# Differential roles of PPARγ vs TR4 in prostate cancer and metabolic diseases

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## Abstract

Peroxisome proliferator-activated receptor γ (PPARγ, NR1C3) and testicular receptor 4 nuclear receptor (TR4, NR2C2) are two members of the nuclear receptor (NR) superfamily that can be activated by several similar ligands/activators including polyunsaturated fatty acid metabolites, such as 13-hydroxyoctadecadienoic acid and 15-hydroxyeicosatetraenoic acid, as well as some anti-diabetic drugs such as thiazolidinediones (TZDs). However, the consequences of the transactivation of these ligands/activators via these two NRs are different, with at least three distinct phenotypes. First, activation of PPARγ increases insulin sensitivity yet activation of TR4 decreases insulin sensitivity. Second, PPARγ attenuates atherosclerosis but TR4 might increase the risk of atherosclerosis. Third, PPARγ suppresses prostate cancer (PCa) development and TR4 suppresses prostate carcinogenesis yet promotes PCa metastasis. Importantly, the deregulation of either PPARγ or TR4 in PCa alone might then alter the other receptor's influences on PCa progression. Knocking out PPARγ altered the ability of TR4 to promote prostate carcinogenesis and knocking down TR4 also resulted in TZD treatment promoting PCa development, indicating that both PPARγ and TR4 might coordinate with each other to regulate PCa initiation, and the loss of either one of them might switch the other one from a tumor suppressor to a tumor promoter. These results indicate that further and detailed studies of both receptors at the same time in the same cells/organs may help us to better dissect their distinct physiological roles and develop better drug(s) with fewer side effects to battle PPARγ- and TR4-related diseases including tumor and cardiovascular diseases as well as metabolic disorders.

## Introduction: the history of cloning/isolation of PPARγ and testicular receptor 4

Peroxisome proliferator-activated receptor γ (PPARγ, NR1C3) was first cloned from *Xenopus* and mice as an orphan nuclear receptor (NR) on the basis of sequence homology to PPARα (NR1C1) (Dreyer *et al.* 1992, Zhu *et al.* 1993), which could induce peroxisome proliferation in rodent hepatocytes (Issemann & Green 1990). Early studies linked PPARγ to adipocyte function because of its high levels of expression in adipose tissues and its ability to stimulate adipocyte differentiation in cultured fibroblasts (Tontonoz *et al.* 1994*a*,*b*). Subsequent studies identified some natural adipogenic regulators, polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA), linolenic acid (LA), and their metabolites, such as 8($S$) hydroxyeicosatetraenoic acid (8(S)-HETE) and 15-deoxy-$\Delta$12,14-prostaglandin $J_2$, (15d-PGJ$_2$) as natural ligands/activators of PPARγ (Forman *et al.* 1995, Kliewer *et al.* 1995, Krey *et al.* 1997). Furthermore, rosiglitazone, one of the thiazolidinediones (TZDs), a class of antidiabetic medicines, was found to bind to PPARγ with a high affinity (Lehmann *et al.* 1995). Since then, more PPARγ selective agonists have been developed to battle type 2 diabetes (Staels & Fruchart 2005).

In contrast, testicular receptor 4 (TR4, NR2C2), another member of the NR superfamily, was initially cloned from human and rat testis (Chang *et al.* 1994), and it shares a high homology (65%) with one of the first identified orphan NRs, TR2 (NR2C1) (Chang & Kokontis 1988, Chang *et al.* 1989). Results from functional studies of TR4 expression patterns (Chang *et al.* 1994, Hirose *et al.* 1994), modulation of target genes (Liu *et al.* 2007), and TR4 knockout ($TR4^{-/-}$) mice (Collins *et al.* 2004, Mu *et al.* 2004, Chen *et al.* 2005, 2008, Zhang *et al.* 2007) indicated that TR4 might play important roles in energy homeostasis as a lipid sensor, as well as in neuronal development and fertility. More importantly, from those extensive *in vivo* function studies, Xie *et al.* (2009) discovered that the PUFA metabolites, 15-hydroxyeicosatetraenoic acid (15-HETE) and 13-hydroxyoctadecadienoic acid (13-HODE) as well as rosiglitazone, could function as ligands/activators to transactivate TR4 to a degree similar to PPARγ. These unexpected results link these two different NRs and raise some very interesting questions. Why does our body need two different NRs that can be activated by similar ligands/activators? Do the ligands/activators bind to them with preferences depending on the context of tissues/cells or diurnal time points? Do they have similar or distinct physiological functions? Do we need to re-evaluate those anti-diabetic drugs that were initially intended to specifically target PPARγ if they actually also activate TR4? Does TR4 activation by those drugs cause any side effects? What are the potential effects on the development of new anti-diabetic medicines?

This review summarizes a comprehensive comparison of PPARγ and TR4 in terms of their sequences, structures, tissue distributions, target genes, ligands/activators, and physiological functions. The potential significance and the possible pathophysiological consequences of the activation of two different NRs by similar ligands/activators will also be discussed.

## Structure analysis and tissue distribution of PPARγ and TR4

### PPARγ and TR4 NR family members

Other than PPARγ, there are two related members in the PPAR family, PPARα (NR1C1) and PPARδ (NR1C2) (Desvergne & Wahli 1999). PPARα is expressed in most tissues, with higher expression levels in brown adipose tissue and liver. PPARγ is mainly expressed in adipose tissue and moderately expressed in the kidney and liver (Kersten *et al.* 2000). PPARδ is ubiquitously expressed in all tissues with the highest expression in the gut and placenta (Bookout *et al.* 2005).

All three PPARs can heterodimerize with retinoid X receptor (RXR) to modulate expression of their target genes (Ferre 2004). The ligand recognition often overlaps among the PPARs. All the three PPARs can be activated by PUFAs with affinities in the following order: PPARα> PPARγ > PPARδ (Bragt & Popeijus 2008).

Three PPARγ transcripts, termed PPARγ1, PPARγ2, and PPARγ3, are derived from the *PPARγ* (*PPARG*) gene by differential promoter usage (Fajas *et al.* 1997). PPARγ1 is broadly expressed and both PPARγ2 and PPARγ3 are highly expressed in adipose tissue. PPARγ3 is also expressed in macrophages. The *PPARγ1* and *PPARγ3* mRNAs yield the same protein product. However, the *PPARγ2* mRNA gives rise to a protein containing an additional 28 amino acids at the N terminus. To date, no functional differences have been found between the PPARγ isoforms.

TR2, which is closely related to TR4, was one of the first identified orphan NRs (Chang & Kokontis 1988). TR2 expression is widespread in various mouse tissues with highest abundance in the testis (Bookout *et al.* 2005). The ligands/activators of TR2 remains unknown, while androgen (Ideta *et al.* 1995) has been reported to repress its expression.

The physiological function of TR2 remains unclear. It was categorized as belonging to the NR cluster that controls lipid metabolism and energy homeostasis by hierarchical, unsupervised clustering based on tissue expression (Bookout et al. 2006). However, TR2 knockout ($TR2^{-/-}$) mice have a normal phenotype, yet TR4/TR2 double knockout ($TR4^{-/-}/TR2^{-/-}$) mice die at the embryonic stage, indicating a functional overlap between TR4 and TR2 (Shyr et al. 2009).

### Sequence homology between PPARγ and TR4

Sequence analyses show that all three members of the PPAR family have a highly conserved DNA-binding domain (DBD) encoding two zinc fingers, and a ligand-binding domain (LBD) in the C-terminal region of the protein. Sequence comparison among these three PPARs using the EMBOSS Pairwise Alignment Algorithms (http://www.ebi.ac.uk/Tools/emboss/align/index.html) found that PPARγ shares an overall 51.6% homology with PPARα (with 84.0% in the DBD and 68.8% in LBD), the while the similarity between PPARγ and PPARδ is 48.4% (with 81.6% homology in the DBD and 72.3% in the LBD) (Fig. 1A).

Sequence comparison between TR4 and TR2 demonstrated that they share a 60.4% identity in overall structure (Fig. 1B), with a 72.4% homology in DBD and a 62.4% homology in LBD.

Amino acid sequence comparison between TR4 and PPARγ found that they share an overall 20.2% identity (134 out of 665 amino acids), with the similarity of their

DBDs reaching 54.2%. The similarity of the LBDs between these two receptors is 21.2%.

Based on the relatively lower sequence homology of LBD between PPARγ and TR4, it is not easy to predict whether these two NRs may similar ligands/activators. For example, the androgen receptor (AR) and estrogen receptor (ER) have 26.3% identity in their LBDs and they do not share their ligands. This interesting question may need detailed 3D structure analyses of PPARγ and TR4 to explain how they can share similar ligands/activators with this low LBD homology.

### Tissue distribution of PPARγ and TR4

PPARγ is expressed in all tissues with the highest expression in white and brown adipose tissues (Bookout et al. 2005), where it is the key orchestrator of the transcriptional cascade during adipocyte differentiation. Its expression is highest postprandially (Vidal-Puig et al. 1996), and its activation leads to upregulation of genes that mediate uptake and trapping of fatty acids. It is known that PPARγ controls the alternative activation of macrophages and improves insulin resistance (Odegaard et al. 2007). Selective activation of PPARγ in skeletal muscle protects mice from diet-induced insulin resistance (Amin et al. 2010). TR4 is ubiquitously expressed in all tissues examined (Bookout et al. 2006), with the highest expression in the CNS and testes. Interestingly, a recent study detected a similar expression pattern of TR4 and PPARγ in female mouse liver hepatocytes, liver endothelial cells, and Kupffer cells (Li et al. 2013), indicating that their expression might be regulated by the same stimuli. On the other hand, patterns of TR4 expression in four major metabolic tissues are rhythmic (Yang et al. 2006), which strongly indicates its importance in regulating metabolic homeostasis. PPARγ also shows a diurnal rhythmic expression pattern in white adipose tissue (WAT) and liver, where its expression peaks at zeitgeber time (ZT)16 and ZT8 respectively, while TR4 expression peaks at ZT4 in both tissues (Table 1).

The distinct diurnal expression pattern of PPARγ vs TR4 might provide the flexibility and specificity that allow these two NRs to function as energy and lipid sensors in response to stimuli to coordinately control metabolism.



**Figure 1**
(A) Protein sequence alignment of PPARα, PPARδ and PPARγ using EMBOSS. (B) Protein sequence alignment of TR2 and TR4 using EMBOSS. (C) Protein sequence alignment of PPARγ and TR4 using EMBOSS.

### Transactivation of PPARγ and TR4 by natural and synthetic ligands

#### Natural ligands: PUFAs and their metabolites

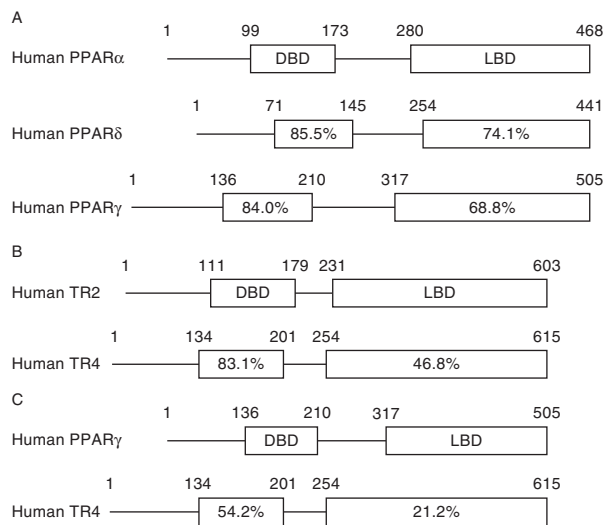PPARγ can be transactivated by a variety of lipophilic ligands, including long-chain PUFAs, such as EPA

**Table 1** The peak expression of TR4 and PPARγ in metabolic tissues. BAT, brown adipose tissue; WAT, white adipose tissue

| Tissues | TR4 | PPAR |
|---|---|---|
| WAT | ZT4 | ZT16 |
| BAT | ZT0 | |
| Liver | ZT4 | ZT8 |
| Muscle | ZT12 | |

(LA (Willson & Wahli 1997, Nagy et al. 1998, Abdelrahman et al. 2005)), and their metabolites, including 9-HODE, 13-HODE, 12-HETE, 15-HETE (Willson & Wahli 1997, Nagy et al. 1998, Abdelrahman et al. 2005) and 15d-PGJ$_2$ (Kliewer et al. 1995). More recently, two other prostaglandins, PG-H(1) and PG-H(2), were also reported to be weak ligands for PPARγ (Ferry et al. 2001).

A metabolite of arachidonic acid, 15d-PGJ$_2$, was first discovered in 1983 (Fitzpatrick & Wynalda 1983), and later in 1995, it was identified as an endogenous ligand for PPARγ (Forman et al. 1995, Kliewer et al. 1995). However, the concentration of 15d-PGJ$_2$ required to stimulate PPARγ is generally in the micromolar range (Powell 2003), and Bell-Parikh et al. (2003) concluded that although 15d-PGJ$_2$ could be generated in vivo, its physiological level is not sufficient to turn on PPARγ. Thus, whether 15d-PGJ$_2$ can be claimed to be an endogenous ligand for PPARγ remains debatable. Nevertheless, 15d-PGJ$_2$ is still the most potent and most commonly used natural agonist for PPARγ to date (Abdelrahman et al. 2005).

TR4 has been an orphan NR without any identified ligand since it was first cloned in 1994 (Chang et al. 1994). Interestingly, Xie et al. (2009) recently found that PPARγ ligands, including TZDs as well as the PUFA metabolites, 15-HETE and 13-HODE, might transactivate TR4 to a level similar to that observed for PPARγ. This discovery was first inspired by the phenotypes found in $TR4^{-/-}$ mice, which display reduced fat mass, smaller adipocyte size, low glucose levels at birth and under fasting conditions, and improved insulin sensitivity (Liu et al. 2007, 2009, Xie et al. 2009, Kim et al. 2011). Further studies showed that TR4 might function as a fatty acid sensor via modulation of several key genes, such as CD36, SCD1, and PEPCK, which are involved in lipid metabolism and insulin sensitivity (Liu et al. 2007, 2009, Xie et al. 2009, Kim et al. 2011). We then screened many CD36 inducers and found that PUFA metabolites, such as 13-HODE and 15-HETE, could induce CD36 expression via transactivation of TR4. Protease peptide mapping assays further demonstrated that trypsin digestion patterns of TR4 were changed in the presence of 13-HODE or 15-HETE, indicating that the receptor

conformation was changed due to the direct binding between TR4 and 13-HODE or 15-HETE (Xie et al. 2009). These ligands/activators were able to transactivate TR4 and PPARγ in a similar manner in the same cells, indicating that both TR4 and PPARγ may function as fatty acid sensors upon activation by these ligands/activators.

**Synthetic ligands: TZDs**

In addition to natural ligands, a wide range of synthetic PPARγ agonists have been developed, including the TZDs, such as rosiglitazone, pioglitazone, ciglitazone, and troglitazone, that were widely used as anti-diabetic drugs (Quinn et al. 2008). In 1997, troglitazone was the first TZD to be approved for the treatment of patients with type 2 diabetes but was withdrawn from the market in 2000 following the emergence of a serious hepatotoxicity in some patients (Booth et al. 2000). Rosiglitazone and pioglitazone also have side effects including increased risk of heart attacks and bladder cancer (BCa) that caused the drugs to be placed under sales restrictions in the USA. TZDs were screened out via modulation of PPARγ transactivation that resulted in the alteration of expression of several key genes involved in glucose and lipid metabolism and energy balance (Hauner 2002). These TZDs may bind to PPARγ closely given their in vivo hypoglycemic potency (Abdelrahman et al. 2005) that increases the ability of insulin to suppress endogenous glucose production resulting in enhanced insulin sensitivity in adipose tissue (Nolan et al. 1994). However, clear evidence also indicate the existence of PPARγ-independent effects of treatment with TZDs (Lecomte et al. 2008, Vandewalle et al. 2008). Finding that TZDs can also transactivate TR4 (Xie et al. 2009) may therefore raise a very interesting question of whether TZDs may yield differential effects via activating these two different NRs.

**Analysis of 3D structure via X-ray crystallography to distinguish ligand specificity of PPARγ and TR4**

X-ray crystallography has greatly improved our understanding of how NRs are activated by the binding of their specific ligands and the subsequent conformational changes (Nolte et al. 1998). The apo-PPARγ LBD possesses a canonical helical sandwich fold as seen in other NRs. Unlike other NRs, PPARγ has an additional α-helix (designated H2), which creates a solvent-accessible channel between H3 and the β-strands, and this channel seems to serve as an entry to the ligand-binding pocket. The rosiglitazone-bound PPARγ has been detected as a homodimer (Nolte et al. 1998), and more recently, as a

heterodimer with RXRα (Gampe et al. 2000), which might be more physiologically relevant. In both cases, only the S-isomer of rosiglitazone was found bound to PPARγ, even though a racemic mixture was used in the crystallization. Consistent with this observation, ligand-binding data also indicate that only the S-isomer of rosiglitazone is a high-affinity ligand for PPARγ.

The overall root-mean-square deviation between PPARγ-LBD and TR4-LBD is 5.57, indicating that the overall conformation of these two proteins is different. Nonetheless, PPARγ-LBD shares common structural characteristics with TR4-LBD. Structural comparison of both apo-PPARγ and apo-TR4 LBD structures reveals that the proximal half of the three layer helices is highly similar, suggesting that the helix sandwich fold is evolutionarily conserved between these two proteins (Fig. 2A).

Despite the similarity of the proximal halves of the LBDs between PPARγ and TR4, the ligand-binding pockets of the two receptors, which are located in the distal half of the LBD, vary greatly. The apo-PPARγ ligand binding cavity has a large T-shape pocket of ∼1300 Å$^3$ (Nolte et al. 1998). Unlike PPARγ, the recently discovered apo-TR4 structure reveals an absence of a ligand-binding pocket (Zhou et al. 2011). The C-terminal part of helix 10 bends and collapses into the space where the ligand-binding pocket should be (Fig. 2B). The large pocket of the PPARs, as compared with that of TR4, allows these receptors to bind to diverse metabolites promiscuously and with a low affinity. Several studies indicate that some essential fatty acids, oxidized lipids, and prostaglandin J$_2$ metabolites can bind to and activate PPARγ at micromolar concentrations (Kliewer et al. 1997). When PPARγ is bound to rosiglitazone, rosiglitazone was seen to occupy 40% of the ligand-binding pocket. Currently, there is no crystal structure of an active TR4 even though Zhou et al. (2011)

have attempted to make a computational model of the active TR4 structure. TR4's ligand binding pocket was estimated to be 560 Å$^3$, suitable for accommodating small ligands (Zhou et al. 2011). Apart from the size of the ligand binding pocket, the shape and hydrophobic/hydrophilic nature of the pocket surface also play important roles in determining the ligand-binding specificity. PPARs have a distinct three-armed Y-shaped conformation, allowing them to bind to ligands with multiple branches or to bind to single-branched ligands such as fatty acids with multiple conformations (Xu et al. 1999). Unlike PPARs, TR4 has been suggested to be activated by retinol (vitamin A) (Zhou et al. 2011). According to the above structural analysis, due to the differences in size and shape of their ligand-binding pocket, PPARs should be regulated by a larger diversity of ligands compared with TR4. The fine molecular mechanism of why TR4 and PPARγ can be mediated by similar ligands is currently unknown due to a lack of more detailed structural information for TR4. Further structural characterization of the TR4 complex with a ligand would be crucial to explain the similarity between TR4 and PPARγ in ligand recognition.

## Transactivation of PPARγ and TR4 by receptor conformation changes via protein modulations

### Phosphorylation

Early studies concluded that phosphorylation/dephosphorylation of NRs by various kinases/phosphatases could result in conformational changes within the receptors to either induce or repress their transactivation (Jiang & Hunter 1999). This was especially important for the study of orphan receptors, including TR4 and PPARγ, before identification of their ligands.

Through peptide mapping and motif scan by Scansite (http://scansite.mit.edu), Kim et al. (2011) found that the serine 351 (S351) phosphorylation site, which is conserved in mouse, human, rat, and chicken TR4, is a potential target of AMPK, and applied an in vitro phosphorylation assay to prove that TR4 could be phosphorylated by AMPK in the presence of AMP. Mutation of S351 completely abolished the phosphorylation of TR4 by AMPK. Suppression of AMPK via compound C led to enhanced TR4 transactivation, whereas the activation of AMPK by AICAR resulted in suppression of TR4 transactivation. Metformin, an anti-diabetic drug, also suppressed TR4 transactivation through AMPK-mediated TR4 phosphorylation (Kim et al. 2011).
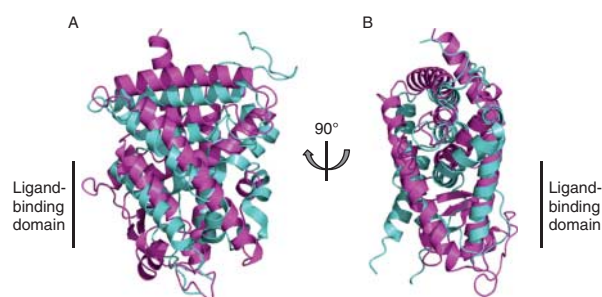


**Figure 2**
(A) Front view overlay of Apo PPARγ (pink) with Apo TR4 (cyan). (B) Side view overlay of Apo PPARγ (pink) with Apo TR4 (cyan). (AF2 domains were removed for a clear view of the ligand binding pockets.).

AMPK could also suppress PPARγ transactivation via phosphorylation of PPARγ in both ligand-dependent and ligand-independent mechanisms (Leff 2003), and TZDs might improve insulin sensitivity via increasing the expression and release of adiponectin, an adipokine that activates AMPK. The details of PPARγ phosphorylation by AMPK, however, remain unclear.

TR4 could also be phosphorylated by MAPK at its AF1 domain in the absence of specific ligands (Huq et al. 2006). MAPK activation by anisomycin causes a nearly complete loss of TR4 transactivation. In contrast, the inhibition of MAPK by PD98059 significantly increases TR4 transactivation. A mass spectrometric analysis of TR4 revealed three MAPK phosphorylation sites, Ser19, Ser55, and Ser68 in its AF1 domain. Site-directed mutagenesis studies demonstrated the functionality of phosphorylation on Ser19 and Ser68 but not Ser55. TR4 functions as a repressor when MAPK mediates the phosphorylation of the AF1 domain through the preferential recruitment of corepressor RIP140. In contrast, TR4 functions as an activator when its AF1 domain is dephosphorylated due to its selective recruitment of the coactivator PCAF (Huq et al. 2006). However, the potential physiological consequences of these MAPK phosphorylations of TR4 remain unclear. Transfection with a dominant negative MEK results in a decrease in the effects of both insulin and TZDs on PPARγ activity, indicating that MAPK is able to modulate PPARγ transactivation (Zhang et al. 1996). In vitro assays demonstrated that ERK2 and JNK were able to phosphorylate PPARγ2 (Adams et al. 1997). The ERK- and JNK-MAPK phosphorylation sites (Camp et al. 1999) were mapped to Ser82 of mouse PPARγ1 and Ser112 of mouse PPARγ2 (Shao et al. 1998) respectively. Substitution of this serine by alanine (S82A) leads to a loss of MAPK activator PDGF-mediated repression of PPARγ activity (Camp & Tafuri 1997). Phosphorylation of human PPARγ1 at this Ser84 results in inhibition of both its ligand-dependent and ligand-independent transactivations, and S84A mutant showed an increase in the AF-1 transactivation of PPARγ (Adams et al. 1997). Mutation of the MAPK Ser112 phosphorylation site in PPARγ2 (S112D) results in a decreased ligand-binding affinity (Shao et al. 1998). With substitution of proline by glutamine at position 115, the human PPARγ becomes constitutively active through the modulation of the MAPK-dependent phosphorylation status of Ser114 (Ristow et al. 1998). Subjects carrying this mutation are extremely obese but surprisingly show a lesser insulin resistance than expected. Overall, these data indicate that prevention of PPARγ phosphorylation may lead to an improvement of insulin sensitivity, which is similar to treatment TZDs that results in the transactivation of PPARγ (Rangwala et al. 2003).

The PKA activator, cholera toxin, could also increase the basal and ligand-induced activity of PPARγ (Lazennec et al. 2000), although details of this activation remain unclear. Treatment with PKA stimulators markedly increased PPARγ activity (Akahoshi et al. 2003). Interestingly, Liu et al. (2009) also found cAMP/PKA signals could induce TR4 expression by triggering C/EBPα and β binding to the selective cAMP response elements located on the TR4 promoter that leads to the modulation of gluconeogenesis.

### Sumoylation

Several laboratories recently reported that PPAR activity can be modulated by sumoylation (Ohshima et al. 2004, Yamashita et al. 2004, Pascual et al. 2005, Jennewein et al. 2008, Lai et al. 2008). Two sumoylation sites have been identified for PPARγ2 in mouse, lysine 107 (K107) in the AF1 domain, and lysine 395 (K395) in the AF2 domain (K77 and K365 in murine PPARγ1 respectively). Sumoylation of PPARγ2 at K107 inhibits PPARγ-dependent gene induction, possibly by recruitment of corepressors (Ohshima et al. 2004, Yamashita et al. 2004). K107 sumoylation of PPARγ has no effect on its trans-repression function. In contrast, sumoylation of PPARγ2 at K395 leads to its recruitment to the promoters of inflammatory genes, where it represses gene transcription by preventing clearance of corepressor complexes (Pascual et al. 2005). The detailed molecular mechanism(s) behind sumoylation-mediated regulation of PPARγ activity need to be established. It was recently discovered that FGF21 increases PPARγ activity by preventing its sumoylation at K107 while it has no effect on K395 sumoylation. FGF21 is required for the effects of rosiglitazone in WAT (Dutchak et al. 2012).

There have been no studies yet regarding the sumoylation of TR4. However, it has been shown that its close relative, TR2, can be sumoylated, resulting in the replacement of coregulators recruited to the regulatory region of its endogenous target gene Oct4 (Pou5f1). Unsumoylated TR2 can activate Pou5f1 to enhance embryonic carcinoma cell proliferation and is localized on the promyelocytic leukemia (PML) nuclear bodies. Increased expression of TR2 may lead to more sumoylation at K238 that may result in release from the nuclear bodies to act as a repressor. Sumoylation of TR2 also induces an exchange of its corepressor RIP140 with the coactivator PCAF, which can then switch TR2 from an activator to a repressor. This involves dynamic

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partitioning of TR2 into Pml-containing and Pml-free pools (Park *et al.* 2007, Gupta *et al.* 2008).

## Acetylation

TR4 can be acetylated at K175 and K176, which attenuates its DNA binding ability to TR4 response elements (TR4REs) on its target genes (Xie *et al.* 2011). The coregulator ARA55 increased TR4 acetylation levels via modulation of HAT enzymes bound to the ARA55–TR4 complex. However, the physiological function of acetylated TR4 remains unclear (Xie *et al.* 2011).

Few results regarding acetylation of PPARγ have been reported. However, COUP-TFII may be able to recruit the SMRT corepressor complex to the first intron of both PPARγ1 and PPARγ2 and maintains the local chromatin in a hypoacetylated state, which may lead to the suppression of adipogenesis (Okamura *et al.* 2009).

## Ubiquitination

The PPARγ protein has a short half-life ($t_{1/2} < 4$ h) in adipocytes (Waite *et al.* 2001). Certain PPARγ ligands stimulate a reduction in PPARγ protein level, and the ligand-dependent degradation of PPARγ by the proteasome is regulated by polyubiquitination (Hauser *et al.* 2000). The AF2 domain of PPARγ is important for ligand-induced downregulation (Hauser *et al.* 2000). Interferon-γ also promotes the ubiquitination and degradation of PPARγ in fat cells (Floyd & Stephens 2002). Interestingly, a recent finding indicates that PPARγ itself can act as an E3 ubiquitin ligase and induce NFκB/p65 ubiquitination and degradation (Hou *et al.* 2012), although no evidence indicates that PPARγ regulates the ubiquitination by itself. To date, there are no reports of ubiquitination studies on either TR4 or TR2.

## Signaling pathways of TR4 vs PPARγ

### Signaling pathways via downstream target genes with conserved DNA response elements

As TR4 and PPARγ share some homology in their DBD and they both can be activated by similar ligands/activators, one might expect that they may also modulate similar downstream target genes via binding to the similar DNA response elements existing in the promoter region of their target genes. TR4 binds to TR4REs, which consist of an imperfect direct repeat (DR) of two consensus sequences, AGGTCA, separated by 0–5 spacing nucleotides (Lee *et al.* 2002). Of the DR sequences recognized by TR4, DR-1, has the highest affinity for TR4 (Kim *et al.* 2003). In contrast to the broad range of TR4REs with various spacing nucleotides, and PPARγ binding sites, PPREs, are generally DRs with one intervening nucleotide (DR-1). Tables 2 and 3 summarize and compare the TR4REs and PPREs that were found in identified target genes.

Although TR4 and PPARγ recognize similar DNA sequences and modulate many similar target genes, there are also distinct target genes that so far are only regulated by either TR4 or PPARγ. For example, SV40 has been

**Table 2** The target genes and binding sites of TR4 and PPARγ

| Target genes | TR4 binding sites | PPARγ binding sites |
|---|---|---|
| *PEPCK* | DR1 (−439/−451) | DR1 (−999/−987) |
| *GATA1* | DR1 | NA |
| *CRBPII* | DR1 | NA (PPARα) |
| Apolipoprotein E (*ApoE*) | DR1 (−319) | DR1 (−623) |
| *HBV* | DR1 | NA |
| *CNTFR* | DR1 | NA |
| *NDUFAF1* | DR0, DR2, DR5 | NA |
| *SV40* | DR2 | NA |
| Rat *P450cc24* | DR3 | NA |
| Rat *α-MHC* | DR4 | NA |
| Oxytocin | DR0, DR4 | NA |
| *S14* | DR4 | NA |
| *RARβ* | DR5 | NA |
| *CD36* | DR1 | DR1 |
| HIV-LTR | DR9 or half site | NA |
| *SCD1* | DR0 | DR1 |
| *FATP1* | DR1 | DR1 |
| Steroid 21-hydroxylase | Half site | NA |
| Rev-Erbα | | DR2 |
| *LXRα* | | DR1−722 and −710 bp |

**Table 3** Nuclear receptors TR4 and PPARγ are fatty acid sensors via modulation of similar/distinct target genes

| Tissues | PPAR target genes | TR4 target genes |
|---|---|---|
| Adipose | ↑Adiponectin, *FABP*, *FATP1*, *CD36*, *PEPCK*, glycerol kinase<br>↓*PAI1*, resistin, *IL6*, TNFα, leptin, *11β-HSD1*<br>↑LPL, acyl-coa synthase, β3-AR<br>↑c-Cbl associating protein, *GLUT4*, *PI3K*, *IRS1*, *IRS2* | NA |
| Liver | ↓*PEPCK* (↑*PEPCK* in adipose tissue)<br>↑*SCD1*<br>↑*GLUT2*, liver type glucokinase (LGK) | ↑*PEPCK*<br>↑*SCD-1* |
| Pancreas | ↑*GLUT2*, *GLUT4* | NA |
| Muscle | ↑*GLUT2*, *GLUT4*, adiponectin, *PI3K*, ↓*PDK4*<br>↓*UCP3* | *Ndufaf1* |
| Macrophage | ↑*CD36*<br>↑*LXRα*, *ABCA1*<br>↓*iNOS*, *IL6*, TNFα, *MCP1*, *IL12*, IFNγ | ↑*CD36* |
| Kidney | ↑Na$^+$ channel | NA |
| Vascular smooth muscle cell | ↑p27$^{Kip1}$ and RB<br>↑AMPK activity | NA |

identified as a TR4 target gene (Lee *et al.* 1995), but not a PPARγ target gene. It is not completely clear whether these two receptors compete for DNA binding, or whether they complement each other in different tissues, at different developmental time points. However, mouse embryos lacking PPARγ are not viable by embryonic day 11 (E11) as a result of severe trophoblast dysmorphogenesis (Barak *et al.* 1999), and mice lacking TR4 also have serious development defects (Collins *et al.* 2004). Based on the phenotypes of $TR4^{-/-}$ and PPARγ knockout mice ($PPARγ^{-/-}$), TR4 and PPARγ seem to have unique properties that are essential for the viability of the organisms.

### Signaling pathways via modulation of interacting coregulators

Coregulators are NR-interacting proteins that modulate the NR function. The downstream signaling pathways of both PPARγ and TR4 are subject to regulation by their coregulators (see details in Table 4).

**NRs as interacting coregulators for TR4 vs PPARγ** TR4 is a unique NR with the capacity to form heterodimers with various other NRs to modulate its transactivation. TR4 forms heterodimers preferentially with TR2 in solution as well as on TR4RE containing DR5. The three leucine residues on helix 10 of TR2 are critical for this dimerization (Lee *et al.* 1998). Coexpression of TR4 and TR2 resulted in a much stronger repression of a DR5-containing reporter than expression of either receptor alone. In the developing testis, TR4 and TR2 are expressed in the same cell populations and exhibit a parallel expression pattern during development (Lee *et al.* 1998).

TR4 interacts with AR, and this heterodimerization prevents TR4 from binding to its target DNA and thus suppresses expression of TR4 target genes. Interestingly, expression of AR target genes was also repressed by the dimerization with TR4 (Lee *et al.* 1999). ARA55 is one of the coregulators that AR and TR4 share (Xie *et al.* 2011). ARA55 enhances AR transcriptional activity but inhibits TR4 activity. We also found that inactivation of ARA55 inhibits the agonist effect of antiandrogens in prostate cancer (PCa) cells (Rahman *et al.* 2003). Does increased TR4 activity induced by ARA55 inactivation play a role here? Further experiments are needed to answer this question. To date, no evidence indicates that PPARγ directly binds to AR. However, it has been found that testosterone inhibits the activity of PPARγ (Du *et al.* 2009) and troglitazone suppresses AR in a PPARγ-independent manner (Yang *et al.* 2007). TR4 can also repress ER-mediated transactivation through direct protein–protein interaction. The interaction between TR4 and ER would suppress the homodimerization of ER and prevent ER from binding to the estrogen response element that results in the suppression of ER target genes and ER-mediated cell proliferation in MCF-7 cells (Shyr *et al.* 2002). A physical interaction between PPARγ and ERα was also observed in MCF-7 cells (Bonofiglio *et al.* 2005). Treatment with 17β-estradiol or a PPARγ agonist, BRL49653, decreased this interaction slightly, whereas an ER inhibitor, ICI182780, strongly inhibited the association. It is still not very clear how this interaction affects the activity of the two receptors. Houston *et al.* (2003) found that in leiomyoma cells, the stimulation of PPARγ signaling inhibited ER-mediated cell growth and gene expression. On the other hand, 17β-estradiol suppressed

**Table 4** PPARγ and TR4-associated proteins

| Associated protein | Effect on TR4 | Effect on PPAR |
|---|---|---|
| RIP140 | Corepressor[198] | |
| AR | TR4 represses AR-mediated transactivation and thus expression of its target genes; AR prevents TR4 from binding to its target DNA[88] | |
| TIP27 | Corepressor | |
| TRA16 | Corepressor | |
| ER | Inhibits the homodimerization of ER and interrupts/prevents ER binding to the estrogen response element[92] | |
| CBP/p300 | Corepressor | Coactivator |
| HDAC | Corepressor | |
| ARA55 | Corepressor | |
| TR2 | Exerts a synergistic biological activity | |
| HNF4 α | TR4 may suppress HBV core promoter activity via the interruption of the HNF4α-induced HBV core promoter by protein–protein interaction with HNF4α | |
| ARA70 | | Coactivator |
| SRC-1,2,3 | | Coactivator |
| PGC-1 | | Coactivator |
| PRIC285 | | Coactivator |
| PBP | | Coactivator |
| PRIP | | Coactivator |
| BAF60c | | Coactivator |
| TRAP220 | | Coactivator |
| NCoR | | Corepressor |
| SMRT | | Corepressor |
| Sirt1 | | Corepressor |

PPARγ-induced adipogenesis and adipocyte-specific gene expression through both ERα and ERβ (Foryst-Ludwig et al. 2008, Jeong & Yoon 2011).

TR4 also functions as a transcriptional suppressor via competing for the same hormone response elements (HREs) that are bound by other NRs, such as RAR/RXR and VDR/RXR. Interestingly, TR4 can also interact with T3R and consequently promoted ligand-induced thyroid hormone receptor α-mediated transcriptional activity and expression of its downstream target genes and cellular functions (Huang et al. 2010).

PPARγ can form a heterodimer with RXRα to bind to promoters of downstream target genes (Kliewer et al. 1992). The heterodimerization with RXR is required for PPARγ DNA binding (Tan et al. 2005). As RXR is a common heterodimeric binding partner for several NRs, PPARγ usually needs to compete with other NRs, including VDR (Wood 2008) or thyroid hormone receptor α (Wang et al. 2005), for a limited amount of the RXR.

A sequence alignment of PPARγ and TR4 revealed a key amino acid Σ residue in the PPARγ heterodimerization motif and this amino acid was changed to an alanine in the TR4 motif (Fig. 3). This Σ residue (E or D) is crucial for forming a salt-bridge with R393 in helix 9 of RXR; thus, this amino acid difference might, in part, explain the lack

of interaction between TR4 and RXR. Structurally, PPARγ and TR4 are restricted to form different dimers, thus resulting in different signaling pathways. There has also been a report that PPARγ can physically interact with the glucocorticoid receptor (GR; Nie et al. 2005), and this interaction resulted in the inhibition of histone H4 acetylation and p65 binding to the eotaxin promoter and the suppression of eotaxin gene transcription. More interestingly, there was a recent report that claimed that TZDs are partial agonists for the GR (Matthews et al. 2009), that can induce GR phosphorylation (Ser211), which was once believed to be a GR ligand-binding-specific effect in both PPARγ-expressing and PPARγ-null cells.

**Coactivators as interacting coregulators for PPARγ and TR4**    Steroid receptor coactivator (SRC-1) was identified as a PPARγ coactivator that can enhance ligand-induced PPARγ transactivation (Zhu et al. 1996, Nolte et al. 1998). PPARγ can also interact with members of the SRC family such as SRC-2 and/or SRC-3 to enhance its transactivation, which results in positive effects on fat storage (Hartig et al. 2011). The absence of SRC-3 may abolish adipocyte differentiation by altering PPARγ-mediated transactivation of genes that are important for lipid storage (Picard et al. 2002). Indeed, in SRC-3$^{-/-}$

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                                    468
PPARγ  459  L  F  A  K  L  L  Q  K  M  T  D  L  R  Q  I  V  T  475
TR4    539  R  L  A  R  I  L  V  R  L  P  A  L  R  L  M  S  S  N  556
                                     ↑
```

**Figure 3**
Sequence alignment of PPARγ with TR4 at heterodimerization motif in N-terminal of helix 10. Arrow indicates the position of the Σ substitution.

mouse embryonic fibroblasts (MEFs), adipocyte differentiation was severely impaired, and re-expression of SRC-3 was able to restore the ability of MEFs to differentiate into adipocytes. At the molecular level, SRC-3 acts synergistically with the transcription factor CAAT/enhancer-binding protein to control the gene expression of PPARγ2.

Subunit 1 of the mediator complex (named Med1 or PBP), which represents a large complex of coactivators, plays an important role in adipocyte differentiation by coactivating PPARγ (Misra et al. 2002). PPARγ-interacting protein (PRIP) also acts as a molecular scaffold that cooperatively enhances PPARγ/RXR-mediated transactivation that results in positive regulation of adipogenesis via binding to the aP2 promoter in adipocytes (Qi et al. 2003).

PGC-1 is an unusual coactivator among known NR coactivators in that its expression is dramatically regulated with respect to both tissue selectivity and the physiological state of the animal (Feige & Auwerx 2007). PGC-1 is also distinct from the known coactivators in that it appears to use different sequence motifs for protein–protein docking, on both sides of the receptor-coactivator pair. Nearly all the known coactivators and corepressors utilize LXXLL sequences to bind to the ligand-regulated helix 12 in the carboxy terminal AF-2 domain. In contrast, PGC-1 utilizes a domain rich in proline residues to bind to a region that overlaps the DNA-binding and hinge region of PPARγ (Puigserver et al. 1998). There had been few identified coactivators for TR4 until Huq et al. (2006) identified a classic steroid receptor family coactivator, p300/CREB-binding protein-associated factor (PCAF). Although originally characterized as a histone acetyltransferase, PCAF also acetylates non-histone transcription-related proteins like p53 (Schiltz & Nakatani 2000). TR4 activity is positively regulated by PCAF through an unknown mechanism. Both WT full-length TR4 and TR4 LBD deletion mutant proteins were found to interact strongly with PCAF, indicating that the LBD of TR4 is not required for the interaction (Huq et al. 2006). Interestingly, the authors also found that the hyperphosphorylated TR4

preferentially recruited corepressors while the hypophosphorylated TR4 recruited coactivators (Huq et al. 2006).

**Corepressors as interacting coregulators for PPARγ and TR4** Formation of the corepressor complex including PPARγ with NCoR and SMRT may result in suppression of transactivation of the PPARγ-mediated deacetylase Sirt1, which may then lead to reduced loss of fat via suppression of lipolysis with a limitation of fatty acid mobilization (Guan et al. 2005). Addition of siRNA for NCoR and SMRT may also result in enhanced adipogenesis via increased PPARγ-mediated pro-adipogenic target gene expression. PPARγ can recruit SMRT and NCoR in the absence of ligands to modulate PPARγ-mediated transcriptional activity (Yu et al. 2005).

The PDZ-binding motif (Miglio et al. 2009) is another ligand-independent PPARγ corepressor that inhibits adipogenesis (Debril et al. 2005). The scaffold attachment factor B1 (SAF-B1) and unphosphorylated RB also function as PPARγ corepressors involved in regulation of adipogenesis (Debril et al. 2005). The unphosphorylated RB can inhibit the clonal expansion phase of adipocyte differentiation by recruiting histone deacetylase 3.

The tribbles homolog 3 (TRB3), containing a single kinase domain without enzymatic activity, downregulates PPARγ transcriptional activities by protein–protein interaction (Takahashi et al. 2008). TRB3 gene and protein expression was increased during adipocyte differentiation concomitantly with an increase in the mRNA levels of CCAAT/enhancer binding protein homologous protein. Forced TRB3 expression in 3T3-L1 cells decreased the mRNA levels of PPARγ-target genes and intracellular triglyceride (TG) levels and TRB3 could also inhibit PPARγ-dependent adipocyte differentiation in PPARγ-expressing 3T3-L1 cells (Takahashi et al. 2008).

TIP27 has two zinc finger motifs and can function as a TR4 corepressor. TIP27 interacts with TR4 through the region spanning Asp39 and Lys79, leading to suppression of TR4-mediated DR1 transactivation. This repression does not involve the inhibition of TR4 homodimerization or

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DR1 binding but may be due to an interruption of interaction between TR4 and its coactivators (Nakajima et al. 2004).

TR4-associated protein (TRA16) is a selective TR4 corepressor with little influence on the transactivation of AR or GR. TRA16 may suppress TR4-mediated transactivation via decreasing TR4 binding to the TR4REs on the target gene(s). TRA16 can also block the interaction between TR4-DBD and TR4-LBD (Yang et al. 2003). These unique suppression mechanisms indicate that TRA16 may function as a novel corepressor to selectively suppress TR4-mediated transactivation.

## The pathophysiological functions of PPARγ and TR4

### Metabolic diseases

The first striking finding revealing the opposing functions of these two receptors comes from studies on their modulation of insulin sensitivity: loss of PPARγ reduced insulin sensitivity (Kubota et al. 1999); in contrast, loss of TR4 increased insulin sensitivity (Kim et al. 2011).

The development of insulin resistance is an early event in the onset and progression of type 2 diabetes mellitus (T2DM) and PPARγ ligand TZDs have been used in T2DM as insulin sensitizers with adipose tissue as the primary target tissue where PPARγ is predominantly expressed. The functional mechanism of TZDs as insulin sensitizers includes the following: i) they can selectively stimulate lipogenic activities in fat cells resulting in greater insulin suppression of lipolysis (Oakes et al. 2001), thus decreasing infiltration of free fatty acids (FFAs) into other tissues leading to the insulin-desensitizing effects of FFAs in muscle and liver (Granberry et al. 2007). ii) TZDs can alter the expression and release of adipokines, such as resistin and TNF-α, that results in alteration of insulin sensitivity (Steppan et al. 2001). Conversely, the level of adiponectin is increased after treatment with TZDs (Maeda et al. 2001). iii) TZDs can enhance the lipid storage capacity of adipose tissue by increasing the number of small adipocytes. This lipogenic effect may lead to a reduction of deleterious lipid accumulation in other insulin-sensitive tissues such as the muscle and liver, thus reducing insulin resistance (Okuno et al. 1998). Since PPARγ may function as a key factor for adipogenesis and a critical determinant of body fat distribution (Tsai & Maeda 2005), T2DM patients treated with TZDs may develop an accumulation of subcutaneous fat with reduced or unchanged visceral adipose tissue volume. This is a positive effect because subcutaneous adipose tissue is metabolically less harmful than visceral adipose tissue despite their tendency to induce weight gain (Bays et al. 2004). In summary, TZDs can ameliorate insulin resistance due to lipotoxicity in obesity- and lipodystrophy-associated T2DM (Unger & Orci 2001). In contrast, $TR4^{-/-}$ mice have reduced fat mass and smaller adipocytes. $TR4^{-/-}$ mice display low glucose levels at birth and under fasting conditions have improved insulin sensitivity, indicating that TR4 is an important modulator of insulin sensitivity and glucose/lipid metabolism. TR4 also controls liver glucose metabolism through transcriptional regulation of phosphoenolpyruvate carboxykinase (PEPCK) (Liu et al. 2007), the key enzyme in gluconeogenesis (Postic et al. 2004). PEPCK expression is reduced in $TR4^{-/-}$ mice, and the hypoglycemia at birth and under fasting conditions in $TR4^{-/-}$ mice is caused by failure to induce PEPCK expression.

In addition to glucose metabolism, TR4 also plays an important role in lipid metabolism by directly regulating the expression of stearoyl-CoA desaturase 1 (SCD-1) (Kim et al. 2011), which is the rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids. SCD-1 activity has been implicated in obesity, diabetes, lipogenesis, β-oxidation, and insulin sensitivity (Cohen et al. 2003, Cohen & Friedman 2004). $TR4^{-/-}$ mice showed reduced fat volume and TG deposition and improved insulin sensitivity that may be partially due to reduced SCD-1 expression. Moreover, TR4 controls fatty acid uptake through transcriptional regulation of fatty acid transport protein 1 (FATP1) in 3T3-L1 adipocytes (Choi et al. 2011).

More questions remain to be answered; for instance, how the newly identified TR4 upstream modulators, such as TZDs, could affect PEPCK and SCD-1 expression, thus altering systemic lipid and glucose metabolism as well as insulin sensitivity.

### Cardiovascular diseases

The second striking finding is that PPARγ attenuates atherosclerosis (Francis et al. 2003) but TR4 might increase the risk of atherosclerosis (Xie et al. 2009) in animal models.

Atherosclerosis is a vascular disease that arises from an imbalance in lipid metabolism and a chronic immune response driven by the accumulation of cholesterol-laden macrophages in the artery wall (Moore et al. 2013). It is the usual cause of heart attacks, strokes, and other cardiovascular diseases. Decreases in atherosclerosis lesions were seen in mouse models after treatment with TZDs (Li et al. 2000). TZDs also reduced inflammatory cytokine production by macrophages (Jiang et al. 1998) and inhibited

monocyte chemotactic protein-1-directed migration of monocytes (Kintscher et al. 2000). PPARγ has been shown to regulate transcription of key genes in different cell types at different stages of the development and progression of atherosclerosis. PPARγ is prominently expressed in activated monocytes/macrophages, including foam cells in atherosclerotic lesions (Neve et al. 2000), which promotes monocyte differentiation and cholesterol efflux in macrophages through upregulation of ABCA1, ABCG1, and apoE (Tontonoz et al. 1998, Chawla et al. 2001). Activation of PPARγ by its ligands (Nicholson & Hajjar 2004) increased the expression of CD36, a class B scavenger receptor implicated in oxLDL uptake. CD36 is not the only scavenger receptor that is influenced by PPARγ. The expression of scavenger receptor class A (SRA) (Misra et al. 2002), utilized in the uptake of modified lipids, is also suppressed by PPARγ (Ahmed et al. 2009). In fact, the in vivo data with either LDL receptor (LDLR) or apoE knockout mice indicate that cholesterol accumulation in macrophages might be prevented by PPARγ activation induced by TZDs (Moore et al. 2001). In addition to macrophages, endothelial cells and smooth muscle cells (SMC) also participate in the development of atherosclerosis by mediating immune cell recruitment and vascular remodeling. PPARγ regulates the function of both these cell types during the development of atherosclerosis.

SMC-specific PPARγ deficiency increased atherosclerosis in male mice. The TZD, pioglitazone, suppresses Ang II-induced atherosclerosis lesions, but the effect is lost in mice with SMC-specific PPARγ deficiency (Subramanian et al. 2010). Furthermore, PPARγ activation ameliorated endothelial cell activation and reduced the following adherence of monocytes to the activated endothelial cells probably through suppressing the diacylglycerol-PKC pathway (Verrier et al. 2004) and inhibiting the expression of vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1) (Dolezalova et al. 2007). Interestingly, recent studies found that CD36 is also a TR4 target gene and thus TR4 might have direct and indirect influences on atherogenesis via regulation of CD36 (Xie et al. 2009). TR4$^{-/-}$ mice have reduced foam cell formation with reduced CD36 gene expression and TR4 could function as a transcriptional factor to directly regulate CD36 gene expression in macrophage cells. Overexpression of CD36 can rescue impaired foam cell formation in macrophages due to reduced expression of TR4, indicating that CD36 is involved in TR4-mediated foam cell formation (Xie et al. 2009). In vitro studies showed that TZDs could activate the CD36 promoter through TR4 via binding to the same response element as PPARγ.

These results indicated that TZDs might modulate atherosclerosis via activation of two different NRs, TR4, and PPARγ, which might lead to differential outcomes in atherosclerosis. Revealing detailed mechanisms by which these two NRs respond to TZDs in mediating lipid metabolism will be beneficial to our understanding of the complexity of the activity and specificity of TZDs.

## Cancer

The third striking finding is the differential influences of these two NRs on the progression of PCa. PPARγ was believed to act as a tumor suppressor in tumors of prostate, breast and colon by inducing apoptosis (Koeffler 2003, Philips et al. 2004, Kim et al. 2007). Yet TR4's roles in PCa progression can be either as an enhancer of PCa metastasis or as a suppressor of PCa development.

PPARγ-mediated apoptosis and cell differentiation have been found to be beneficial in the chemotherapy of different types of cancers including prostate, breast, and colon (Koeffler 2003, Philips et al. 2004, Kim et al. 2007). Moderate anticancer activities of PPARγ ligands with minimal toxicities have been observed in patients with liposarcomas and PCa, possibly due to decreased expression of cyclin D1 and increased expression of p21waf1 (Wang et al. 2001, Wei et al. 2008, Lyles et al. 2009). In particular, troglitazone-treated patients showed significant induction of terminal adipocyte differentiation and reduction in Ki-67 biological marker levels for cell proliferation (Demetri et al. 1999). Surprisingly, some or many of these effects may occur independently of PPARγ, which may raise the interesting question of whether the TZDs may also modulate tumor growth via a different NR, such as TR4.

TR4 acts as a caretaker tumor suppressor that suppresses PCa initiation through promoting DNA repair and maintaining genome integrity. TR4$^{-/-}$ mice developed prostatic intraepithelial neoplasia (PIN) yet their WT littermates showed normal prostate histology. PTEN$^{+/-}$/TR4$^{+/-}$ mice and not PTEN$^{+/-}$/TR4$^{+/+}$ mice developed PIN and formed PCa tumors at 15 months (Lin et al. 2014). Interestingly, a survey of PCa samples also found that one allele of the TR4 gene is deleted in 9% of PCa patients (SJ Lin, CY Lin, K Izumi, E Yan, X Niu, DR Yang, N Wang, Li G and C Chang, unpublished observations). These results indicate that TR4 is a tumor suppressor gene in the early development of PCa (Lin et al. 2014). However, in vitro migration/invasion assays and in vivo mouse studies also found that TR4 promoted migration/invasion of various PCa cells via modulation of CCL2/CCR2 axis, indicating that TR4 can enhance PCa metastasis (XF Ding,

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DR Yang, SO Lee, YL Chen, LQ Xia, SJ Lin, SC Yu, YJ Niu, GH Li and C Chang, unpublished observations).

Importantly, Lin and colleagues (SJ Lin, DR Yang, N Wang, M Jiang, H Miyamoto, G Li and C Chang, unpublished observations) found that knocking down TR4 suppressed cell proliferation and overexpression of TR4 promoted cell proliferation in PPARγ-knocked-out (mPrE$^{-/-}$) cells. In contrast, they also found that knocking down TR4 promoted cell proliferation and overexpression of TR4 suppressed cell proliferation in PPARγ-naïve (mPrE$^{+/+}$) cells (Lin et al. 2014). Similar results were also obtained showing that knocking down TR4 significantly suppressed PCa tumorigenesis in mPrE$^{-/-}$ cells. In contrast, overexpression of TR4 promoted prostate tumorigenesis in mPrE$^{-/-}$ cells (SJ Lin, DR Yang, N Wang, M Jiang, H Miyamoto, G Li and C Chang, unpublished observations). Results of experiments using a xenografted PCa mouse model also demonstrated that knocking down TR4 or overexpression of TR4 in mice with xenografted PCa mPrE$^{-/-}$ cells might lead to altered PCa progression (SJ Lin, DR Yang, N Wang, M Jiang, H Miyamoto, G Li and C Chang, unpublished observations). Together, these results indicated that the existence of PPARγ might influence the effects of TR4 on PCa progression.

Lin and colleagues tested whether the existence of TR4 might also affect PPARγ influences on prostate cancer progression, and found that rosiglitazone could promote CWR22Rv1 cell growth significantly after TR4 was knocked down using silenced TR4 (CWR22Rv1-siTR4). In contrast, rosiglitazone showed little effect on control CWR22Rv1 cell growth compared with control scrambled TR4 (CWR22Rv1-scr) (SJ Lin, CY Lin, K Izumi, E Yan, X Niu, DR Yang, N Wang, Li G and C Chang, unpublished observations). They also confirmed these differential in vitro phenotypes using another PCa cell line, C4-2. Similar results were also obtained when they applied a colony formation assay in both PCa CWR22Rv1 and C4-2 cells (SJ Lin, CY Lin, K Izumi, E Yan, X Niu, DR Yang, N Wang, Li G and C Chang, unpublished observations). Importantly, results from a xenografted PCa mouse model using rosiglitazone-treated CWR22rv1-scr and CWR22rv1-siTR4 cells also showed that mice with CWR22Rv1-siTR4 xenografts had bigger tumors and more metastases than mice with CWR22Rv1-scr xenografts. Together, these results indicated that rosiglitazone has adverse effects on PCa progression in vitro and in vivo when TR4 is knocked down, which might indicate that the effects of rosiglitazone on PCa progression might vary and might be dependent on the status of the TR4 expression in PCa (SJ Lin, CY Lin, K Izumi, E Yan, X Niu, DR Yang, N Wang, Li G and C Chang, unpublished observations).

## Other diseases including neurological diseases, aging, mitochondrial diseases, and reproduction

**Neurological diseases**  PPARγ has positive roles in neuroprotection and neurological improvement following ischemic injury (Uryu et al. 2002) and TR4 promotes nervous system development (Chen et al. 2005, Zhang et al. 2007).

TR4 shows the highest expression in the CNS among all tissues and the expression of TR4 correlates with the process of neurogenesis, indicating a role for TR4 in the nervous system during development.

TR4$^{-/-}$ mice exhibited behavioral deficits in motor coordination starting during the postnatal stages. Analysis of the postnatal and adult TR4$^{-/-}$ mice cerebellum revealed gross abnormalities in foliation. TR4$^{-/-}$ mice cerebellar cortex shows decreased lamination, including reduction in the thickness of both the molecular layer (Pascual et al. 2005) and the internal granule layer (IGL) (Chen et al. 2005). This may be caused by disrupted granule cell proliferation within the external granule cell layer (Hankey et al. 1999), delayed inward migration of post-mitotic granule cells, and increased apoptosis during cerebellar development (Chen et al. 2005). In addition, development of Purkinje cells was abnormal in the postnatal TR4$^{-/-}$ mice cerebellum, as indicated by aberrant dendritic arborization (Chen et al. 2005, 2007).

In addition to the abnormal cerebellum development, myelination was reduced significantly in TR4$^{-/-}$ mice forebrains and the differentiation of glial cells, in particular oligodendrocytes, was abnormal in TR4$^{-/-}$ mice in early developmental stages (Zhang et al. 2007), indicating that TR4 is required for proper myelination in the CNS and is particularly important for oligodendrocyte differentiation and maturation in the forebrain regions. Further analysis reveals that CC-1-positive (CC-1$^+$) oligodendrocytes are decreased in TR4$^{-/-}$ mice forebrains (Zhang et al. 2007). Mechanical dissection revealed higher Jagged1 expression levels in axon-fiber-enriched regions in TR4$^{-/-}$ mice forebrains, suggesting a more active Notch signaling in these regions that correlates with previous reports showing that Notch activation inhibits oligodendrocyte differentiation (Zhang et al. 2007).

PPARγ agonists can prevent neuronal death resulting from N-methyl-D-aspartate (NMDA) excitotoxicity in brain in vitro or in vivo (Zhao et al. 2006). Bordet et al. (2006) believed that PPAR activation induces a decrease in neuronal death by preventing inflammation and oxidative damage implicated in cerebral injury. PPARγ agonists also inhibit macrophage and microglial activation, which

contributes to degenerative, ischemic, or inflammatory processes that eventually lead to neuronal death (Kielian & Drew 2003). PPARγ agonists are also able to inhibit the entry of inflammatory cells into the CNS from the periphery by inhibition of chemokines, adhesion molecules, and metalloproteinases (Kielian & Drew 2003). Administration of the PPARγ agonists, troglitazone or pioglitazone, dramatically reduced infarction volume and improved neurological function following transient middle cerebral artery occlusion in rats.

**Aging**    PPARγ itself is not yet directly linked to aging, but as it improves insulin resistance, hyperlipidemia and glycemic levels (Lee et al. 2003), very often the results of aging, it in turn may help to increase longevity. Loss of TR4 leads to retarded development and aging (Lee et al. 2011).

Aging is commonly associated with metabolic syndromes and because the activation of PPARγ by TZDs improves insulin resistance, hyperlipidemia, and glycemic levels, it constitutes an important part of therapy for metabolic syndromes. This therapy might help to increase longevity, even though it may not directly affect the process of biological aging. Additionally, aging is commonly associated with low-grade chronic inflammation. As PPARγ has long been known to be an anti-inflammatory molecule that suppresses NF-κB, AP-1, and STAT and decreases production of cytokines and chemokines, this effect of PPARγ may be an added benefit increasing longevity (Zhang & Zheng 2008). PPARγ can also increase klotho mRNA and protein expression (Zhang et al. 2008), an important protein involved in aging (Kurosu et al. 2005). All these data indicate that PPARγ is a key factor influencing aging.

$TR4^{-/-}$ mice exhibited an early onset of premature aging phenotypes that are associated with increased oxidative stress and genome instability (Lee et al. 2011). At the cellular level, a rapid cellular growth arrest accompanied by elevated intracellular reactive oxygen species (ROS) and more DNA damage were observed in $TR4^{-/-}$ MEFs. Restoring TR4 to $TR4^{-/-}$ MEFs reduced ROS levels and single-strand DNA breaks, which lead to a slower cellular decay, indicating that the acceleration of aging in $TR4^{-/-}$ mice might stem from oxidative DNA damage via loss of functions of DNA repair systems mediated by TR4.

**Mitochondrial diseases**    PPARγ promotes mitochondrial biogenesis (Miglio et al. 2009) and TR4 deficiencies lead to impaired mitochondrial oxidative phosphorylation (Liu et al. 2011).

Through PGC-1α, an important regulator of mitochondrial biogenesis (Wu et al. 1999), PPARγ agonists promoted biogenesis of functional mitochondria (Miglio et al. 2009). PGC-1α is rapidly induced under conditions of increased energy demands such as cold, exercise, and fasting (Ventura-Clapier et al. 2008). Mitochondrial biogenesis and respiration are stimulated by PGC-1α through the powerful induction of NRF1 and NRF2 gene expression. PGC-1α lacks DNA-binding ability but interacts with and co-activates numerous transcription factors including NRFs on the promoter of mtTFA (Rasbach & Schnellmann 2007).

$TR4^{-/-}$ mice suffer from mitochondrial myopathy and exhibit muscle weakness and exercise intolerance. An abnormal mitochondria accumulation as evidenced by ragged red fibers found in $TR4^{-/-}$ soleus muscle biopsies. In addition, increased serum lactate level, decreased mitochondrial ATP production, and decreased electron transport chain complex I (NADH: ubiquinone oxidoreductase; EC 1.6.5.3) activity were found in skeletal muscle tissues of $TR4^{-/-}$ mice. Restoring TR4 could rescue the reduction of mitochondrial ATP generation capacity and complex I enzyme activity in $TR4^{-/-}$ cells. Screening complex I structure/ancillary genes revealed reduced mRNA expression of NDUFAF1, a complex I assembly factor, in tissues of $TR4^{-/-}$ mice. Mechanism studies proved that TR4 transcriptionally regulates NDUFAF1 and addition of NDUFAF1 into $TR4^{-/-}$ cells restored mitochondrial ATP generation capacity and complex I activity (Liu et al. 2011).

**Reproduction**    Female $PPARγ^{-/-}$ mice are infertile (Minge et al. 2008), while both male and female $TR4^{-/-}$ mice have reduced fertility (Collins et al. 2004, Chen et al. 2008).

PPARγ is required for the attachment of embryos to the endometrium for the development and function of the placenta (Barak et al. 1999). Strong PPARγ expression has been detected in the trophectoderm and inner-cell mass of the blastocysts. PPARγ inactivation leads to impaired placental vascularization, resulting in the death of the embryo. Pregnancy failure can be prevented by replacing PPARγ-deficient placental cells with WT cells (Barak et al. 1999). PPARγ ligands are currently used to treat type II diabetes and also to attenuate the secondary clinical symptoms frequently associated with insulin resistance, including polycystic ovary syndrome (PCOS). Several laboratories have described strong expression of PPARγ in ovarian granulosa cells, and glitazones modulate granulosa cell proliferation and steroidogenesis in vitro.

All these recent data raise new questions about the biological actions of PPARs in reproduction and their use in therapeutic treatments of fertility troubles such as PCOS or endometriosis (Froment et al. 2006).

Male $TR4^{-/-}$ mice are subfertile, which might be due to reduced sperm production at various stages (Mu et al. 2004) and have delayed spermatogenesis in the first wave of spermatogenesis from stages X to XII. But $TR4^{-/-}$ and $TR4^{+/+}$ mice show no differences in sperm morphology and cauda epididymis sperm motility. Histological examination of testis sections shows degenerated primary spermatocytes and some necrotic tubules in $TR4^{-/-}$ mice. Taken together, these findings indicate that TR4 may play essential roles in normal spermatogenesis in vivo (Mu et al. 2004).

TR4 also plays essential roles in normal female folliculogenesis and ovarian functions (Chen et al. 2008). Female $TR4^{-/-}$ mice display subfertility, with prolonged and irregular estrous cycles, reduced size and weight of ovaries, as well as fewer or no preovulatory follicles and corpora lutea. More intensive granulosa apoptosis was found in ovaries of $TR4^{-/-}$ mice. The normal serum-luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations in female $TR4^{-/-}$ mice after superovulation ruled out the possibility that the subfertility of $TR4^{-/-}$ mice is due to deficiency in the pituitary gonadotropin axis. Mechanical dissection revealed that LHR is a direct $TR4$ target gene and that its expression in ovaries is reduced in female $TR4^{-/-}$ mice, indicating that TR4 might play essential roles in normal folliculogenesis by modulating LHR signals (Chen et al. 2008).

## The advantage of having two distinct receptors activated by similar ligands

Why does our body need two distinct NRs that can be activated via similar ligands/activators? The questions remain to be answered as to when, where, and how these two receptors are activated in response to ligands/activators with delicate temporal and special regulation of metabolism functions.

The distinct tissue distribution pattern of PPARγ and TR4 might provide a hint. Take the metabolic system for example: PPARγ is mainly expressed in adipose tissue and controls adipocyte differentiation and function, while its role in liver is relatively limited (Gavrilova et al. 2003). In contrast, the role of TR4 in liver has been clearly demonstrated (Liu et al. 2007), yet its function in adipose tissue remains unclear. Recently, Choi et al. (2011)

reported that TR4 facilitates fatty acid uptake into 3T3-L1 adipocytes via upregulation of the FATP1 gene, resulting in lipid accumulation in adipocytes. This indicates that TR4 also plays a key role in adipose physiology, although further study will be required to define its role in adipose tissue (Choi et al. 2011). Both PPARγ and TR4 have been shown to be involved in skeletal muscle functions (Hevener et al. 2003, Liu et al. 2011). Therefore, it is possible that these two receptors are needed for proper function in response to ligands in different tissues.

Another explanation is the timing of their expression. Previous studies show that the peak expression of TR4 and PPARγ is different in metabolic tissues (Table 1). In WAT, TR4 peaks at ZT4 while PPARγ peaks at ZT16 and in liver TR4 peaks at ZT4 while PPARγ peaks at ZT8. The cause and the regulatory mechanism(s) behind these cycles of expressions are not clear yet, but the expression peaks of these two receptors at different times might allow each receptor to dominate the downstream events of the ligand at different time points.

Another possibility is that even when TR4 and PPARγ are expressed in the same tissue at the same time, they sometimes might counteract with or cooperate with each other, which allows the flexibility to add to the complexity of ligand effects. This might be the answer to the undisclosed mystery of how the ligands can keep the subtle balance for the regulation of the complicated downstream metabolic pathway networks.

## The effects on the pharmaceutical industry/drug development

The withdrawal of troglitazone from the market because of liver toxicity has led to concerns of the safety of the other TZDs. Because of this, the FDA recommends liver enzyme checks for this rare but potentially catastrophic complication every 2–3 months in the first year of TZDs therapy.

Furthermore, two newer TZDs, Avandia (rosiglitazone) and Actos (pioglitazone), have been subject to significant restrictions or warnings by the FDA (Harris 2010, FDA 2011). The major warning for Actos use is that long-term treatment may increase bladder cancer (BCa) risk. Yang et al. (2013) also recently found that PPARγ amplification was increased dramatically in BCa tissue compared with normal urothelium (38.1 vs 4.3%) and in tumors with lymph node metastasis compared with those without metastasis (75.0 vs 15.4%). Human BCa 5637 cells with strong PPARγ expression had better cell migration and invasion abilities than the BCa UMUC-3 cells with weak

PPARγ expression. Knocking down PPARγ in BCa 5637 cells led to decreased cell migration and activation of PPARγ with TZDs promoted their migration and invasive ability, indicating that PPARγ and its ligand TZD may play a positive role in promoting BCa progression (Yang *et al.* 2013).

Other side effects of TZDs include edema, anemia, body pain, fatigue, weight gain, jaundice, headache, stomach upset and the worst, heart failure. Therefore, TZDs should be prescribed with both caution and patient warnings about the potential for water retention/weight gain and other side effects, especially in patients with decreased ventricular function (NYHA grade III or IV heart failure).

As the current insulin sensitizer, TZDs can activate their downstream targets through both TR4 and PPARγ; this might be part of the reason behind the drug side effects. In this scenario, treatment with TZDs can activate both TR4 and PPARγ signals, and thus alter their interactions with each other in the body, to either strengthen each other on some occasions or counteract each other on other occasions, all of which might be dependent on the cellular context. Although the detailed mechanisms need further exploration, the side effects of TZDs might be partly explained by our unexpected findings, showing the unbalanced regulation of PPARγ and TR4, which has not been previously acknowledged (SJ Lin , K Izumi, H Miyamoto, N Wang and C Chang unpublished observations).

Considering the physiological importance of both receptors in mediating the metabolic syndrome and their affinity for similar ligands/activators, it might be necessary to test both PPARγ and TR4 response when developing the new generation of PPARγ-targeting anti-diabetic drugs. A better characterization of each of these two NRs and the interaction between them might help to reduce the side effects and gain better potency.

## Summary and future perspectives

Early studies indicated a role of PPARγ in adipocyte function because of its high expression in adipocytes and its ability to stimulate adipocyte differentiation of cultured fibroblasts (Tontonoz *et al.* 1994*a*,*b*). Subsequent studies identified a natural adipogenic regulator, PUFA and its metabolite, 15d-PGJ$_2$ as ligands/activators of PPARγ (Forman *et al.* 1995, Kliewer *et al.* 1995, Lehmann *et al.* 1995). Several PPARγ agonists, including members of the TZDs family, such as the antidiabetic drug rosiglita-zone, were then developed to battle metabolic diseases.

Previous *in vitro* and *in vivo* studies indicated that TR4 plays essential roles in postnatal growth, neural development, spermatogenesis, metabolism, skeletal muscle function, and bone remodeling (Collins *et al.* 2004, Mu *et al.* 2004, Chen *et al.* 2005, Kim *et al.* 2005, Zhang *et al.* 2007). TR4 has long been viewed as an orphan receptor, until the recent discovery that the PPARγ ligands/activators, such as PUFAs and its metabolites, 15-HETE and 13-HODE, as well as rosiglitazone, could also transactivate TR4 to a similar degree to their activation of PPARγ (Xie *et al.* 2009). This result further confirmed previous findings that TR4 is important in insulin sensitivity, glucose metabolism (Liu *et al.* 2007), and lipid metabolism (Kim *et al.* 2011).

Most of previous studies regarding TR4's role in metabolism focus on the liver, while the action of TR4 in adipose tissues is largely unknown. After the identification of PUFAs, 15-HETE and 13-HODE, as the new TR4 ligands/activators, we need to look into the role of TR4 in adipose tissues, considering the high abundances of the ligands/activators there. Also, future studies on the effects of TZDs in tissue-specific TR4-knockout mice will improve our understanding of how TZDs work and TR4's physiological and pathological roles in response to TZDs.

Although sharing similar ligands/activators, TR4 and PPARγ could trigger similar or distinct downstream signals, depending on the context. While TZDs improve insulin sensitivity through the activation of PPARγ and its downstream pathways in adipose tissues, they might trigger some side effects in other tissues by the unexpected activation of TR4. Thus, it is extremely important to fully understand the details of the spatial and temporal action of TZDs on TR4 and PPARγ, the respective consequent downstream events following their activation, and the interaction between these two signaling networks. With this knowledge in hand, the drug industry might be able to improve drug specificity and reduce side effects of the old TZDs drugs possibly by limiting the 'bad' effects of TR4. It might be possible to develop new therapeutic approaches for metabolic and other diseases by targeting either TR4 or PPARγ in specific tissues.

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