatment, [260], indicating that PPARγ activation and deacetylation have a combined effect increasing PPARγ browning activity.

These residues' structural proximity might explain this positive crosstalk among S273 phosphorylation and K268/K293 acetylation. S273 is buried within the grooved, lined by K268 and K293, and it was suggested that the acetylation state of these two residues could induce conformational changes in the protein structure, which affect access to related kinases or phosphatases, to the phosphorylation site of S273 [260]. In some cases, deacetylation of proteins was reported to increase their phosphorylation [258, 259]; however, in this case, it was suggested that mainly K293 acetylation would induce conformational changes allowing S273 phosphorylation. This hypothesis is addressed because K293 acetylation mimetic mutant was reported to increase S273 phosphorylation and their deacetylated and dephosphorylated states were shown to have the same effects inducing browning of WAT and decreasing lipogenesis [260].

Nevertheless, another work showed that deletion of SIRT1 from adipocytes leads to increased PPARγ acetylation under short-term (5 weeks) and long-term (15 weeks) HFD, but S273 phosphorylation levels, which were higher in the SIRT1-KO animals, decreased after long-term HFD compared to wild-type animals [210]. These results indicate that more research is necessary to understand the fine-tuning modulation of PTM crosstalk regulating PPARγ functions in metabolic syndrome.

21.3.5 Positive of Crosstalk of PPARγ S112 Phosphorylation-Dependent SUMOylation of K107

A phosphorylation-dependent SUMOylation motif (PDSM) ΨKxExxSP has been identified in NRs, including PPARγ and thyroid hormone receptor (TRβ) [126]. In PPARγ2, S112 phosphorylation promoted a phosphorylation-dependent SUMOylation of K107, as demonstrated by decreased SUMOylation in the presence of phosphorylation defective mimetic S273A [353]. S112 and K107 are close in the PDSM, and the proposed model is that S112 phosphorylation induces a conformational change in PPARγ that allows for the SUMO-1 SUMOylation of K107 [323]. Both phosphorylation and SUMOylation at these residues cause a decrease in the receptor activity, with impaired coactivator and increased corepressor recruitment, and activation of the adipogenic gene expression pathway [122, 135, 150, 287, 291, 353]. The blockage of these PTMs resulted in the improvement of insulin sensitivity [150, 265].

21.4 Modulation of PPARs PTMs

Modulations of PPAR posttranslational modifications (PTMs) were reported in the literature in two major ways. The most common is through the use of a PPAR ligand that binds in the LBD and induces conformational changes that allow or decrease the occurrence of a PTM. Another way described in the literature for PTM characterization is using an activator or inhibitor of the protein responsible for the PTM addition. However, as will be discussed further in this topic, kinases, acetylases, and other proteins involved in the addition of a posttranslational modification at PPARs are also involved in modulating other pathways. Therefore, pharmaceutical inhibition or activation of these effectors seems not to be the best strategy for the specific modulation of PPARs PTMs.

On the other hand, the use of PPARs LBD ligands to inhibit or promote a specific PTM could lead to undesired side effects resulting in receptor activation or inhibition, for example, the use of rosiglitazone for the inhibition of PPARγ S273 phosphorylation. Aiming to block the S273 phosphorylation effect of insulin resistance,

the agonist rosiglitazone activates the receptor, leading to adipogenic and osteoclastic effects. This constant activation of PPARγ by rosiglitazone in diabetic patients resulted in the side effects of weight gain and bone loss, which resulted in the removal of the drug in the treatment of T2D. In this topic, we are going to present the molecules that can regulate the addition of PTM in PPARs, either by promoting structural changes in the receptor LBD or by activating/inhibiting the effectors proteins of these modifications. Moreover, we are going to discuss the positive and negative aspects of each modulation, discoursing about the perspectives of the clinical use of molecules to modulate PPAR function through PTM regulation.

21.4.1 Modulation by PPAR Ligands

In this section, we are going to focus on ligands that bind in the LBP of the PPAR-LBD and promote conformational changes, interfering directly or indirectly in the PTMs occurrence. The binding site cavity for the three PPAR isotypes is very similar and is located in their protein cores [75, 254]. The Y-shaped LBP is mainly formed by hydrophobic residues and presents a large volume of ~1300 Å3, which allows the interaction of single and multiple branched ligands in different conformations [59]. This pocket is flanked by helix (H) 3, 5, 7, and 10 and by an antiparallel beta-sheet. The space between H3 and beta-sheet is the ligand-entry site, whereby different ligands can access the PPAR LBP, promoting structural changes mostly in H12.

21.4.1.1 PPARα

In PPARα, three PTMs seem to have their presence regulated by PPAR ligand: a polyubiquitination enhanced by WY-14643 [102]; a K185 SUMOylation, in the hinge domain, reduced in the presence of GW-7647 [257]; and K358 SUMOylation, in the LBD, increased in the presence of WY-14643[185]. No sites for the polyubiquitination by MDM2 were identified, but this modification at the A/B domain of PPARα decreased its basal activity, promoting its degradation, and this process was enhanced by WY-14643 [102].

Both SUMOylations promoted repression of PPARα, as defective mutations of K165A and K358A increased the receptor activity compared to the native protein. This inhibited activity could be explained by increased recruitment of NCoR in the case of K165 and GA-binding proteins and histone deacetylases in K385 [185, 257]. However, these two SUMOylations differ regarding the effect of PPARα agonists. Treatment with GW-7647 reduced specifically PPARα SUMOylation; however, it is not clear if ligand binding impairs the SUMOylation of PPARα or promotes its deSUMOylation [257]. On the other hand, treatment with WY-14643 increased PPARα SUMOylation because the agonist induces conformational changes in the LBD, in which K358 is presented at the surface and therefore available for

SUMOylation, in contrast to the antagonist-induced conformation, in which case K358 is hidden [185]. This agonist-induced conformational change that exposes K358 for acetylation might also hide K165, protecting it from SUMOylation.

Regarding the others PPARα PTMs, ligands such as oleic acid and WY-14643 were reported only to modulate the activity of the receptor bearing phosphorylations at S12/S21 and S73, and studies analyzing the effects of the ligands in the modification per se are still required [17, 147, 300]. S12/S21 phosphorylation increased basal PPARα transactivation, which was enhanced in the presence of the ligand oleic acid or insulin [17, 147]. Downregulation of these phosphorylations was observed in TTD mice, which showed downregulation of *Cyp4a1* and *Pacoth* in the liver [55]. On the other hand, phosphorylation of S73 was reported to decrease PPARα activation induced by ligand WY-14643 [127]. Downregulation of *Cyp4a12* and *Cpt1a* was observed in BVRA KO mice bearing increased S73 phosphorylation [127]. These results indicate that S12/S21 and S73 phosphorylations have antagonist effects in hepatocytes.

However, since S12/S21 and S73 phosphorylations are in the AF-1 domain, an activation domain independent of ligand, it might be improbable that ligands bound to the LBD could induce conformational changes to inhibit or increase the occurrence of phosphorylation on AF-1 domain. On the other hand, conformational modifications or other allosteric-like effects might happen, but further studies in this field are necessary to elucidate these mechanisms.

21.4.1.2 PPARδ/β

Until now, few PTMs are related to the subtype PPARδ/β. Unlike most nuclear receptors that are degraded upon ligand binding, PPARδ/β ligands (L-165,041, GW501516, and PGI2) were reported to inhibit the ubiquitination of this receptor, thereby preventing its degradation [97, 275]. In this case, the ligand-mediated ubiquitination might be influenced by PPARδ/β protein levels, as was observed in mouse fibroblasts transfected to overexpress PPARδ/β, where the agonist GW-501516 strongly inhibits the ubiquitination and degradation of PPARδ/β. However, this effect was not observed at moderate protein levels, indicating that the process is not influenced by the ligand presence, but by the protein level [275].

21.4.1.3 PPARγ

As mentioned before, the PPARγ is the most studied isotype due to its role and relevance in obesity, diabetes, and other metabolic disorders. Ligand effect on PTM modulation was not reported for modifications occurring at AF-1 and DBD (phosphorylations at S46, S51, Y74, and Y78 and PPARγ1 acetylations at K154/K155). However, the influence of ligands in PTMs that occur in the LBD was extensively reported, especially for phosphorylation at S273 [45].

Rosiglitazone is the PPARγ ligand used for many years for T2D treatment since it acts as an insulin sensitizer by blocking phosphorylation at S273, whereas its full agonism activates the nuclear receptor and promotes the transcription of genes related to adipogenesis. However, its use causes side effects, such as weight gain and others related to fluid retention and cardiac hypertrophy. Thus, this molecule was removed from the market [45, 93, 339]. Besides this, rosiglitazone is still used as a classical ligand for PPARγ function studies, mainly on in vitro and in vivo assays.

The only PPARγ phosphorylation reported to be modulated by rosiglitazone is at the residue S273 [45]. Treatment with this and other PPARγ ligands blocks S273 phosphorylation and results in improving insulin sensitivity, as will be discussed in detail in the next topic.

Rosiglitazone treatment also decreased K268/K293 acetylation in a SIRT1-dependent manner [260]. Furthermore, acetylation defective animals for both K268R/K293R, treated with rosiglitazone, maintained the insulin-sensitizing, glucose-lowering response to TZDs, while not showing the TZDs adverse effects on fat deposition, bone density, fluid retention, and cardiac hypertrophy [171]. The crosstalk among S273 unphosphorylated state and K268/K263 deacetylation indicates that treatment with rosiglitazone promotes insulin sensitivity improvement due to inhibition of S273 phosphorylation, with no collateral effects of PPARγ activation, which may be the resulted of the K268/K293 deacetylated state. Drugs that inhibit both S273 phosphorylation and K268/K293 acetylation could be interesting to the treatment of T2D and obesity.

Regarding SUMOylations, rosiglitazone was reported to enhance this PTM at K395 [12, 245] and to activate PPARγ SUMOylation in HK-2 cells [199]. Ligand-dependent increase in K395 SUMOylation might be explained by structural analysis: crystal structures of apo and rosiglitazone-bound forms of PPARγ1 indicate that K365 is oriented toward the interior of the LBD in the apo form but is solvent-exposed in the rosiglitazone-bound form, allowing for covalent attachment of SUMO [245]. However, rosiglitazone and another ligand, GW1929, negatively regulate SUMOylation of K395 by intramolecular communication between the C-terminal LBD and the N-terminal AF1 domain [67].

Rosiglitazone and other TZD ligands (troglitazone and pioglitazone), although increasing PPARγ activity, were reported to enhance the receptor ubiquitination and degradation [119, 158], but interestingly pioglitazone was also reported to inhibit EGFR/MDM2 signaling-mediated PPARγ degradation, suppressing cancer cell chemoresistance [289].

21.4.1.4 Modulation of PPARγ S273 Phosphorylation

Phosphorylation of S273 is one of the most studied PPARγ PTM due to its involvement in insulin resistance in obese and diabetic humans. As mentioned before, rosiglitazone is a member of the TZD class, together with pioglitazone and troglitazone, acting as PPARγ agonists and binding directly in the LBD of the protein, stabilizing

H12, and recruiting coactivators that will promote expression of PPARγ target genes. It was also reported that rosiglitazone and pioglitazone were able to block the S273 phosphorylation [45], although this effect has not been reported yet to troglitazone. This inhibition, mainly by rosiglitazone, was the cause of the improvement of insulin sensitivity, reducing fasting glucose and insulin levels, and rosiglitazone was used for many years for the treatment of T2D. However, treatment with this drug resulted in several side effects, which are related to the strong PPARγ activation caused by this molecule, which leads to the expression of genes related to adipogenesis, promoting weight gain, increase on hepatic steatosis, and fluid retention, among others side effects [45, 92, 204]. Moreover, phospho-defective (S273A) $PPAR\gamma^{A/A}$ did not show innately any of the TZD-associated side effects (bone loss, fluid retention, and increase in adipocyte size) [108], corroborating the hypothesis that these effects are associated with PPARγ full agonism.

It is important to highlight this phosphorylation mechanism, which involves strong interaction between the kinase, specifically CDK/p25, and their specific substrate [65, 247]. Two elements guide this mechanism: the first one is the recognition of the phosphorylation motif by the catalytic site of the kinase, and the second, mainly involved in S273 phosphorylation, is the substrate recruitment, involving an increase of encounters between the enzyme and the substrate through distal docking sites [65, 311]. In PPARγ there is a noncontiguous recognition site (K261, K263, and K265), located at the H2'-H3 loop of the LBD region, being essential for CDK5 and PPARγ interaction [273]. However, these residues seem not to be involved in inhibiting S273 phosphorylation by PPARγ ligands, since these molecules do not interact with the H2'-H3 loop [273]. It was described that another residue, I341, may be involved in this process, mainly due to a structural shift promoted by ligand interaction with the receptor, stabilizing H2′ and part of the H2-H2′ loop and impacting its association with CDK5 [273]. Here it is shown PPARγ LBP ligands that promote insulin sensitization, with lower activation and, except for a few cases, experimentally confirmed blocking of S273 phosphorylation.

YR4-42 In 2019, a PPARγ agonist was reported to block S273 phosphorylation: YR4-42, a tetrahydroisoquinoline derivative [137]. This ligand showed weaker affinity and equivalent activation of PPARγ compared to pioglitazone [137]. In 3 T3-L1 adipocytes, YR4-42 promoted fewer lipids droplets than TZDs, same triglyceride levels as the control group, and blockage of S273 phosphorylation. Moreover, through a diet-induced obese (DIO) mouse model, it was shown that YR4-42 could control blood glucose and improves insulin sensitivity, with results similar to pioglitazone, also decreasing serum triglycerides, total cholesterol, and FFA, with lower body weight and an improvement on hepatic steatosis. Regarding gene expression, it was observed upregulation in genes involved in glucose metabolism and on thermogenesis, as *Cidea* and *Ucp1* [137].

WSF-7 Another PPARγ agonist was discovered at the end of 2019, called WSF-7 (5,5,7-trimethyl-3-(p-tolyl)- 3,3a,4,5,6,7-hexahydro-4,6-methanobenzo[c]isoxazol-7-ol), which is derived from natural monoterpene α-pinene [365]. This molecule

was detected by a screening for PPARγ agonists with the capacity of inhibiting S273 phosphorylation. In vitro studies demonstrated its ability of binding to the LBD of this receptor and activate it, also promoting adipogenesis, but it is less potent than rosiglitazone. Moreover, this ligand upregulated adiponectin expression and its oligomerization, increased insulin-stimulated glucose uptake, and SLC2A4 protein expression. They also confirmed its effects on inhibiting S273 phosphorylation in 3 T3-L1 cells, indicating WSF-7 as a potential insulin sensitizer and drug for T2D treatment [365].

EPA-PC and EPA-PE Sea cucumber phospholipids were also detected as PPARγ agonists, mainly the phosphatidylcholine (EPA-PC) and phosphatidylethanolamine (EPA-PE) ones, binding to this receptor with high affinity, as well as to PPARα [316]. In vitro assays demonstrated that these compounds promoted adipocyte differentiation and lipid accumulation, promoting an increased expression of *Pparg, Fabp4, Fas*, and *Cebpa*. EPA-PC and EPA-PE also activated hepatic fatty acid β-oxidation in HepG2 cells. In addition, in vivo experiments revealed that EPA-PC and EPA-PE treatment slightly decreased S273 phosphorylation, but increased the protein expression of CD36 and FABP4, also suppressing the increase in iWAT and eWAT weight, reducing adipocytes size and lipid droplets. These molecules ameliorate glucose intolerance and insulin resistance in mice, with a significant reduction in triglycerides, cholesterol, and non-esterified fatty acids (NEFA), suggesting its role on improving metabolism and as a new therapeutic approach on T2D treatment [316].

In order to avoid the undesirable side effects of PPARγ full activation (adipogenesis, bone loss, etc.), other researchers are focusing on molecules that act as S273 blockers, but not as receptor full activators, acting as partial agonists or non-agonists. Some of them are presented here, showing their physiological roles related to this PTM inhibition (Fig. 21.9).

nTZDpa In this context, in 2003, 5-Chloro-1-[(4-chlorophenyl)methyl]-3-(phenylthio)-1H-indole-2-carboxylic acid (nTZDpa) was first described as potent and selective PPARγ partial agonist, antagonizing the effects of full agonists [23]. On in vitro assays, this molecule treatment reduced the lipid content of fully differentiated adipocytes, causing alterations in *Fabp4* expression. In a few experiments in vivo with DIO mice, nTZDpa effects on attenuating insulin resistance and hyperglycemia, decreasing weight gain, and increasing adiponectin expression were also observed [23]. After, in 2010, when S273 phosphorylation was reported, this molecule was described as a blocker of PPARγ phosphorylation [45].

2-BABAs After that, in 2004, it was reported another class of molecules, the 5-substituted 2-benzoylaminobenzoic acids (2-BABAs), which binds to PPARγ without direct interaction with H12, although activating the receptor, herein being classified as partial agonists [239]. Among them, the compound BVT.13 was evaluated in ob/ob mice, in which, although resulting in a significant reduction in fasting plasma glucose, triglycerides, plasma insulin, and FFA, the treatment led to weight

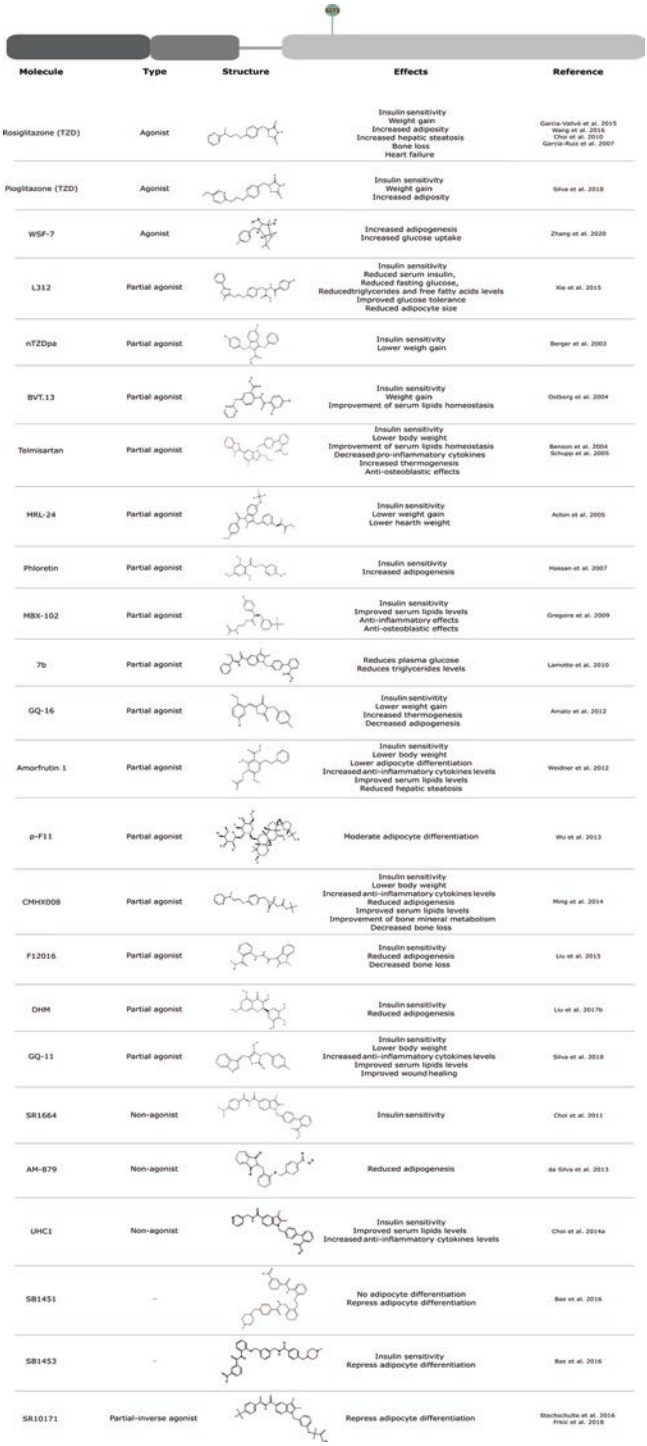


Fig. 21.9 PPARγ ligands that prevents S273 phosphorylation and their main effects. Here is listed only ligands with retrieved structural information

gain [239]. This compound was further investigated in vitro, where the ligand showed moderate transcriptional activity and confirmed its lack of interaction with H12 [33], also acting as a blocker of S273 phosphorylation [45].

TEL In 2004, an angiotensin II receptor blocker called telmisartan (TEL), was reported as a partial agonist of PPARγ [22, 283], and molecular docking analysis indicated that TEL interacts with the receptor by H3, H6, and H7, making strong hydrophobic interactions, and it does not appear to make contact with AF-2 region or histidine adjacent regions [22]. Although in vitro assays showed that TEL promoted adipogenesis in 3 T3-L1 cells, this result was not compared to tosiglitazone's effects on differentiation [22]. In adipocytes, TEL upregulates PPARγ target genes related to adipogenesis but in a less extent than rosiglitazone and decreased *Cfd* expression [166, 283]. Cellular assays also demonstrated that TEL upregulates thermogenic genes, did not have anti-osteoblastic activity, and decreased S273 phosphorylation levels [166]. In the cellular model of insulin resistance condition through TNFα treatment, TEL was able to reduce the TNFα- increased PPARγ S273 phosphorylation, reverse the decrease on glucose uptake, partially restore expression levels of *Adipoq, Cfd*, leptin (*Lep*), and *Slc2a4*, and decrease the expression of *Fabp4* [74].

TEL treatment in three DIO rodent models (C57BL/6 J mice, OLETF rats, and Male Sprague-Dawley rats) and one obese diabetic yellow agouti Avy/a mice resulted in lower weight gain and decrease in glucose, insulin, and lipids levels [22, 166, 283, 367]. Besides, in Avy/a mice, TEL did not affect the volume and structure of trabecular bone, with no fat accumulation in the marrow, and the combination of TEL and rosiglitazone treatments resulted in partial protection against bone loss [166]. TEL treatment in diabetic mice also induced the expression of beige markers, increased oxygen consumption, and carbon dioxide production, increasing respiration rate and confirming its action on energy expenditure [166]. Moreover, this molecule also provoked a decrease of pro-inflammatory cytokines and leptin levels, as well as an increase in adiponectin and a significant reduction in insulin resistance in OLETF rats [367]. Preliminary human studies showed that TEL improved insulin resistance in hypertensive and T2D, whereas no significant changes observed in adiponectin were upregulated only in high doses of TEL body weight, fasting plasma glucose, and plasma lipids levels [224, 321].

MRL24 A benzoyl indole called MRL24 was discovered in 2005 as a poor agonist of PPARγ with significant anti-diabetic effects, reducing weight gain, heart weight, and glycemia, when compared to rosiglitazone, in db/db mice [1]. This ligand also improved insulin sensitivity and glucose tolerance, as well as fasting insulin levels on mice fed HFD, mostly due to its action on reducing CDK5-mediated phosphorylation [45].

Phloretin In 2007, based on researches focused on the effects of flavonoids and chronic diseases, the chalcone phloretin was identified as PPARγ ligand [117]. Using 3 T3-L1 cells, it was observed that phloretin treatment resulted in an increase

on lipid accumulation, an increase on the triglycerides content, and in adiponectin expression and secretion, together with an increase on the expression of adipogenic markers, such as *Pparγ, Cd36,* lipoprotein lipase *(Lpl),* and *Cebpa* during the process of differentiation, indicating its effects on adipogenesis and suggesting its possible role on insulin sensitivity [117]. Later on, microarray analysis of phloretin treatment on adipocytes identified an upregulation of genes associated with carbohydrate and lipid metabolism, as well as of genes encoding adipokines and transcriptional regulators associated with adipocyte phenotype, confirming the previous results [118].

Phloretin also affected the insulin signaling pathway, mainly by increasing phosphor-Akt and phosphor-GSK3β, despite no effect on the AMPK pathway, revealing its adipogenesis role to the PI3K-AKT signaling pathway [292]. In vivo experiments with C57BL BKS-DB mice showed that treatment with phloretin increased food consumption with no effect on body weight, decreasing blood glucose and cholesterol levels and improving glucose tolerance. It was also observed an increase in PI3K and AKT's activity on mouse adipose tissue, besides an increase in proteins of adipogenic markers, as SLC2A4 (former GLUT4) and CD36 confirming the in vitro results [292].

Other in vivo experiments were performed to evaluate phloretin's effects on glucose metabolism, and it was observed that the treatment with this flavonoid protects mice from HFD-induced obesity, with no weight gain, loss of fat mass, and smaller WAT, suppressing lipid accumulation on this tissue. It was also detected an increase in *Adipoq* expression, decreased fat content on the liver due to a reduction in the expression of PPARγ, and a decrease in glycemia and an improvement in insulin sensitivity [6]. Molecular docking and molecular dynamics analysis showed that the PPAR-phloretin complex was formed by three hydrogen bonds and six hydrophobic interactions, suggesting that phloretin was effectively bound to PPARγ [174]. All these positive effects of phloretin on glucose homeostasis and insulin sensitivity, as well as its binding site in an activated conformation of PPARγ, suggest that this ligand decreases S273 phosphorylation [174]; however, more experiments are required to confirm the phosphorylation modulation.

MBX-102 Another PPARγ partial-agonist group, MBX-102, was described in 2009 [104]. This compound belongs to the halofenate compounds family, which are a racemic mixture of (−)- and (+)-[2-acetoaminoethyl (4-chlorophenyl) (3-trifluoromethylphenoxy) acetate]. These compounds have already been clinically tested in the 1970s, revealing its actions as hypolipidemic and hypouricemic agents [13]. MBX-102 is an enantiomer of halofenate, a pro-drug ester that is wholly modified in vivo by nonspecific serum esterases to the mature free form MBX-102 acid, which is the circulating form of the molecule. In vitro assays showed its capacity to bind to PPARγ and activate it in a dose-dependent manner, but with lower efficiency than rosiglitazone, also having the ability to antagonize rosiglitazone-dependent PPARγ activation. It was shown that the interaction of MBX-102 with LBD of PPARγ occurs distinctly from TZDs. Moreover, this ligand has the capacity of dis-

placing the corepressors NCoR and SMRT and has a reduced ability to recruit coactivators, explaining its partial agonism [104].

Regarding the physiological effects of MBX-102, in vitro studies using 3 T3-L1 adipocytes revealed its ability to enhance insulin-stimulated glucose membrane translocation and a decreased ability to stimulate adipocyte differentiation [104]. In vivo studies with three T2D rodent models (ob/ob and db/db mice and Zucker fatty diabetic (ZDF) rats) demonstrated that the treatment with this ligand promoted a reduction on fasting plasma glucose [41, 104]. ZDF treated with MBX-102 also showed reduced plasma insulin levels and increased glucose infusion rate and glucose disposal rate on the hyperinsulinemic-euglycemic clamp, suggesting its role on insulin sensitivity. Long-term treatment with MBX-102 revealed an improvement in glucose tolerance and an increase in adiponectin levels, with no increase in body and heart weight, side effects observed with rosiglitazone treatment [104]. Another study using ZDF rats revealed positive effects of MBX-102 on reducing triglycerides, FFA, and cholesterol levels [41].

MBX-102 also reduced osteoblastic differentiation in vitro and decreased the levels of LPS-stimulated pro-inflammatory cytokines (such as monocyte chemoattractant protein-1 (MCP-1) and interleukin 6 (IL-6) in mouse primary peritoneal macrophages, revealing its promising therapeutic potential on the treatment of T2D [104], also acting as a blocker of S273 phosphorylation [45]. MBX-102 anti-diabetic effects were addressed in humans in phase 2 and 3 clinical trials (identifiers NCT00814372, NCT00353587), but up to 2020, no results were published yet.

7b In 2010, a potent PPARγ partial agonist, called 7b, was developed after modifications on TEL structure and showed a high affinity to the NR and binding mode that differs from the full agonists [178]. On this molecule, the molecule's carboxylic acid binds at the opposite end of the active site and hydrogen bonds with R288 and S342, allowing the amide group to donate and accept hydrogen bonds to S289 and Y327, respectively. This compound was used for in vivo assays with ZDF rats, being evaluated as suitable for chronic administration. A reduction in plasma glucose and triglycerides levels was observed with fewer side effects than full agonists, but more experiments are necessary to confirm this compound's efficacy and confirm if this molecule may act as a blocker of S273 phosphorylation [178].

GQ-16 Two years later, other PPARγ partial agonist, GQ-16, was reported to reverse the impairments on insulin signaling that HFD promotes [7]. GQ-16 treatment increased insulin receptor expression, insulin receptor substrate 1, and protein kinase B, among others. In addition, HFD mice showed an improvement in insulin sensitivity due to an increase in glucose disappearance rate (KITT) and glucose tolerance, with a lower increase in body weight and a decrease in fat mass. It was showed that this molecule could block S273 phosphorylation in a concentration-dependent manner, binding to PPARγ in an axial orientation, parallel to H3, making no contact with residues of H12. This interaction protects the lower half of LBD, stabilizing the H11-12 loop, H3, and β-sheet/S273 regions, more efficiently than rosiglitazone does [7].

Mice treated with GQ-16 exhibited a slight decrease in hepatic triglycerides and attenuation on adipocyte hypertrophy and a decrease in interscapular BAT, with small lipid droplets. Interestingly, this ligand promoted an increase on the expression of *Ucp1*, *Cidea*, and *Prdm16* in BAT and epididymal WAT, which are genes related to thermogenesis, indicating the potential role of this molecule on the treatment of T2D and obesity [52].

Amorfrutins In 2012, another group of molecules belonging to the family of natural products, amorfrutins, was described as partial-agonist PPARγ. They were identified by screening a library containing 8000 pure compounds, which revealed 90 potential PPARγ ligands. Amorfrutins, a family of isoprenoid-substituted benzoic acid derivatives without any stereocenters, were selected as structurally new molecules with a high binding affinity to the NR [343]. It was reported that these compounds induce partial recruitment of coactivators, as CBP, PGC1α, TRAP220/DRIP, and PRIP/ RAP250, as well as disrupt the recruitment of the corepressor NCoR. It was also shown that these molecules interact with PPARγ LBD by contact between H3 and the β-sheet, stabilizing these structures [343].

Regarding the physiological effects of amorfrutins, in vitro assays using 3 T3-L1 adipocytes showed an upregulation of PPARγ target genes, as *Fabp4, Slc2a4*, and LXRα (*Nr1h3*), but in a lower degree than rosiglitazone. Moreover, it was observed a less pronounced adipocyte differentiation and an upregulation of genes involved in cholesterol biosynthesis, fatty acid elongation, and oxidation, in contrast to a downregulation of inflammatory genes. Besides these results, in vivo studies using DIO C57BL/6 mice revealed that the treatment with amorfrutin 1 reduced insulin resistance, enhanced glucose tolerance, and decreased plasma triglycerides, FFA, insulin, and glucose in the same levels as rosiglitazone did. Although it promoted an increase in food intake, body weight gain was significantly reduced, which was related to an increase in plasma levels of thyroxine (T4), a marker of increased energy expenditure [343].

The anti-diabetic effects of amorfrutin 1 were also evaluated in db/db mice, showing no weight gain, with a reduction in plasma insulin levels, as well as glucose, triglycerides, and FFA compared to vehicle. As S273 dephosphorylation was described as a mechanism for improvement on insulin sensitivity, this parameter was checked, and it was observed a reduction in S273 phosphorylation on WAT of DIO mice, increasing the expression of gene related with this PTM, as *Nr1d2, Selenbp1, Adipoq,* and *Cfd*. In the liver of DIO mice, amorfrutin 1 treatment reduced hepatic triglycerides and induced FAO by upregulating *Fabp4, Pgc1a*, and *Cpt1a* compared to a vehicle with reduced TNFα protein concentration and higher glycogen content. Besides, amorfrutin 1 leads to decreased inflammation and macrophage accumulation in the liver and WAT. All these effects could indicate the potential action of amorfrutins on glucose metabolism and lipid profile, improving insulin sensitivity and acting as a molecule against T2D [343].

p-F11 In 2013, pseudoginsenoside F11 (p-F11), an ocotillol-type ginsenoside isolated from the roots and leaves of *Panax quinquefolium* L. (American ginseng), was

identified in a screening for partial PPARγ agonists. p-F11 was described with moderate adipogenic activity in vitro [347]. In a gene reporter assay, p-F11 was shown to dose-dependently increase PPARγ activation, although higher concentrations of p-F11 promoted an activation increase smaller than lower concentrations of rosiglitazone, corroborating its action as a partial agonist. This ligand increased the mRNA expression and protein level of both PPARγ and adiponectin, as well as adiponectin oligomerization and secretion in 3 T3-L1 adipocytes, indicating that this adiponectin upregulation is through PPARγ activation during the differentiation of 3 T3-L1 preadipocytes. Regarding S273 phosphorylation, p-F11 decreased this PTM occurrence in 3 T3-L1 adipocytes in the same extent than rosiglitazone [347].

CMHX008 In the following year, other PPARγ partial agonist was discovered through docking methods. CMHX008 presents a different binding mode to interact with the receptor, forming hydrophobic interactions with L255, I281, M348, and I341 in the receptor entrance [219]. Compared to rosiglitazone, this molecule showed, in vitro, a decreased activation of PPARγ, reduced adipocyte differentiation and lipid accumulation, and an increase on *Adipoq, Slc2a4,* and *Fabp4* expression and on adiponectin secretion [219].

To confirm these effects, in vivo studies were performed using DIO mice, in which CMHX008 treatment showed a decrease in body weight, together with a reduction in adipocyte size and WAT weight and reduction on triglycerides and LDL-cholesterol levels [219]. These animals also improved glucose tolerance with reduced glycemia and reduced insulin plasma levels, indicating CMHX008 antidiabetogenic effects. These effects were justified as a result of S273 phosphorylation blockage and alteration on the ability of PPARγ to interact with coactivators, promoting differential recruitment of the receptor to the promoter of its target genes [219].

Regarding inflammation, DIO mice treated with CMHX008 showed reduced serum IL-6 and TNFα and increased IL-10, a cytokine with anti-inflammatory properties [219]. CMHX008, as well as with rosiglitazone, switched macrophage polarization from pro-inflammatory M1 to anti-inflammatory M2 dominant [219]. Studies to compare the effects of CMHX008 with rosiglitazone, concerning one of its well-known side effects, bone mass loss, showed that CMHX008 promoted a decrease in trabecular bone, but in a small proportion. Moreover, treatment with this molecule displayed a more mineralized matrix during differentiation into osteoblasts [132]. Altogether, these results indicate the effects of CMHX008 in glucose, lipid, and bone mineral metabolism.

L312 In 2014, another PPARγ partial agonist was described as a potent molecule on insulin sensitivity with low side effects. It was called L312 ((S)-2-(4-chlorobenzamido)-3-(4-(2-(5- methyl-2-phenyloxazol-4-yl)ethoxy)phenyl) propanoic acid), and it had a similar affinity to PPARγ-LBD as pioglitazone with a less extent transcriptional activity [349]. In vitro assay demonstrated its effects on the recruitment of CBP coactivator and NCoR displacement, besides a weak adipogenic activity. Moreover, in vivo experiments revealed that L312 improves insulin resis-

tance in a dose-dependent manner and improves glucose tolerance, reducing serum insulin, fasting glucose, triglycerides, and FFA levels. This molecule also reduced adipocyte size and results in a lower increase on body weight when compared to pioglitazone. All these effects were related to the inhibition of S273 phosphorylation in vitro and in vivo, attenuating the expression of several genes regulated by this PTM, which makes L312 a potential drug on T2D treatment [349].

Chelerythrine In 2015, a molecule called chelerythrine, which is derived from *Chelidonium majus* (greater celandine) and is already used as a medical therapy with antiviral, antitumor, antifungal, and anti-inflammatory activity, was revealed as a selective modulator of PPARγ [368]. It was shown that this compound has weak activity on PPARγ-LBD but exhibited a high binding potency, promoting the recruitment of coactivators as SRC1 and PGC1α, and displacement of SMRT corepressor with less efficiency than rosiglitazone, being considered a partial agonist. In vivo experiments demonstrated its effects on improving glucose tolerance and insulin sensitivity with no weight gain. In addition, chelerythrine promoted a reduction on serum glucose, insulin, and cholesterol levels and a decrease on the expression of *Cd36*, *Il1b*, interferon gamma (*Ifng*), and *Tnfa*. Despite its lower transcriptional activity, this molecule exhibited a higher capacity on blocking S273 phosphorylation when compared to rosiglitazone, which confirms its potential role on the treatment of diabetes and obesity [368].

F12016 Another PPARγ partial agonist and an S273 phosphorylation blocker, F12016, was reported in 2015. This benzamide derivate is structurally different from TZDs, and it was shown to promote the transcriptional activity of the receptor through the binding to its LBD, but with moderate intensity when compared to rosiglitazone [193]. This interaction includes binding through two hydrogen bonds, a π-π stacking interaction, and several van der Waals forces with surrounding amino acids, such as C285, M364, I326, L330, M329, and I281 [193]. F12016 showed moderate activation of PPARγ, with impaired coactivator and improved corepressor recruitment compared to rosiglitazone, suggesting its partial agonism [193].

The effects of F12016 in vitro using 3 T3-L1 and hepatocytes showed that this compound enhanced insulin-stimulated glucose uptake, increasing insulin sensitivity and promoting glucose transport. Besides, F12016 presented low potency to induce the formation of lipid droplets, also reducing triglycerides content inside the cells [193]. As expected from partial agonists, F12016 differentially regulates a set of genes involved in adipogenesis, decreasing expression of *Fabp4* and *Cd36*, among others, and increasing the expression of *Adipoq*. Still using cell models, researchers found that this molecule caused less reduction of bone cells' calcification than rosiglitazone, suggesting F12016 would cause less osteoporosis. Besides this, in the KK-Ay murine diabetes model, F12016 promoted a reduction in fasting glucose levels and improved glucose tolerance and insulin sensitivity, with no weight gain. In conclusion, this ligand demonstrated various advantages as insulin sensitizer without showing side effects [193].

DHM Still, on the PPARγ of partial agonists class, many flavonoids are reported to act on glucose and lipid metabolism. One example is dihydromyricetin (DHM), described in 2016, that promoted less weight gain than rosiglitazone in ZFD rats and significantly reduced fasting blood glucose, improving the insulin/glucagon ratio [194]. Moreover, DHM reduced insulin resistance by 50% in comparison to rosiglitazone. On lipid profile, it was observed an improvement on serum lipids levels compared to control and a significant reduction on visceral and total fat mass, reducing adipocytes size more efficiently than rosiglitazone, also promoting an increase in adiponectin protein levels in adipose tissue [194]. DHM reduced PPARγ phosphorylation in vivo more potentially than the rosiglitazone [194]. In 3 T3-adipocytes, DHM also showed a decrease in lipid accumulation and the expression of the adipogenic marker *Fabp4*, and in combination with dexamethasone, DHM increased glucose uptake by 90% in cells, also improving adiponectin and FGF21 secretion by adipocytes [195]. In normal adipocytes, DHM treatment decreased CDK5 activation and ERK phosphorylation, reducing insulin-resistant adipocytes, through the prevention of S273 phosphorylation, further elucidating the mechanisms of anti-diabetic properties of DHM [195].

GQ-11 In 2018, the compound GQ-11 was described as a partial agonist of PPARα and PPARγ. Using docking studies, it was shown that this molecule interacts with the hydrophobic residues F282 and L469 of PPARγ arm I and forms a hydrogen bond with S289, also interacting with PPARα [295]. The pharmacological effects of this molecule were evaluated in LDL receptor-deficient mice (LDLr−/−) fed on a diabetogenic diet, showing positive effects on insulin sensitivity, decreasing fasting glucose and insulin levels, and improvement of glucose tolerance compared to control. Furthermore, GQ-11 treatment resulted in lower body weight and did not modify adipose mass, differing from pioglitazone [295]. On PPARγ modulation, GQ-11 is involved in improving adipokines levels, as adiponectin, with a concomitant increase on the expression of *Slc2a4*, followed by a decrease in serum leptin. This molecule also affects the animals' inflammatory state, increasing IL-10 in adipose tissue, with a decrease in MCP-1, suggesting its influence on local and systemic inflammation. Related to lipid profile, GQ-11 treatment promoted a decrease in VLDL cholesterol levels and serum triglycerides, with an increase in HDL-cholesterol levels compared to control [295]. GQ-11 was also reported as an anti-diabetic compound because it induces the upregulation of anti-inflammatory cytokines and growth factors gene involved in tissue repair in db/db and non-diabetic mice. This ligand improved wound closure in db/db mice compared to control or pioglitazone groups, increasing collagen deposition and decreasing macrophage infiltration in this lesion [295].

Another possible approach to modulate PPARγ action would be to prospect molecules that act as non-agonists of PPARγ. These compounds should bind to the receptor but do not promote its transcriptional activity, and some of them were reported to block S273 phosphorylation, promoting insulin sensitivity.

SR1664 In this context, in 2011, after discovering compound 7b, some analogs of this molecule were developed, of which the most efficient one was SR1664 [46]. In silico docking studies revealed that SR1664 increases the conformational mobility of C-terminal end of H11, a helix that abuts H12, which could be explained by the interaction of phenyl substituted nitro group of the molecule with hydrophobic side chains of H11, such as L452 and L453 of the loop N-terminal to H12 [46]. Following this, it was evaluated its capacity to recruit cofactors and DNA binding ability, observing that SR1664 did not influence SRC1 recruitment or the occupancy of PPARγ [46]. On the first in vitro assays with SR1664, its effects on adipocyte differentiation were evaluated, resulting in no changes in lipid accumulation or the fat cells' morphology, with little or no change on fat cell gene expression. It is already known that TZDs affect bone formation, and, using MC3T3-E1 cells, it was seen that SR1664 did not affect the extent of calcification or the expression of osteoclastic genes [46].

Subsequently, the anti-diabetic properties of SR1664 were analyzed in vivo using DIO mice, and the treatment promoted a decrease in S273 phosphorylation, as well as on glucose and fasting insulin levels, with an improvement on insulin sensitivity, without changing body weight. These same results were observed in a more severe animal model, ob/ob mice, confirming the beneficial effects of SR1664 on glucose metabolism and insulin sensitivity, with no side effects due to its non-agonism characteristics [46].

AM-879 Another PPARγ non-agonist, discovered in 2013 on a study that used a structure-based strategy to search for new ligands, was AM-879 [61]. On the initial screening, this molecule was selected due to its ability to ensure increased thermal stability for the receptor over the unbound one, proving its capacity to bind to the PPARγ. After, in vitro transactivation assays presented its capacity to decrease the basal transcription of this receptor [61, 272]. Further, this ligand did not favor coactivator recruitment, did not induce corepressor release, and did not induce a significant increase in lipid accumulation or adipocyte differentiation in adipogenesis assays, decreasing expression levels of *Adipoq*, *Cfd*, and *Cd36* [272]. Finally, this study showed that AM-879 reduced S273 phosphorylation more effectively than rosiglitazone, indicating its potential anti-diabetic role and the requirement of further studies [272].

UHC1 Another PPARγ non-agonist, described in 2014, was UHC1, which showed in vitro assays not to stimulate lipid accumulation and not increase some classical adipogenic markers but influencing the expression of genes regulated by S273 phosphorylation [47]. In addition, in vivo studies with HFD mice showed that UHC1 induced an improvement in glucose tolerance and reduced fasting glucose and insulin levels. On lipid profile, UHC1 promoted a reduction in serum triglycerides, cholesterol, and FFA and upregulated the expression adiponectin and adipsin. Regarding the inflammation process, UHC1 showed a potential role in the inflammatory process, as it inhibited the TNFα-stimulated pro-inflammatory responses in 3 T3-L1 adipocytes and reduced the mRNA levels of the pro-inflammatory cytokine *Il6* and

increased the levels of anti-inflammatory markers as *Il10* and arginase in HFD mice. All these data indicate that UHC1 exerts potent anti-diabetic effects, positively influencing inflammation, without causing side effects as TZDs [47].

SB1451 and SB1453 Afterward, in 2016, two new molecules were synthesized based on previous compounds that bind to PPARγ and block S273 phosphorylation: SB1451 and SB1453 [15]. These ligands contain hydrophilic piperazine moieties attached to the other benzene rings to improve their solubility. In vitro assays confirmed their ability to block CDK5-mediated S273 phosphorylation. Further, these molecules have shown a low activation of the receptor and did not trigger adipogenesis in 3 T3-L1 cells. To evaluate the anti-diabetic effects, DIO mice model showed that SB1453 was more effective in reducing S273 phosphorylation than SB145, as well as altered the expression of 10 out of 17 affected genes by this PTM and improvement on glucose tolerance. Possible side effects of SB1453 were studied, and no significant changes were seen on the markers for heart failure (natriuretic peptide B) and hypertrophy (myosin heavy chain β). Finally, the crystal structure of SB1453 with PPARγ-LBD showed that this compound was covalently bound to C313 on H3 and occupied the hydrophobic region between H3 and β3-β4 sheets, which is closely related to the inhibition of S273 phosphorylation [15].

SR10171 An inverse agonist of PPARγ was also described, binding to the receptor as an agonist but promoting an opposite response. SR10171 is considered a partial inverse agonist since it partially represses the NR's basal transcriptional activity [301]. This molecule reduces S273 phosphorylation, resulting in induced pro-osteoclastic activity, increased osteoclastogenesis, and cortical bone thickness, at the same time that it enhances insulin sensitivity in DIO mice [87, 301]. In addition, this same ligand promotes preferential recruitment of corepressors than coactivators, due to its interaction with H12 of the receptor, favoring the antagonist conformation [87].

21.4.2 Modulating the Activity of Enzymes Responsible for PTMs

In order to characterize a PPAR PTM, its addition or removal is often modulated by an activator or inhibitor of the enzyme responsible for the modification. Although useful in isolated systems such as in vitro cell culture or knock-in/knockout animals, this approach may not correlate well in the clinical trials. Modulation of a PPAR PTM occurrence requires a fine-tuning adjustment to avoid unspecific activation or blockage of other signaling pathways and to avoid undesirable side effects.

In this topic, strategies used in the research laboratories to inhibit or stimulate the addition of a PTM in PPAR using PTM enzyme modulators will be presented.

Moreover, we are going to discuss the applicability of this strategy in the clinic to modulate the PPAR function.

21.4.2.1 Phosphorylation

Phosphorylation in PPARα seems to have antagonistic effects as increased S12/S21 and decreased S73 phosphorylation lead to increased activity of the receptor, resulting in a protective effect for hepatic steatosis [55, 128]. However, the reports with kinases and phosphatases activators and inhibitors to modulate these phosphorylations are rare. In the only report of kinase modulation, S12/S21 phosphorylation is inhibited by MAPK inhibitor PD98059, decreasing the receptor activity [147]. In order to stimulate the PPARα activation and its protective effect in hepatic steatosis, it would be interesting to induce S12/S21 phosphorylation and/or to reduce the one at S73. GSK3β phosphorylates the later serine, and the phosphatase that mediates this dephosphorylation was not yet described [127].

PPARγ phosphorylations can be increased upon stimulation of the kinases MAPK, ERK, and CDK5 pathways with growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), TGFβ, insulin, prostaglandin F2α (PGF2 α), TNFα, cellular stress (UV, TPA, and anisomycin), IL-6, or FFAs [2, 37, 45, 135, 270, 361]. PPARγ phosphorylation in the LBD (S273) does not change transcriptional activity, but inhibition of this phosphorylation increases insulin sensitivity, whereas PPARγ phosphorylation in AF-1 and DBD decreases the receptor activity (S112, Y74, Y78, S16/S21). Therefore, with the objective to revert the insulin resistance mediated by S273 phosphorylation without activating the NR and inducing weight gain, hepatic steatosis, and bone loss, a good strategy would be to block the kinase or activate the phosphatase responsible to respectively phosphorylate and dephosphorylate the S273 residue, while promoting the phosphorylation of serines and tyrosines present in AF-1 and DBD. However, these residues are phosphorylated by different kinases: casein-kinase II (S16/S21 in PPARγ1), c-SRC (Y78 PPARγ2), EGFR kinase (Y74 PPARγ1), and MAPK, CDK7, CDK9, and ERK for S112 in PPARγ2, making this a hard approach.

Some protein kinases, such as WEE1 and MEK, are specific and perhaps phosphorylates only one or two distinct protein targets [320]. However, many other protein kinases have a broader specificity and are likely to phosphorylate hundreds of distinct proteins within cells.

Casein-kinase II (CK2), which phosphorylates PPARγ1 S16/S21, is a ubiquitous serine/threonine kinase that has over 100 potential physiological targets [192], including growth-related proteins, NOPP140, tumor protein p53, Fas-associated factor-1 (FAF-1), topoisomerase II and CD5, and potential CK2 regulators such as fibroblast growth factor-2 (FGF-2) [9, 28, 29, 80, 144, 186, 263]. Due to its role in cell fate determination in cancer cells, there is an increasing interest in the development of CK2 modulators for cancer therapies [103, 111, 261].

Y78 phosphorylation can be inhibited with a c-SRC kinase inhibitor, PP2, aggravating insulin resistance in obese mice and dysregulating the gene expression of

cytokines and chemokines in adipocytes [49]. However, the non-receptor tyrosine kinase c-SRC, the first proto-oncogenic protein ever described [208, 330], is ubiquitously expressed in all cell types, and pharmaceutical modulation of its activity can interfere in other pathways which regulate cell growth, differentiation, and cytoskeletal regulation [77, 313].

Differently, EGFR is a transmembrane receptor for EGF with a tyrosine kinase domain and member of the ErbB family of receptors [362]. The kinase domain of EGFR can cross-phosphorylate tyrosine residues of other receptors with which it is aggregated, for example, PPARγ 1 Y74 [350], and can be itself activated in that manner.

PPARγ S112 was shown to be phosphorylated by several kinases (MAPK, CDK7, CDK9, ERK), and treatment with a MEK inhibitor, U1026, reduced phosphorylation and the receptor degradation [83].

The best strategy to modulate insulin resistance would be to only block S273 phosphorylation, with a CDK5 or ERK inhibitor, or to promote its dephosphorylation with the activation of PPM1A phosphatase. For example, treatment with roscovitine, a selective CDK5 inhibitor, prevents S273 phosphorylation [45]. However, CDK5 does not seem to be a simple drug target for specific PPARγ modulation because its pharmaceutical activation could deregulate the different pathways coordinated by this kinase, resulting in undesirable side effects.

CDK5, unlike other members of the cyclin-dependent kinases family, is not typically activated upon binding with cyclin and does not require T-loop phosphorylation for activation. CDK5 is activated by binding of p35 or its cleaved form p25 [65, 129, 247]. Additionally, CDK5 has functions in both terminally differentiated and proliferating cells [206] and, as others CDKs, is highly expressed in mitotic cells [285]. CDK5 plays a vital role in the central nervous system but also has a function in the immune system by increased interferon γ-induced programmed death-ligand 1 (PD-L1) expression, in insulin secretion, in angiogenesis promotion, in cell cycle by increasing expression of cyclins and other CDKs, and in cancer progression [294]. Due to its biological and clinical relevance in multiple cell types, CDK5 presents an attractive therapeutic target for treating various conditions such as diabetes, cancer, and neurodegeneration. Two CDK5 inhibitors are in clinical trials for cancer treatment, dinaciclib and seliciclib, whereas roscovitine, widely experimentally used to inhibit CDK5 activity, is being intensively examined as clinical cancer therapeutics [26, 85, 345].

To modulate the phosphate addition without interfering in the other targets of CDK5 phosphorylation, a different approach has been proposed: the development of ligands or peptides targeting the interface region between PPAR and this kinase [273]. Computational and biophysical analysis of PPARγ and CDK5 structures resulted in a model of interface interaction, which was validated with single point mutations experiments with purified proteins and in cell culture. At PPARγ, the CDK5 phosphorylation occurs in a noncontiguous motif, where P0 is S245, P + 1 is P246, P + 2 is F247, and K261 structurally occupies P + 3 position [273]. These computational analyses identified PPARγ K261, K263, and K265 as anchor

residues in the CDK5/p25 interaction, and single point mutations of these lysines resulted in decreased interaction with CDK5, decreasing the NR phosphorylation [273]. These results suggest that inhibition of CDK5-mediated phosphorylation of PPARγ with ligands that bind in the LBP might occur due to conformational changes in the receptor H2′ (residues 254 to 259), which loses its flexibility after ligand binding, probably blocking CDK5 anchorage to its recognition interface. It was hypothesized that peptides targeting the residues in the interaction interface could also block PPARγ phosphorylation, being an attractive therapeutic approach to treat T2D. This approach could also be expanded to the other PPARs PTMs, with the use of computational biology and biophysics experiments being used to propose and validate sites for protein anchorage to promote acetylation (and deacetylation), SUMOylation, phosphorylation, and other PTMs, which further the development of inhibitors of these specific interactions and with specificity to the desired PPAR isotype.

21.4.2.2 Acetylation/Deacetylation

In order to map and study PPARγ acetylation, the acetyl addition was promoted by treatments with acetyltransferase CBP or with HDAC inhibitors like trichostatin A (TSA) and nicotinamide (NAM), which is also a SIRT1 inhibitor [260, 314], suggesting that basal levels of PPARγ acetylations are very low.

The importance of PPARγ acetylation state in the balance between the beneficial and adverse effects of TZDs raises scientific interest in targeting this modification together with S273 phosphorylation. Deacetylation of PPARγ was promoted with purified deacetylase together with deacetylase co-factor nicotinamide adenine dinucleotide (NAD), and in cells with RSV, an activator of the deacetylase SIRT1, resulting in decreased expression of adipogenic genes [260, 314]. Consequently, inhibition of SIRT1 by NAM treatment prevented PPARγ deacetylation [314].

The modulation of SIRT1 seems an interesting approach to maintain the deacetylated state of PPARγ in a research laboratory isolated set up of experiments. However, treating obese patients with SIRT1 activator would be seen with caution once this deacetylase regulates many other relevant pathways in humans. SIRT1 is a member of the sirtuin family and can deacetylate various substrates and is, therefore, involved in a broad range of physiological functions, including control of several cardiometabolic and aging-related pathways [262, 340]. Besides PPARγ, other SIRT1 substrates are the tumor suppressor protein p53, members of the Forkhead box factors regulated by insulin/Akt (FoxO) family, hairy and enhancer of split 1 (HES1), hairy/enhancer-of-split related with YRPW motif 2 (HEY2), COUP-TF-interacting protein 2 (CTIP2), p300, PGC1α (PPARγ coactivator), and NF-κβ [106, 215, 253, 352].

Because of its many substrates, among other effects, SIRT1 is reported to regulate energy and lipid homeostasis, hepatic lipid homeostasis [352], DNA damage

repair and genome integrity [338], and chronic inflammation [281]. In these last two examples, insulin sensitivity and lipid accumulation in adipocytes regulate PPARγ activity [249, 250, 305].

SIRT1 has a controversial effect on cancer; it is upregulated and serves as a tumor promoter in human prostate cancer [138], acute myeloid leukemia [31], and primary colon cancer [191, 201, 303]. However, it is downregulated in other cancer types such as glioblastoma, bladder carcinoma, prostate carcinoma, and ovarian cancers when compared to the corresponding normal tissues [338], and its overexpression, in these cases, reduces tumor development [64, 81, 107].

Because of its involvement in many pathways, SIRT1 does not seem a simple drug target for specific PPARγ modulation through deacetylation. However, the use of sirtuin modulators has been described to treat diabetes, fatty liver diseases, obesity-induced insulin resistance, and inflammation [190, 340].

Promising SIRT1 activator compounds include the natural polyphenol RSV and SRT1720 [76, 133, 218, 296]. Due to the RSV low bioavailability, some derivatives were developed to improve this characteristic and have already been tested in clinical trials [328]. resVida®, a nutraceutical formulation of RSV, demonstrated beneficial effects in healthy obese men, decreasing intrahepatic lipid content, circulating glucose, triglycerides, alanine-aminotransferase, and inflammation markers, and mimicking the effects of calorie restriction [317]. However, another nutraceutical formulation, Longevinex®, did not modify blood pressure, insulin resistance, lipid profile, or inflammatory markers [89]. SRT501, a commercial micronized RSV formulation, enhanced mitochondrial biogenesis, improved metabolic signaling pathways, and blunted pro-inflammatory pathways in mice fed a high-calorie diet [296]. Moreover, it was shown to lower blood glucose and to improve insulin sensitivity in patients with T2D in a Phase IIa trial [133], requiring further clinical studies.

SIRT1 activators structurally unrelated to RSV were also developed, and some of them, for example, SRT1720, activate the deacetylase more potently than the former [76]. Its therapeutic potential to treat insulin resistance and diabetes was tested in three in vivo models of T2D, and it was able to promote insulin sensitization via metabolic adaptations simulating low energy levels [76, 218, 296]. Administration of SRT1720 reduced fed glucose levels, partially normalizing elevated insulin levels, and reduced fasting blood glucose to near normal levels in mice on HFD, strongly protecting mice from DIO and insulin resistance by enhancing oxidative metabolism in skeletal muscle, liver, and BAT [76, 218, 296].

Another synthetic SIRT1 activator, SRT2104, was tested in a Phase IIa trial in patients with metabolic, inflammatory, and cardiovascular diseases and was shown to significantly attenuate LPS-induced IL-6 and interleukin 8 (IL-8) release and activation of coagulation [328]. The cardiometabolic effects were also evaluated in a clinical trial with T2D, where treatment with SRT2104 resulted in weight loss and deterioration in glycemic control [235].

Nevertheless, these compounds' tissue-specific effects need to be carefully evaluated to avoid undesirable side effects due to the broad spectrum of pathways modulated by SIRT1.

21.4.2.3 SUMOylation

PPARs SUMOylations decreases the activity of the receptors in all isoforms. For PPARβ/δ, SUMOylation physiological effects are not clear yet [168], whereas PPARα-reduced activity is related to hepatic steatosis [185, 257] and PPARγ-decreased activity is desired in the context of metabolic disorders since its activation leads to adipogenesis [216, 353]. Increased deSUMOylation of PPARγ was observed in a report describing the receptor ligand modulating deSUMOylation effects by targeting a SUMO-specific protease, SENP2. Treatment with saturated FA, like palmitate, led to NF-κβ-mediated increase in the expression of SENP2, resulting in increased PPARγ deSUMOylation, and consequently increased PPARγ activity and upregulation of some target genes, such as *Fabp3* and *Cd36* [51].

SUMOylation is a PTM that regulates several biologic processes, including transcription, cell cycle, DNA repair, and innate immunity [24, 284]. This modification is involved with the immune system and inflammatory responses, cancer progression, and Alzheimer's disease, and the modulation of SUMO addition has been described for many therapeutics claims [86, 155, 207, 356].

Inhibition of SUMOylation has been achieved by several natural products, such as ginkgolic and anacardic acids, curcumin, α-lipoic acid, and flavone 2-D08 (flavonoids) [207]. Ginkgolic acid [90], kerriamycin B [91], davidiin [308], and tannic acid [307] are natural products confirmed to inhibit SUMO E1 by blocking formation of SUMO E1–SUMO intermediate. A SUMO E2 protein, UBC9, is increased in many cancers [284], including advanced melanomas, head and neck tumor, lung tumor, HCC, colon cancer, breast cancer, and glioblastoma [4, 44, 51, 182, 225, 226, 318, 346, 354]. UBC9 inhibition is interesting as anticancer therapeutics and GSK145A (doi: https://doi.org/10.1089/adt.2012.501), 2-D08 (2′,3′,4′-trihydroxyflavone) [162], and spectomycin B1, an antibiotic against gram-positive bacteria [298], have been described as UBC9 inhibitors. SUMO-activating enzymes (SAE) 1/2 inhibitors, such as ML-792, have been shown to potently inhibit SUMOylation with a promising application in treating MYC-amplified malignancies [121, 279]. PIAS1 is a SUMO E3 which enhances the SUMOylation of many proteins, including PPARγ. Although it has a potential drug target for cancer therapy, and perhaps obesity-related diabetes, no small-molecule inhibitor has been designed so far for its inhibition [356].

Topotecan, a drug with approval of the US Food and Drug Administration (FDA_ for the treatment of several cancers (e.g., small cell lung cancer, cervical, ovarian) [32, 256], is primarily a DNA topoisomerase I inhibitor; however, it also modulates the SUMOylation status of this protein [68, 222].

Another strategy to modulate the SUMOylation state is to inhibit the deSUMOylates SENPs. Except by SENP2, which is decreased in bladder cancer [309] and HCC [288], other SENP members, such as SENP1, SENP3, and SENP5, are upregulated in various cancers, including neuroblastoma, multiple myeloma, gastric cancer, oral squamous cell carcinoma, and breast cancer [351, 356, 371]. Experimentally, SENP1 inhibitors have exhibited anticancer activities in vitro, including benzodiazepine-based peptidomimetic covalent compounds,

SUMO-derived peptide-based covalent inhibitors, noncovalent 2-(4-chlorophenyl)-2-oxoethyl 4-benzamidobenzoates, 1-[4-(N-benzylamino) phenyl]-3-phenylureas, triptolide, and Momordin Ic [356], showing that SENP1 could serve as a drug target for developing new cancer therapeutics.

SENP2, reported to deSUMOylate PPARδ/β and PPARγ, also regulates SUMOylation levels of the tumor suppressor p53 and ERK5 [173], and it could serve as a drug target to atherosclerotic plaque formation [123] and hepatocellular carcinoma cell growth [288] and cardiac dysfunction [159].

21.4.2.4 Ubiquitination

All PPAR isoforms suffer ubiquitination, and this PTM, in general, regulates the protein level and decreases receptor activity by targeting for protein degradation. The ubiquitin proteasome system (UPS) is composed by sequential actions of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3), which promote ubiquitin addition in internal lysine residues or, more uncommon, in the N termini of target proteins, directing them to proteasomal degradation [363]. There are reports of modulation of this PTM in PPAR by either inhibiting the ubiquitin ligases or the proteasome machinery. Starting with the beginning of the UPS cascade, inhibition of the E1 by E1-I leads to an inhibition of ubiquitination that returns PPARγ protein to control levels even in the presence of rosiglitazone, which usually induces ubiquitin-mediated degradation of PPARγ [158].

UPS is also involved in regulating proteins involved in several pathways, being the dysfunction of its components observed in many pathological disorders, including cancers, cardiovascular diseases, viral diseases, neurodegenerative disorders, and congestive heart failure [19, 348]. For this reason, selective inhibition of the UPS components has significant therapeutic potential. At the beginning of the UPS cascade, inhibitors such as E1-I were developed to inhibit E1 Ub activating enzymes. PYR-41, an irreversible pyrazone derivative inhibitor, was identified to selectively inhibit ubiquitin-like modifier-activating enzyme 1 (UBA1), without affecting E2 and E3 proteins [355]. PYR-41 also partially inhibits NEDD4 [344]. TAK-243, an inhibitor to UBA1, was in 2019 in a clinical trial (phase 1) for advanced solid tumors [140, 217]. Although E1 inhibitors have shown efficacy, an obvious drawback of such compounds is that they influence generically many proteins/cellular networks, which renders their toxicity [348].

The second step of the ubiquitination cascade is controlled by E2 Ub conjugating enzymes (~20), which have been linked to head and neck carcinoma [203], lung cancer [113], and tumor formation [324]. CC0651 is a highly selective inhibitor of the CDC34 E2 enzyme, and its treatment caused accumulation of the cell cycle inhibitor p27 and cyclin E in cells and inhibited proliferation of human cancer cells [39].

At the last step of ubiquitination, E3 Ub ligases transfer the Ub from E2 to the substrate, and there are many kinds of these enzymes since E3 ligases can be divided

in N-end rule family E3α, homologous to the E6AP carboxyl terminus (HECT) domain family (~30), multi-subunit complex family (e.g., SCF complex and anaphase-promoting complex (APC)), and the fascinating new gene (RING) finger ligases (~600), which contains MDM2 and CRL4B [73, 348]. These RING ligases can be separated into single subunit ligases, which can be targeted by enzymatic inhibitors, and into multi-subunit RING finger ligases, which should be targeted in their substrate-specific adaptor bound to the catalytic core. This strategy resembles the one described to inhibit CDK5 phosphorylation: the use of small molecule inhibitors that disrupts the protein-protein interaction between that adaptor and its target protein; however, such specificity can be challenging to achieve.

Among several E3 ligases inhibitor, we are going to focus on molecules described to inhibit proteins involved in PPAR ubiquitination. Nutlin-2 is an inhibitor that disrupts the interaction between MDM2 and p53 by binding directly to the interface of MDM2-p53 contact [326]. Therefore, this inhibitor stabilizes p53 and has a significant anti-tumor effect and has advanced to clinical trials for solid tumors and leukemia [53]. Four MDM2 inhibitors were under clinical trials in 2020: avadomide (CC-122) [267], iberdomide (CC-220) [25], APG-115 [266], and CGM097 [130]. Inhibitors for other E3 ligases that target PPARs were also described: HS-152 inhibitor of SMURF1 [315] and small-molecule covalent inhibitors of NEDD4–1 [151].

Another approach to modulate reversible ubiquitination is to target deubiquitinating enzymes (DUBs) (~100); such inhibitors have been developed to target severe acute respiratory syndrome coronavirus (SARS-CoV), the papain-like protease DUB PLpro [72, 268], USP1 [43], USP7 [42, 271], and USP14 [180].

In the final steps of the UPS cascade, the use of proteasome inhibitors experimentally prevented PPAR degradation. For example, treatment with MG132, a proteasome inhibitor, has demonstrated to increase the level of ubiquitinated PPARα and PPARγ and to inhibit their degradation, therefore increasing their activity [27, 119, 157, 158]. This treatment also partially prevented the decrease of PPARγ levels after C168 S-nitrosylation, suggesting that for this modification, the proteasome-dependent degradation might account for the impaired PPARγ stability [358]. Moreover, the use of the PS341, a selective proteasome inhibitor, inhibited PPARδ/β proteolysis, increasing the half-life of the DNA-bound receptor and therefore increasing its activity [97]. The 26S proteasome is a 2.4 MDa multifunctional ATP-dependent proteolytic complex, which degrades a large variety of cell proteins and is essential for many cellular regulatory mechanisms, that includes cell cycle progression, by the proteasomal degradation of cyclins and inhibitors of CDKs [165], transcriptional regulators (such as c-JUN, E2F-1, and β-catenin) [124], and kinases (such as SRC and protein kinase C (PKC)) [114, 200], terminating specific signal transduction cascades. Furthermore, the ubiquitin-proteasome pathway also plays an essential role in immune surveillance [142], muscle atrophy [221], regulation of metabolic pathways [109, 227], acquisition of long-term memory [40], inflammatory response [212, 242], and in the regulation of circadian rhythms [230] and tumor progression [124, 240].

Due to its involvement in several pathways, inhibition of the 26S proteasome results in a decrease of overall rates of protein breakdown in cells [57, 276], increasing the levels of ubiquitin-conjugated protein, as well as of misfolded and damaged proteins [79, 213, 280, 329]. The long-term exposition to proteasomal inhibitors is toxic for most cells and leads to death by apoptosis [70, 198, 214, 277]. Nonetheless, these undesirable side effects can have a bright side. For example, the accumulation of unfolded polypeptides incites the expression of heat shock proteins, which protects the cells against toxic conditions, including increased temperature or oxygen radicals [181]. Moreover, the ability of some inhibitors to inhibit cell proliferation and selectively induce apoptosis in proliferating cells, together with their ability to inhibit angiogenesis [70, 238], makes these molecules attractive candidates as anticancer drugs [164]. For example, PS341, a potent selective 26S proteasome inhibitor, was the first drug (generic name Bortezomib) targeting the UPS approved by the FDA in 2003, and it is used for patients with multiple myeloma [148]. Two other drugs received FDA approval, carfilzomib (PR-171) [161] and ixazomib [176], and three others were in clinical trials in 2020: oprozomib, delanzomib, and marizomib [36, 176, 255, 348].

21.4.2.5 S-nitrosylation

S-nitrosylation modifications were identified in the AF-1 and DBD of PPARγ and were reported to decrease the receptor activity. There is one report about modulation of PPARγ denitrosylation by inhibiting the GSNOR with 4-[[2-[[(2-cyanophenyl) methyl]thio]-4-oxothieno-[3,2d] pyrimidin-3(4H)-yl]methyl]-benzoic acid [38]. This blockage of GSNOR led to decreased adipocyte differentiation and a decreased expression of PPARγ target genes involved in adipocyte differentiation, indicating that the observed effects are due to the maintenance of the S-nitrosylated state of C139.

S-nitrosylation is a ubiquitous mediator of nitric oxide (NO) signaling and, therefore, is a PTM that occurs in many proteins involved in several physiological processes, including neuronal development and survival, blood pressure regulation, smooth muscle constriction, G-protein-coupled receptor (GPCR) signaling, and endothelial permeability [125, 286, 306]. Dysregulation of this PTM may compromise cell function and cause neurodegenerative diseases, heart failure, and dystrophic-like phenotype in the muscle [21, 101, 231].

To modulate the effects of excessive S-nitrosothiols (SNOs) formation and SNO-proteins, one therapeutic approach would be pharmacological inhibition of NO synthases (NOS) and/or application of antioxidants. Some NOS inhibitors and antioxidant treatments showed limited clinical trial results due to the nonspecificity of NOS inhibitors to NOS isoforms and mixed outcomes with antioxidants in neurodegenerative disorders [231]. Another approach would be to regulate protein denitrosylation with GSNOR modulators as it was reported that GSNOR inhibitor treatment seems to maintain the S-nitrosylated state of PPARγ C139 [38]. However,

in the case of PPARγ, S-nitrosylation would be interesting to use as GSNOR activator to increase denitrosylation and decrease PPARγ adipogenic effects.

However, both approaches have the limitation of being unspecific for the SNO-protein target. About 3000 SNO-proteins have been described both under physiological and pathological conditions [112, 241], making it difficult to modulate an SNO addition in one specific protein with a modulator of a promiscuous enzyme.

21.4.2.6 O-GlcNAc Addition

Generally, an increase in O-linked beta-N-acetylglucosaminylation (O-GlcNAc) is observed during adipocyte differentiation [134, 142], and this differentiation can be blocked by a general decrease in intracellular O-GlcNAc modification induced by pharmacological inhibition of glutamine by fructose-6-phosphate amidotransferase (GFAT) [134, 142]. The compound 6-diazo-5-oxo-L-norleucine (DON) inhibits GFAT, and it is commonly used to decrease intracellular O-GlcNAc modification level [142]. On the other way of O-GlcNAc PTM modulation, treatment with the O-GlcNAcase inhibitor, NButGT, increased O-GlcNAc modifications of PPARγ1 in 3 T3-L1 adipocytes, reducing the nuclear receptor transcriptional activity [145]. However, it must be considered that reduced intracellular levels of O-GlcNAc may affect other glycosylation reactions [335, 366], for example, exacerbating the side effects of Alzheimer's disease and frontotemporal dementia and parkinsonism [359].

O-GlcNAc levels work as a nutrient sensor, and glycosylation reactions regulate not only PPARγ activity in adipocytes but virtually all functional classes of protein since O-GlcNAc addition modulates nearly every cellular process, including signaling, transcription, translation, cytoskeletal functions, and cell division [359]. O-GlcNAc levels are critical in chronic diseases of aging, including diabetes, cancer, neurodegeneration, and cardiomyopathies [78, 205, 233, 244]. Only two proteins, glycosyltransferase OGT and the antagonistic one OGA, regulate O-GlcNAc addition in many proteins in the human body. Their modulation by ligands can deregulate the desired protein target and several other proteins that suffer this modification.

21.5 Perspectives

PPARα has been reported to be a target of phosphorylation, SUMOylation, and ubiquitination. Apart from S12/S21 phosphorylation, all PTMs were reported to decrease the activity of the receptor. Modulation of PTMs with PPARα ligands was described: WY-14643 enhanced polyubiquitination and K358 SUMOylation in the LBD, whereas GW-7647 reduced K185 SUMOylation in the hinge domain.

PPARδ/β is target only of ubiquitination in non-specified residues, signaling for degradation, and SUMOylation at K104, where deSUMOylation of this residue was

reported to increase the receptor activity. Modulation of ubiquitination was reported with PPARδ/β ligands (L-165,041, GW501516, and PGl2), which prevented the ubiquitination of this receptor, thereby decreasing its degradation.

Most efforts have been applied in studying PPARγ due to its involvement in adipogenesis, energetic metabolism, and insulin resistance induced by obesity. Until now, many residues were identified to suffer PTMs, such as phosphorylation, acetylation, SUMOylation, ubiquitination, nitration, S-nitrosylation, and glycosylation. PPARγ acetylation mimetics showed the same effects as the native protein, whereas its deacetylation decreases lipogenic differentiation and promotes the expression of "browning genes" and adiponectin in WAT. SUMOylation, S-nitrosylation, nitration, and O-GlcNAcylation decrease transcriptional activity and so does PPARγ phosphorylation in AF-1 and DBD (S112, Y74, Y78, S16/S21). Although PPARγ phosphorylation in the LBD (S273 phosphorylation) does not change transcriptional activity compared to wild type, the occurrence of this modification is associated with insulin resistance and the recruitment of corepressors. TZDs molecules are PPARγ agonists that received FDA approval to treat type 2 diabetes by targeting the receptor and blocking phosphorylation of S273. However, adverse cardiometabolic effects were reported after rosiglitazone (trade name Avandia) use, and in 2010 it was withdrawn from the market in the UK, Spain, Brazil, and India and some other countries later. The reports of TZDs side effects were associated with full PPARγ activation, and therefore research efforts have been focused on the development of a ligand able to block S273 phosphorylation without fully activating the receptor. In our knowledge, 21 PPARγ ligands (partial agonists and inverse agonists) were reported experimentally to block this phosphorylation with none or reduced side effects compared to TZDs; however, none of them reach and succeed in clinical trials.

Other approaches have been explored to modulate PTMs, for example, the use of modulators of enzymes responsible for the addition or removal of modifications. Nevertheless, as said before, these enzymes target several proteins in many cellular pathways, and its general modulation could have the side effect of dysregulating other cellular mechanisms instead of a specific protein. Despite this nonspecificity, some clinical trials and approved drugs for modulation of SIRT1, CDK5, MDM2, and 26S proteasome at the same time, are under studies.

Until this moment, no treatment has been approved to modulate PPARs PTMs, besides PPARγ S273 phosphorylation. However, the research and understandings of PPARs PTMs and their modulation have primary importance for improving therapeutics' development with more specificity and fewer side effects. For example, it is already known that an ideal drug for diabetes targeting PPARγ should inhibit S273 phosphorylation without a full activation of the receptor. However, recent reports suggest that inhibition of K268/K293 acetylation could prevent the collateral side effects triggered by rosiglitazone activation of PPARγ.

Besides the development of PPAR ligands that inhibit certain PTMs from having the desired effect of insulin sensitization, new drugs for this claim can be developed approaching to inhibit an addition of a PTM by blocking the protein-protein

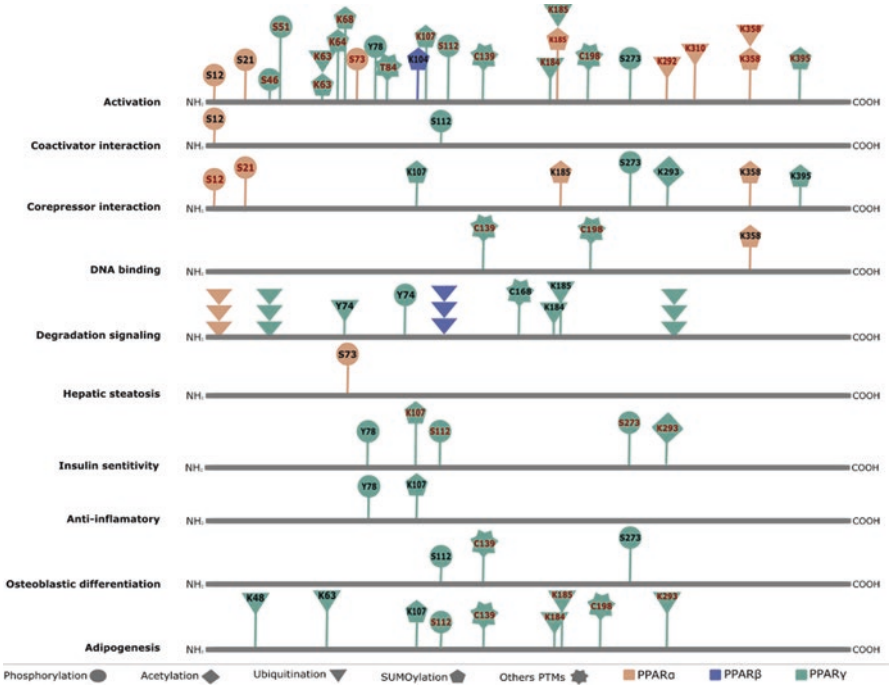


Fig. 21.10 Schematic representation of PPARs posttranslational modification sites grouped by their roles. For PPARγ, the amino acid number is representative of the PPARγ2 isoform

interface with molecules designed specifically for the desired target. Finally, the understanding of PTMS modulation of PPARs is not restricted to the scope of metabolic diseases, as the strategies learned from this receptor can be applied to the development of modulators for other proteins that undergo PTM (Fig. 21.10).

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