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PPARγ and the Global Map of Adipogenesis and Beyond

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Abstract

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-dependent transcription factors which functions as a master regulator of adipocyte differentiation and metabolism. Here we review recent breakthroughs in the understanding of PPARγ gene regulation and function in a chromatin context. It is now clear that multiple transcription factors team up to induce PPARγ during adipogenesis, and that other transcription factors cooperate with PPARγ to ensure adipocyte-specific genomic binding and function. We discuss how this differs in other PPARγ-expressing cells such as macrophages, and how these genome-wide mechanisms are preserved across species despite modest conservation of specific binding sites. These emerging considerations inform our understanding of PPARγ function as well as adipocyte development and physiology.

Keywords

PPARγ; adipogenesis; chromatin; genome-wide analyses; transcriptional network

PPARγ as a master regulator of adipocyte biology

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor (NR) superfamily of ligand-activated transcription factors (TFs) that regulate essential aspects of biology from development to metabolism [1–3]. PPARγ is required for adipocyte differentiation, regulation of insulin sensitivity, lipogenesis, and adipocyte survival and function [1, 4, 5] (Box 1). The structure of PPARγ and its mechanism of binding to DNA are similar to those of a number of other NRs (Box 2). Synthetic PPARγ agonists have emerged

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as important pharmacologic agents in diabetes management, however their use has been limited due to serious side effects caused by off-target PPARγ activation in non-adipose tissues [6] (Box 3). Novel strategies involve selective targeting of PPARγ in adipose tissue, for instance with compounds that modulate PPARγ activity by targeting posttranslational modifications of the receptor. Thus, elucidating the gene- and tissue-selectivity of its actions could lead to the development of novel PPARγ compounds that maintain efficacy while reducing side effects. Accomplishing this would involve defining the PPARγ transcriptional network and putative regulatory elements in their specific chromatin contexts and in different cell types. Integrating these data with gene expression profiling under conditions that affect PPARγ levels or activity may reveal cell-type specific PPARγ transcription networks that potentially could be targeted in tissue-selective ways.

TEXT Box 1

Early studies of PPARγ in adipogenesis

Many studies on PPARγ have utilized adipocyte cell lines, such as 3T3-L1 cells, which are derived from mouse embryonic fibroblasts and can be induced to undergo adipocytic differentiation using a cocktail of dexamethasone, a cAMP elevating agent (3-isobuthyl-1-methylxantine), and insulin [90, 91]. Early studies demonstrated that PPARγ is induced during adipogenesis [92, 93] and that PPARγ is necessary and sufficient for adipocyte differentiation [94], thereby establishing PPARγ as a master regulator of adipogenesis [1]. *In vivo*, a whole-body PPARγ knockout in mice is embryonic lethal due to placental defects, while mice with chimeric PPARγ expression have shown that embryonic stem cells lacking PPARγ cannot contribute to fat formation [95, 96]. Targeted fat-specific PPARγ deletion results in various abnormalities, including reduced white and brown fat, decreased adipocyte gene expression, and fatty liver [1, 97] and recently a more efficient fat-specific knockout revealed a critical role for adipocyte PPARγ in all adipose depots including mammary gland, bone marrow, and skin [98]. PPARγ is also required for the survival of adult adipocytes as evidenced by a conditional knockout model [99].

TEXT Box 2

Structure of PPARγ

The PPARγ gene is transcribed from alternative promoters giving rise to two protein isoforms, PPARγ1 and the longer PPARγ2, which is almost exclusively expressed in adipocytes [3]. PPARγ contains an N-terminal domain involved in ligand-independent activation function (AF1); a DNA binding domain (DBD), and a C-terminal ligand-binding domain (LBD) containing the ligand-dependent activation function 2 (AF2) [1] (Box 2: Figure I).

PPARγ binds as an obligate heterodimer with members of the retinoid X receptor (RXR) family at consensus binding sites consisting of imperfect direct repeats of the sequence AGGTCA separated by a single base pair (DR1 elements) (Box 2: Figure I) [4]. The crystal structure of the DNA-bound PPARγ-RXR heterodimer in the presence of ligand

as well as coactivator peptides provides structural support for the functional findings that initially characterized the PPARγ domains [18].

PPARγ binds to its cognate binding site even in the absence of ligand. The unliganded state of PPARγ favors interactions with NR corepressor (NCoR) and silencing mediator for retinoid and thyroid receptors (SMRT), which recruit chromatin-modifying enzymes such as histone deacetylases, to repress transcription. Conversely, in the presence of ligand, a conformational change in the PPARγ LBD favors interactions with coactivators such as steroid receptor coactivators (SRCs), histone acetyltransferases (HATs) such as CBP and P300, and the Mediator complex, which ultimately promotes gene transcription [1]. Finally, the N-terminal AF1 domain is important for agonist-independent recruitment of HATs and Mediator [100, 101] (Figure I).

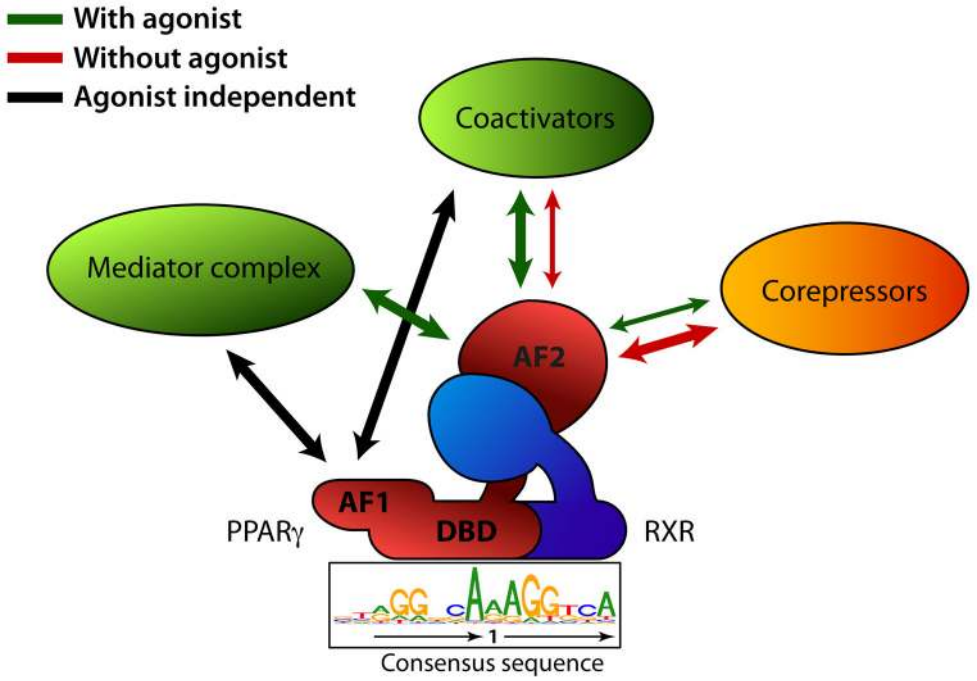

Figure I for TEXT BOX 2. PPARγ domain structure and mechanism of binding to DNA

The main structural domains of the PPARγ protein are shown, including the N-terminal activation function 1 (AF1) domain, the DNA binding domain (DBD), and the C-terminal AF2 domain. PPARγ binds as a heterodimer with RXR to DNA sequences that conform to a consensus motif containing two imperfect direct repeats of the sequence AGGTCA, separated by a single nucleotide (DR1). The shown graphical representation of the PPARγ:RXR binding motif is based on the position weight matrix in the JASPAR database generated on PPARγ ChIP-seq data from 3T3-L1 cells [13]. Also shown is the conserved portion of the 5′ extension of the consensus motif. In the presence of agonist, the PPARγ AF2 domain facilitates agonist-dependent recruitment of coactivators and Mediator in exchange for corepressors, leading to increased expression of target genes. In the absence of agonist, the AF2 associates more strongly with corepressors. The AF2 domain is also responsible for agonist binding and heterodimerazation with RXR. The

AF1 domain is mediates agonist-independent recruitment of coactivators and the Mediator complex.

TEXT Box 3

Ligands of PPARγ

A number of lipid metabolites have been implicated as PPARγ activators, including poly-unsaturated fatty acids (FAs), eicosanoids, and the prostagladin, 15-deoxy-Δ12,14-prostaglandin J2; however, these molecules have low affinity for PPARγ or exist at low levels in adipocytes, making their physiological relevance as endogenous PPARγ ligands uncertain [102]. Thus, the physiologically relevant PPARγ ligand(s) remain(s) unknown.

PPARγ is the pharmacologic target of thiazolidinedione (TZD) drugs, which act as potent insulin sensitizers by increasing peripheral glucose disposal, decreasing fasting free FA concentrations, increasing circulating adiponectin concentrations, and decreasing pro-inflammatory cytokines [6]. However, TZDs have a number of unwanted side-effects, including weight gain, fluid retention, and bone loss, that have limited their wide-spread clinical use [6]. Additionally, meta-analyses of clinical trials and post-marketing drug-surveillance studies have implicated TZDs in increasing the risk of heart failure [103], myocardial infarction [104], and bladder cancer [105]. Although recent studies have questioned the increase in risk of myocardial infarction in particular [106], these concerns have prompted new warnings and restrictions for TZD administration, reinforcing the need for new generations of PPARγ ligands. A promising new approach to pharmacologic PPARγ targeting uses compounds that inhibit post-translational modifications of PPARγ that are associated with insulin resistance [107].

Here we discuss how the PPARγ transcriptional network is established during adipogenesis. We describe the molecular mechanisms underlying cell-type specific PPARγ actions, and offer insights into promises and pitfalls of translating discoveries made in murine systems to PPARγ biology in humans.

Establishment of the PPARγ transcriptional network during adipogenesis

Adipogenesis has been studied extensively *in vitro,* in particular using the murine 3T3-L1 preadipocyte cell line (Box 1) [7–10]. Based on these *in vitro* studies it appears that adipogenesis proceeds through the activation of at least two waves of TFs (Figure 1). The first is induced directly by the adipogenic cocktail, and includes TFs such as C/EBPβ and −δ as well as the glucocorticoid receptor (GR), signal transducer and activator 5A (STAT5A), and cAMP-responsive element-binding protein. These factors in turn activate TFs of the second wave, which initiate the adipocyte gene program [8, 11]. PPARγ and C/EBPα appear to play the most prominent roles in this second wave as demonstrated by loss-of-function studies [4].

PPARγ binding during adipogenesis

Over the last decade techniques such as chromatin immunoprecipitation (ChIP) combined with whole genome microarrays (ChIP-chip) and deep sequencing (ChIP-seq) have enabled genome-wide mapping of TF binding and patterns of histone modifications. These techniques have greatly changed our view on transcriptional regulation. Genome-wide profiling of PPARγ and RXR binding in 3T3-L1 adipocytes has demonstrated that PPARγ:RXR bind to thousands of sites in mature adipocytes [12–16]. Bioinformatic analysis of the DNA sequence of the binding regions from these genome-wide studies have confirmed a degenerate DR1 element with a conserved 5′ flanking sequence conforming to the depicted position weight matrix (Box 2, Figure I) as the primary binding sequence recognized by PPARγ:RXR [12, 13]. This consensus sequence is close to the one initially determined based on alignment of a limited number binding sites and is consistent with reports demonstrating that the carboxyl-terminal extension of the PPARγ DBD directly interacts with the 5′ flanking sequence [17, 18] and facilitates binding of PPAR:RXR heterodimers to DR1 elements that are imperfect matches to the consensus [19, 20].

Consistent with the finding that PPARγ can bind to chromatin also in the absence of agonists (Box 2), the genome-wide binding pattern of PPARγ in adipocytes does not change dramatically in response to synthetic agonists. However, binding of PPARγ to many preexisting binding sites in 3T3-L1 is enhanced in response to acute treatment with rosiglitazone. This enhanced binding of PPARγ correlates with increased recruitment of the mediator subunit 1 and expression of nearby genes, indicating that enhanced PPARγ recruitment plays a role in the activation of PPARγ targets in response to rosiglitazone in adipocytes [21].

The majority of PPARγ binding sites are also occupied by RXR, consistent with early findings that RXR is an obligate heterodimerization partner of PPARγ. The time-course of PPARγ binding during adipogenesis follows the induction in PPARγ protein levels, whereas RXR is already bound to many sites in the undifferentiated state, probably as heterodimer with PPARδ or other NRs [13]. PPARγ binding sites are strongly enriched in the vicinity of genes that are induced during differentiation [12–16, 22], such as genes involved in FA and glucose metabolism. This indicates that PPARγ is directly involved in establishing the metabolic program during adipogenesis.

Although PPARγ binding is enriched in the proximal promoter region of induced genes, only a small fraction (< 10%) of the PPARγ binding sites is located close to promoters. Instead, many sites are found in distal intergenic regions and about half of the binding sites are found in intronic regions. This distribution of binding sites parallels that of many other TFs [23–26]. It is important to note that proximity to a particular promoter is not proof of involvement in the regulation of the corresponding gene. Results from analyses based on chromatin conformation capture technologies indicate that binding sites far away from the promoter and in some cases embedded in other genes may loop to the promoter, indicating that these distant sites are important for the regulation of the gene [27–30]. However, genome-wide interaction maps from adipocytes are not yet available, and proximity to the transcription start site is currently the best indicator linking binding sites to regulated genes.

Overlap between PPARγ and C/EBPα

During adipogenesis PPARγ cooperates with the other major adipogenic TF, C/EBPα. These two TFs mutually induce the expression of each other [1] and genome-wide profiling of binding sites have demonstrated that 30–60% of PPARγ binding sites in murine and human adipocytes are also bound by C/EBPα [12, 22]. Furthermore, there is a striking and almost complete overlap between C/EBPα and C/EBPβ binding sites in mature adipocytes [12], indicating that C/EBP homo- and heterodimers might bind interchangeably. The functional importance of co-localization of PPARγ and C/EBP has been unclear; however, recent results demonstrate that the two TFs facilitate the binding of each other to chromatin at least in part through chromatin remodeling and assisted loading [31]. This facilitated binding is associated with synergistic coactivator recruitment and synergistic activation of nearby adipocyte genes.

Despite the high degree of cooperativity between PPARγ and C/EBPα in adipocytes, some adipocyte genes are clearly more dependent on C/EBPα than on PPARγ, and vice versa. Intriguingly, whereas treatment of adipocytes with PPARγ agonists generally leads to induction of the genes that display a high PPARγ dependency, several C/EBPα dependent genes are repressed [21]. The mechanism for this repression remains to be clarified, but it may involve selective recruitment of corepressors to C/EBPα binding sites [32]. In addition, more recent studies suggest that PPARγ ligands repress transcription by redistributing coactivators from TFs other than PPARγ, including AP1 and C/EBPs, to PPARγ at its activated gene targets [33].

Shaping the chromatin landscape of adipocytes

Transcription factor access to DNA is limited by the wrapping of DNA around nucleosomes. Open, partially nucleosome-free chromatin regions are therefore much more accessible to TFs than closed nucleosome-dense regions. The factors that drive this nucleosome positioning are the chromatin remodeling complexes, which are recruited to specific chromatin regions either by interactions with proteins such as sequence-specific TFs, or directly to specific histone modifications [34]. Thus, chromatin remodeling occurs both upstream and downstream of TF binding, and regions undergoing remodeling are therefore likely to represent important ‘action points’ in the genome. By using techniques such as DNAse I hypersensitive sequencing (DHS-seq), one can obtain an unbiased genome-wide map of regions of open chromatin. These regions are likely to represent sites in the genome bound by TFs and other DNA interacting proteins [35–39].

Recently, examination of adipocyte differentiation using DHS-seq has generated the first genome-wide map of chromatin remodeling during a developmental process and shown that the chromatin structure is dramatically remodeled at very early stages of adipogenesis in 3T3-L1 cells [40]. With the level of sequencing depth and the stringent threshold used to identify high-confidence DHS regions in that study, approximately 10,000 open chromatin sites were identified in unstimulated preadipocytes, whereas there are more than three times as many DHS regions at four hours following addition of the adipogenic cocktail. While many of these sites are only transiently open, a large fraction of sites persists in the mature adipocytes, indicating that these early remodeled sites are also functionally important in the

mature adipocytes. A final group of sites are remodeled later during differentiation along with the induction of the second wave of TFs (Figure 1). An independent study using formaldehyde-assisted isolation of regulatory elements (FAIRE)-seq also found profound differences between the chromatin structure of 3T3-L1 preadipocytes and mature adipocytes [41].

The early remodeling is driven by the concerted action of multiple TFs that bind to transcription factor 'hotspots' in a cooperative manner [40]. C/EBPβ appears to be particularly important, since this factor binds to almost all hotspots. Furthermore, binding of C/EBPβ precedes chromatin remodeling for about one third of the hotspots identified in this study, indicating that C/EBPβ is able to bind to relatively 'closed' or only partially open chromatin (Figure 1). Importantly, knockdown of C/EBPβ, GR or STAT5A, also reduces recruitment of other early acting TFs to hotspots, indicating that TFs mutually facilitate binding of each other to hotspots [11, 40].

An intriguing question is whether hotspots are primed prior to activation with the adipogenic cocktail. Preliminary data indicate that many hotspots are already marked by active histone marks and C/EBPβ binding in the preadipocyte stage [40]. Consistently, the Rosen laboratory showed that 77% of PPARγ binding sites found in mature adipocytes are located in regions that appear to be preprogrammed in unstimulated preadipocytes, i.e. marked with chromatin marks characteristic of active enhancers, H3K4me1/2 and H3K27ac [16]. The H3K4 methyltransferases MLL3 and MLL4 interact with C/EBPβ and associate with enhancers during differentiation of brown adipocytes [42]. Thus, C/EBPβ may confer active histone marks to the preprogrammed adipocyte enhancers in part by recruiting MLL3 and MLL4. Notably, many of the preprogrammed PPARγ binding sites display an open chromatin structure in multiple cell types, indicating that these sites may constitute ubiquitous enhancers engaging PPARγ, whereas sites established later during adipogenesis appear to mediate adipocyte specific functions of PPARγ [43]. Adipogenesis is also associated with removal of repressive histone marks at adipocyte genes. Thus, in 3T3-L1 preadipocytes, the entire PPARγ locus as well as many other adipogene loci, are marked by H3K9me2, and this mark is removed in response to the adipogenic cocktail [44].

It is clear from these studies that active histone marks at preprogrammed sites *per se* are not sufficient for gene activation. Enhancer activation appears to require the cooperative action of multiple transcription factors binding to the same region (i.e. transcription factor hotspots) thereby leading to chromatin remodeling [40]. This may also be important for removal of repressive histone marks on genes. Interestingly, recent results indicate that in addition to transcription factor cooperativity at the level of hotspots, cooperativity between hotspots in so-called super-enhancers is also of major importance for reprogramming of the genome [45].

From adipocyte cell lines to primary adipocytes

Investigation of PPARγ binding profiles from primary adipocytes differentiated *in vitro* showed that there are many more binding sites in primary adipocytes compared to 3T3-L1 adipocytes [46]. Reassuringly for the 3T3-L1 model system, the majority of PPARγ binding

sites in 3T3-L1 adipocytes are also found in primary *in vitro* differentiated adipocytes, and presumably play key roles in activating the core adipocyte gene program. However, there are also large numbers of sites that are only detected in primary adipocytes and are found in a closed chromatin configuration in 3T3-L1 adipocytes [46].

Adipose tissue in mammals can be categorized into two major subtypes: white adipose tissue (WAT), which stores excess metabolic energy as triglycerides, and brown adipose tissue (BAT), which is specialized to oxidize FAs and release energy as heat. Interestingly, white adipose tissues from different anatomical locations also differ in expression of distinct gene programs, and have important differences in physiologic properties [47–49]. Comparison of genome-wide PPARγ binding profiles in primary *in vitro* differentiated preadipocytes derived from inguinal, epididymal WAT and interscapular BAT depots, revealed that the majority of the identified sites were occupied by PPARγ in all tissues with relatively similar binding intensities [46]. Likewise, PPARγ binding profiles from epididymal WAT, and interscapular BAT depots are very similar [50]. However, in addition to these common sites both studies found a subset of PPARγ binding sites that are highly depot selective [46, 50]. Importantly, depot-specific PPARγ binding correlates with tissue-specific gene expression, indicating that PPARγ is not only involved in general adipocyte differentiation but also plays a role in depot-selective gene expression [46]. Notably, the depot-selective binding sites identified in *in vitro* differentiated adipocytes recapitulate the depot-selective patterns observed *in vivo*, indicating that depot-selective preprogramming of the mesenchymal stem cells has already taken place before the isolation, and is sustained even under the *in vitro* differentiation conditions [46]. An intriguing question is what factors are responsible for establishing and maintaining this preprogramming. Most likely the combined expression of depot-selective TFs maintains an open chromatin structure at depot-selective sites, similarly to what is observed in non-adipocyte cell types as will be discussed below. As an example of such a factor, Early B cell factor-2 (EBF2) was recently shown to co-localize with PPARγ and facilitates PPARγ binding to BAT-specific binding sites during differentiation of brown adipocytes. Ectopic expression of EBF2 during *ex vivo* differentiation of adipocyte precursors from the stromal vascular fraction of inguinal WAT activates the brown adipocyte gene program [50]. Future investigations are likely to identify similar depot-selective factors directing PPARγ binding.

Cell-type specific binding of PPARγ and cooperating factors

Although PPARγ is most abundant in adipocytes, it is expressed at low levels in various non-adipocyte cell types, where it can regulate metabolism or mediate unwanted side effects of TZDs [6], in addition to functions that are not yet fully understood. Macrophages are of particular interest because of their known roles in the pathogenesis of obesity, insulin resistance, and atherosclerosis [51]. Although PPARγ is not necessary for macrophage differentiation or phagocytic activity [52, 53], it is required for establishing an anti-inflammatory phenotype in adipose tissue macrophages known as alternative activation [54]. Alternatively activated macrophages are found in the adipose tissue of lean mice, whereas in the setting of obesity and insulin resistance there is a switch towards a pro-inflammatory macrophage phenotype known as classical activation [55–59]. Interestingly, myeloid-specific PPARγ deficiency in mice leads to impaired alternative macrophage activation,

diet-induced obesity, insulin resistance, and glucose intolerance [54, 60]. Additionally, PPARγ is expressed in macrophage-derived foam cells in atherosclerotic lesions [55], and low-density lipoprotein receptor knockout mice with PPARγ-deficient hematopoietic cells have elevated levels of atherosclerosis [61]. Correspondingly, *in vitro* studies have shown that PPARγ can regulate both oxidized LDL uptake [55] and reverse cholesterol efflux [61].

Although a small number of target genes responsible for these effects, including the scavenger receptor CD36 and the ABC transporter ABCA1, were elucidated in early studies, it is only recently that PPARγ binding in macrophages has been investigated in a systematic way using ChIP-seq [62, 63]. These studies have demonstrated that in mouse and human macrophages, PPARγ binding occurs at DR1 sites with RXR, while indirect DNA-independent recruitment as in the context of transrepression [64, 65] does not appear prominent, arguing that the latter binding mechanism may be employed only under specific circumstances such as stimulation with pro-inflammatory cytokines. Comparison of PPARγ binding profiles in adipocytes and macrophages has revealed that PPARγ binding is largely cell-type specific, with macrophage-unique binding occurring near genes with functions in immune defense as well as cytokine/chemokine-mediated signaling. In contrast, the small amount of overlap that exists in binding locations between the cell types occurs near metabolic genes [62]. Taken together, these findings provide a molecular mechanism for a number of functional studies implicating PPARγ in the function of alternative macrophages [54, 66, 67] and bone marrow derived dendritic cells [68, 69], in addition to its well-established role in lipid metabolism.

Attempts to determine what drives cell-type specific PPARγ recruitment have led to the identification of tissue-specific TFs with which PPARγ co-localizes and cooperates on a genome-wide scale. For example, whereas adipocyte PPARγ sites are located in proximity to C/EBPα/β binding, in macrophages PPARγ tends to co-localize with the hematopoietic factor PU.1 in addition to C/EBPs [62, 63]. PU.1 is the lineage-determining factor for monocyte differentiation [70, 71], suggesting that in macrophages the binding of PPARγ is subservient to this master regulator, whereas PPARγ itself is the lineage determinant for adipocytes. Intriguingly, expression of PU.1 in adipocytes led to expression of macrophage genes and a global reduction of PPARγ binding to its adipocyte sites, yet was insufficient to recruit PPARγ to most sites that it occupies in macrophages, suggesting that other macrophage-specific factors are required [72]. Indeed, the ability of PPARγ to be recruited to macrophage specific enhancers may be programmed early in differentiation, as it is in adipocytes. In fact, there is evidence that, in a given cell type, PPARγ binding sites that are specific to a different cell type are kept inaccessible through repressive mechanisms. In particular, examination of the chromatin context in the vicinity of PPARγ sites shows that in adipocytes, macrophage-specific binding sites are contained in chromatin with low DNA accessibility and histone modifications characteristic of heterochromatin such as H3K9Me2, H3K27Me3, and hypoacetylation [62] (Figure 2). This repressive chromatin environment is likely to be established early during cell differentiation and may span large DNA domains [73].

Other organ systems where PPARγ binding may need to be investigated with the help of next-generation sequencing technology include the kidney, bone, liver, and the vasculature.

A number of renal cell types play roles in the fluid retention side effects of TZDs, at least partially through increasing expression of the epithelial Na^+ channel in the medullary collecting duct, leading to enhanced sodium absorption [74, 75]. However, there is evidence that additional genes in the collecting duct epithelium as well as other locations in the kidney, such as the proximal tubule, may be responding to TZDs [76–78]. Thus, it is likely that PPARγ regulates multiple pathways in various renal cell types that collectively contribute to sodium and water retention in the kidney, but also account for the renoprotective effects of PPARγ in the setting of type II diabetes, which remain poorly understood from a mechanistic perspective [79].

In bone, PPARγ activation with TZDs promotes bone resorption through combined effects on osteoblast suppression and osteoclast activation, ultimately increasing the risk of bone fractures [80]. While PPARγ deficiency in mesenchymal stem cells promotes osteoblast differentiation at the expense of adipogenesis [80, 81], its absence in osteoclast progenitors leads to dysregulated osteoclastogenesis and osteopetrosis [82]. Thus elucidating cell-type specific PPARγ binding in these tissues may improve understanding of its pharmacologic activation, although an important caveat with this approach is that identification of binding sites does not necessarily indicate the target genes and whether binding is functional. Answering such questions requires a systems approach to TF biology that integrates binding data with profiling of gene transcript abundance, epigenomic profiling, as well as emerging novel approaches that allow high-throughput assessment of the functionality of large numbers of binding sites [73, 83].

Comparison of the PPARγ network in mouse and humans

The vast majority of PPARγ binding studies discussed above have been performed using mouse model systems, and until recently, it had been assumed that sequence conservation would be an accurate predictor of conserved PPARγ binding events across species. However, three studies in adipocytes and one in macrophages have challenged this notion, demonstrating predominantly species-unique genomic localization of PPARγ binding [16, 22, 63, 84]. Nevertheless, important principles delineated in murine model systems, such as the identity of direct gene targets, the co-localization with cell-type specific TFs and the association with active chromatin states, appear to be evolutionarily conserved.

Interspecies comparisons of PPARγ binding have been performed through PPARγ ChIP-seq in human and murine adipocyte cells, followed by conversion of the resulting binding regions to the orthologous genome using pre-computed genome alignments [16, 22, 63, 84]. The different types of outcomes of such comparisons include binding regions that are (i) shared between the species (conserved binding), (ii) lost or gained from one species to the other (species-specific binding), (iii) absent at the orthologous position in one species although a binding site associated with the same gene is present in a different genomic position (conserved gene regulation), or (iv) not convertible between genomes (Figure 3). Using this approach, all of the studies agree that only a minority of mouse binding sites are shared with human adipocytes, ranging between 9% [84] and 30% [16].

Compared to species-specific sites, shared sites have been found to have greater conservation, higher ChIP-seq enrichment signal, and to be more strongly associated with cognate PPARγ motifs and marks of active chromatin. However, none of these features has sufficient predictive value to discriminate shared from species-unique sites [16, 22, 63, 84]. Shared sites are also more likely to demonstrate co-localization with C/EBPα and to contain C/EBP motifs [22], suggesting that there may be increased selective pressure for retention of sites where co-localizing C/EBPs can facilitate the recruitment of PPARγ [85].

Several studies have investigated the mechanisms underlying evolutionary TF binding divergence. The low level of binding retention does not appear to be the result of a change in the DNA specificity of PPARγ, since its binding motifs constructed *de novo* from ChIP-seq data in mouse and human cells appear identical [16, 22, 63, 84]. However, it has been shown that differences in binding of other TFs between mouse and human are indeed driven by sequence rather than changes in the nuclear environment. Specifically, recruitment of hepatocyte nuclear factor (HNF) 1α, HNF4α, and HNF6 in livers of model mice carrying human chromosome 21 largely recapitulates the binding of these factors and the associated active chromatin marks observed in human livers on chromosome 21 [86]. Correspondingly, the loss or gain of binding events is frequently associated with mutations in the cognate binding motifs of TFs, most commonly substitutions, with fewer insertions and deletions, and this is thought to represent neutral evolution rather than selective pressure [87]. Additionally, several studies have shown that up to 30% of species-specific binding events occur in species-unique repetitive elements such as transposons, which presumably have inserted in a given genome after its evolutionary divergence from the other species [16, 88].

In contrast to the low level of retention of PPARγ binding sites between human and mouse adipocytes, there is 50–60% conservation of putative PPARγ target genes [84], consistent with what has been reported for other TFs [88, 89] (Figure 3). As described in prior sections, putative PPARγ target genes are identified by assigning PPARγ binding regions from genome-wide sequence data to differentially expressed genes following siRNA mediated PPARγ depletion [84] or induction of adipogenesis [16, 22]. PPARγ target genes that are shared between mouse and human are more likely to contain shared binding sites, i.e. conserved binding at orthologous genomic locations in both species, although the majority of binding sites associated with target genes represent turn-over events [16, 22, 84]. Shared genes are also more likely to contain a large number of PPARγ binding regions in proximity of their transcription start sites, to be associated with large changes in active chromatin marks and in gene expression when PPARγ levels are altered, and to comprise metabolic pathways known to be regulated by PPARγ. Taken together, such data indicate that the master regulator functions of TFs such as PPARγ are evolutionarily conserved through genetic mechanisms that ensure redundancy even in the face of evolutionary loss of the majority of binding locations.

Concluding Remarks and Future Perspectives

Over the past decade our understanding of the biology of PPARγ regulatory networks has expanded dramatically with the help of powerful genome-wide techniques such as ChIP-seq and DHS-seq. Genome-wide binding profiles of PPARγ have been mapped in several cell

types, revealing first of all, that PPARγ binds to thousands of sites in the genome many of which are located far from proximal promoters. Secondly, these studies have shown that PPARγ is recruited to different sites between different cell types, and even between adipocytes from different anatomical depots, showing that PPARγ binding is highly context dependent. Finally, the exact binding locations of PPARγ are not well conserved between mice and humans; however, importantly, the gene networks regulated by PPARγ are similar, speaking to the conserved function of PPARγ.

One of the big challenges in genome-wide studies of TF binding is to link binding to function. Although in some cases PPARγ regulates the expression of the gene whose promoter is nearest to its binding sites, this may not be the case for many binding events. Abundant evidence indicates that enhancers may loop not only to the nearest promoter but may also loop to promoters far away, underscoring the limitations of assigning binding sites to genes solely based on proximity. Future assignments will need to take these chromosomal interactions into consideration, in *cis* as well as in *trans*. Furthermore, although technically challenging, it will be ultimately critical to mutate the binding sites to demonstrate their biological function.

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Glossary

Hotspot	Genomic region occupied by multiple transcription factors in a given cell type
Position weight matrix	Probabilistic representation of DNA sequence motifs generated based on alignment of several transcription factor binding sites
ChIP-seq	Chromatin immunoprecipitation (ChIP) coupled with deep sequencing to investigate protein-DNA interactions on a genome-wide scale
DHS-seq	DNAse I hypersensitivity (DHS) assays coupled with deep sequencing to investigate chromatin accessibility on a genome-wide scale
FAIRE-seq	Formaldehyde assisted isolation of regulatory elements (FAIRE) coupled with deep sequencing

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Highlights

- PPARγ is directly involved in regulating the majority of adipocyte genes
- Binding of PPARγ to genomic targets depends on cooperating transcription factors
- Cell type specific programming of the chromatin landscape determines PPARγ binding
- Regulatory networks of PPARγ are evolutionary conserved but binding sites are not

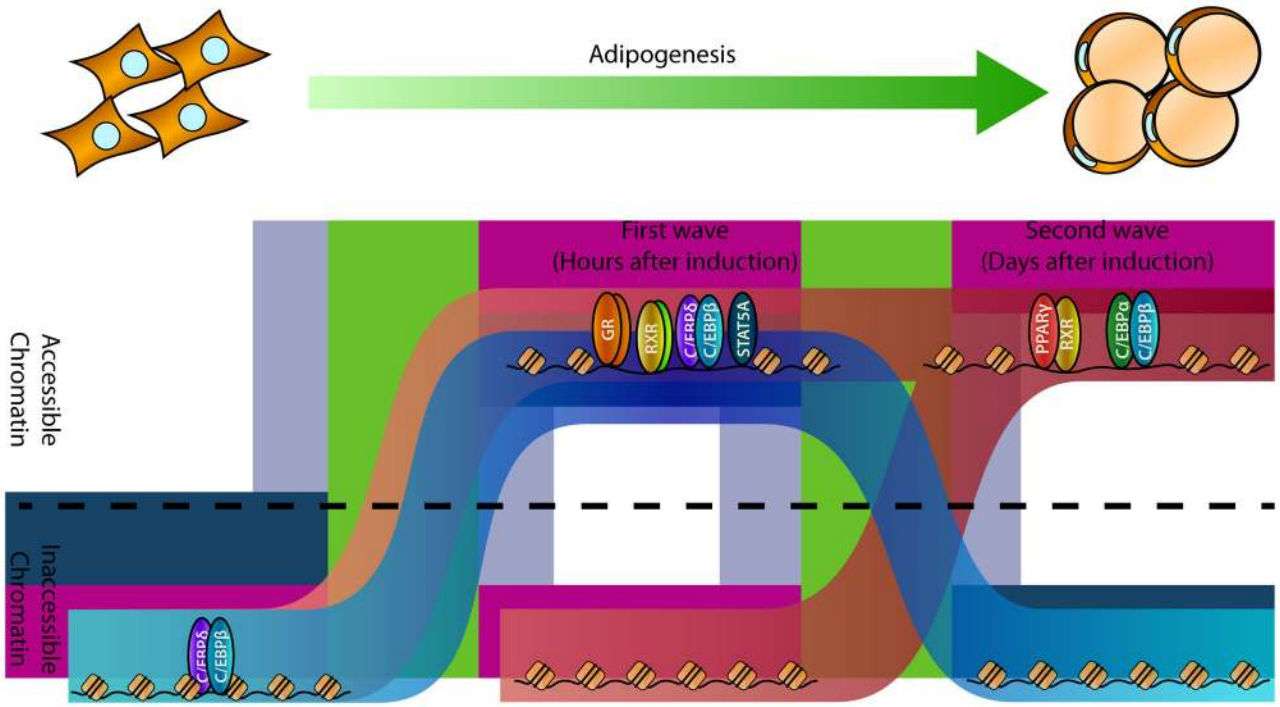

Figure 1. Model for the establishment of the PPARγ transcriptional network during adipogenesis

Adipocyte differentiation proceeds through two waves of TF activation: factors in the first wave are induced by the adipogenic cocktail and collectively activate the second wave of TFs including PPARγ and C/EBPα. This process is associated with changes in chromatin accessibility such that a large number of sites become open (i.e. DNase I hypesensitive) in "hotspots" where TFs bind cooperatively. Such accessibility may be transient during early adipogenesis or persistent in mature adipocytes depending on the TFs that occupy the hotspots. Notably, C/EBPβ is able to bind to relatively 'closed' chromatin at the earliest stages of differentiation.

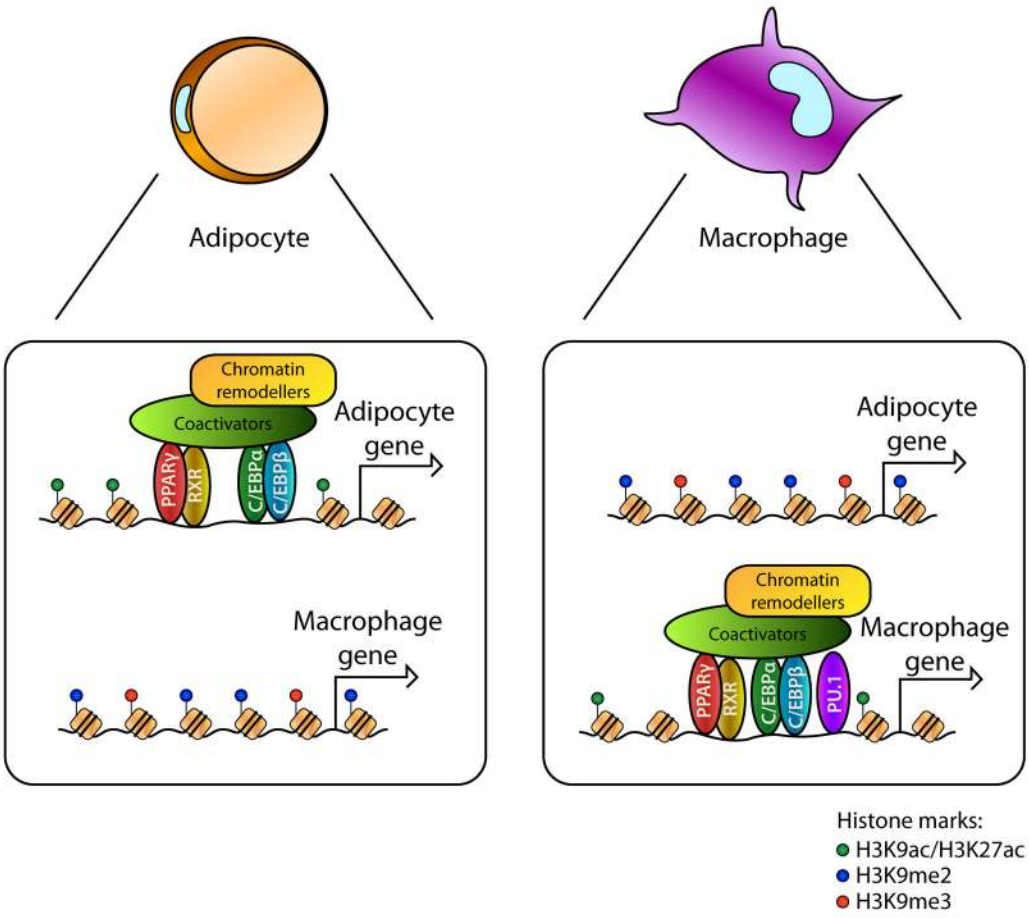

Figure 2. Model for the cell-type specific recruitment of PPARγ

The ability of PPARγ to access binding sites in the genome is limited within a given cell type, and may be defined by repressive mechanisms like chromatin silencing and active mechanisms such as cell type-specific recruitment of co-localizing TFs. For example, genes that are uniquely bound by PPARγ in macrophages ("macrophage genes") contain features of chromatin silencing in adipocytes, such as histone 3 lysine 27 trimethylation (H3K27me3) and histone 3 lysine 9 dimethylation (H3K9me2), that make PPARγ binding sites in these regions inaccessible. In contrast, genes that are regulated by PPARγ in adipocytes (adipocyte genes) have greater DNA accessibility (DNase I Hypersensitivity) and signatures of active chromatin such as histone acetylation (H3K9ac/H3K27ac). The establishment of such putative enhancers may be partially due to binding of TFs that facilitate the recruitment of PPARγ such as C/EBPα/β in adipocytes and PU.1 and C/EBPα/β in macrophages, and ultimately collaborate in recruiting coactivators and chromatin remodelers.

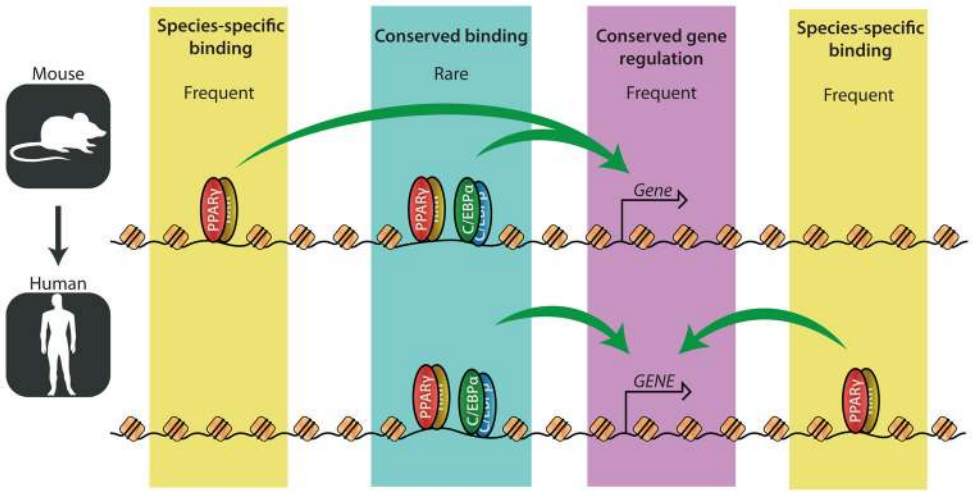

Figure 3. Interspecies conservation of the PPARγ transcriptional network

Comparisons of PPARγ binding between mouse and human reveal extensive evolutionary conservation of regulatory networks but limited conservation of binding events. A binding event is "conserved" when a ChIP-seq peak is detected at orthologous sequences in both species, or "species-specific" when a ChIP-seq peak is detected only in one species. PPARγ binding sites with nearby C/EBPα binding are more likely to be conserved between species than sites only binding PPARγ. Gene regulation is considered "conserved" when a given gene is associated with PPARγ binding in both species, irrespective of whether binding sites are conserved or species-specific.