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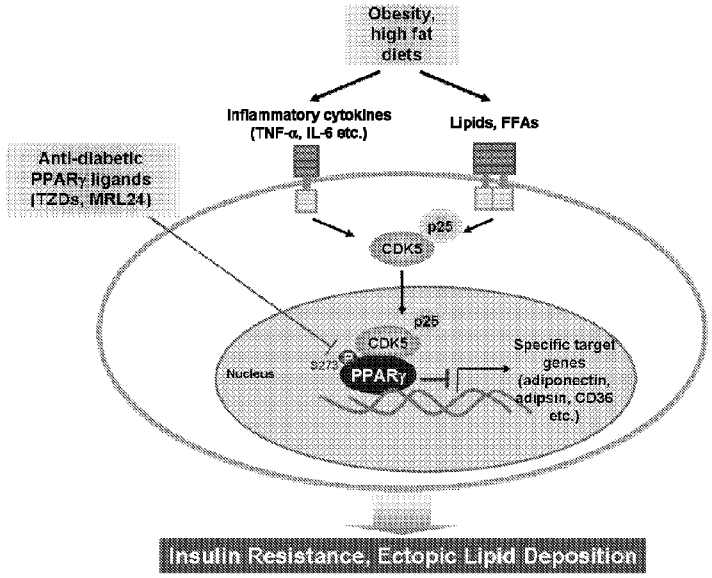
(54) Title: COMPOSITIONS,KITS, AND METHODS FOR IDENTIFICATION, ASSESSMENT, PREVENTION, AND THERAPY OF METABOLIC DISORDERS

Figure 13



(57) Abstract: The invention provides methods and compositions for selectively promoting anti- metabolic disorder activity over classical PPAR gamma activation through modulation of PPAR gamma phosphorylation (e.g., Ser-273 phosphorylation of murine peroxisome proliferator activated receptor gamma (PPAR gamma) 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog, including a human). Also provided are methods for preventing, treating, or predictiving responsiveness of therapies for metabolic disorders in a subject through selective inhibition of such PPAR gamma phosphorylation. Further provided are methods for identifying compounds that are capable of modulating such PPAR gamma phosphorylation.

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COMPOSITIONS, KITS, AND METHODS FOR IDENTIFICATION, ASSESSMENT, PREVENTION, AND THERAPY OF METABOLIC DISORDERS

Cross-Reference to Related Applications

This application claims the benefit of priority to U.S. Provisional Application No. 61/336,483, filed on January 22, 2010, U.S. Provisional Application No. 61/341,455, filed on March 31, 2010, and U.S. Provisional Application No. 61/399,975, filed on July 21, 2010; the entire contents of each of which are expressly incorporated herein by reference.

Government Support

Work herein was supported under Grant No. DK31405, awarded by the National Institutes of Health. The U.S. government may therefore have certain rights in this invention.

Background of the Invention

Metabolic disorders comprise a collection of health disorders or risks that increase the risk of morbidity and loss of qualify of life. For example, diabetes, obesity, including central obesity (disproportionate fat tissue in and around the abdomen), atherogenic dyslipidemia (these include a family of blood fat disorders, *e.g.*, high triglycerides, low HDL cholesterol, and high LDL cholesterol that can foster plaque buildups in the vascular system, including artery walls), high blood pressure (130/85 mmHg or higher), insulin resistance or glucose intolerance (the inability to properly use insulin or blood sugar), a chronic prothrombotic state (*e.g.*, characterized by high fibrinogen or plasminogen activator inhibitor-1 levels in the blood), and a chronic proinflammatory state (*e.g.*, characterized by higher than normal levels of high-sensitivity C-reactive protein in the blood), are all metabolic disorders collectively afflicting greater than 50 million people in the United States. In addition, the number of people afflicted with metabolic disorders correlates with increase in age, affecting more than 40 percent of people in their 60s and 70s.

Although treatments for such metabolic disorders do exist, they generally suffer from severe side effects. For example, agonists of the peroxisome proliferator-activated receptor gamma (PPAR gamma) nuclear receptor transcription factor, such as rosiglitazone and pioglitazone, have received regulatory approval for the treatment of type 2 diabetes in the United States and Europe. However, side effects or conditions that can be provoked or aggravated by such thiazolidinedione and non-thiazolidinedione PPAR gamma agonists,

including weight gain, fluid retention, peripheral edema, and pulmonary edema, limit the clinical utility and safety of such compounds. Thus, while there are several strategies for treating metabolic disorders, such as obesity predisposition, insulin resistance, high blood pressure, dyslipidemia, etc., the molecular basis for controlling such disorders without provoking or aggravating side effects is unclear, making diagnosis or prognosis of these metabolic disorders problematic and the design of therapeutic agents to treat them quite difficult. Accordingly, there is a great need in the art to identify molecular regulators of metabolic disorders, including the generation of diagnostic, prognostic, and therapeutic agents, such as orally active small molecules, that in turn regulate such molecular regulators so as to effectively control metabolic disorders in subjects.

Summary of the Invention

The present invention, at least in part, is based on the discovery that peroxisome proliferator activated receptor gamma (PPAR gamma), the master regulator of lipid metabolism in adipose tissue, is directly phosphorylated by cyclin dependent kinase 5 (cdk5) (*e.g.*, Ser-273 phosphorylation of PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog), and that this covalent modification causes a pathological pattern of gene expression associated with increased body weight, obesity, insulin resistance, and other metabolic disorders, as described herein. In addition, as described herein, PPAR gamma ligands that directly inhibit Ser-273 phosphorylation of PPAR gamma can reverse such metabolic disorders independently of classical PPAR gamma agonist activation. Accordingly, specific inhibition of phospho-Ser-273 on PPAR gamma can eliminate undesirable side effects observed with classical PPAR gamma agonists in the context of diagnosing, prognosing, and treating metabolic disorders, including diabetes and obesity. Other features and advantages of the invention will be apparent from the following detailed description and claims.

Accordingly, the present invention provides methods for identifying a compound which inhibits Ser-273 phosphorylation of murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog comprising contacting a sample comprising said murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog with a test compound and determining the ability of the test compound to inhibit said Ser-273 phosphorylation of murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog thereby identifying a

compound which selectively inhibits said Ser-273 phosphorylation of murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog. In one embodiment, the sample is selected from the group consisting of *in vitro*, *ex vivo*, and *in vivo* samples. In another embodiment, inhibition of Ser-273 phosphorylation of murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog is determined by analyzing the amount of Ser-273 phophorylated PPAR gamma relative to total PPAR gamma and comparing the ratio to a control (*e.g.*, such as the ratio from treatment with rosiglitazone under standard conditions). In still another embodiment, the method further comprises a step of determining whether the test compound directly binds said murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog.

In another aspect, methods are provided for identifying a compound that binds PPAR gamma and which selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation in a cell type, the method comprising, determining whether the compound binds PPAR gamma and comparing the amount and/or activity of a marker in a first sample of the cell type maintained in the presence of the test compound, wherein the marker is selected from the group of markers listed in Table 1 or 2, to the amount and/or activity of the marker in a second sample which is a control, wherein a significantly higher amount and/or activity of a marker listed in Table 1 in the first sample relative to the second sample indicates that the test compound selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation in the cell type and/or wherein a significantly lower amount and/or activity of a marker listed in Table 2 in the first sample relative to the second sample, indicates that the test compound selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation in the cell type. In one embodiment, the cell type is selected from the group consisting of: preadipocytes, mature white adipocytes, brown adipocytes, monocytes, and macrophages. In another embodiment, the first and/or second sample is selected from the group consisting of *in vitro*, *ex vivo*, and *in vivo* samples. In still another embodiment, the first and/or second sample is obtained from an animal model of a metabolic disorder. In yet another embodiment, the first and/or second sample is selected from the group consisting of tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, and bone marrow. In another embodiment, the first and second samples are portions of a single sample obtained from a subject. In still another embodiment, the second sample comprises

cells of the same cell type as the first sample maintained in the absence of the test compound. In yet another embodiment, the second sample comprises cells of the same cell type as the first sample treated with rosiglitazone. In another embodiment, a significantly higher amount and/or activity comprises upregulating the amount and/or activity of the marker listed in Table 1 at least 25% relative to the second sample. In yet another embodiment, a significantly lower amount and/or activity comprises downregulating the amount and/or activity of the marker listed in Table 2 at least 25% relative to the second sample. In still another embodiment, the amount of the marker is compared, for example, wherein the amount of the marker is determined by determining the level of protein expression of the marker (*e.g.*, using a reagent which specifically binds with the protein such as an antibody, an antibody derivative, and an antibody fragment, such as an antibody that binds to PPAR-gamma and an antibody that binds to a peptide comprising a consensus cdk5 phosphorylated site). In yet another embodiment, the level of expression of the marker in the sample is assessed by detecting the presence in the sample of a transcribed polynucleotide or portion thereof (*e.g.*, mRNA or a cDNA and/or further comprising amplifying the transcribed polynucleotide) or is assessed by detecting the presence in the sample of a transcribed polynucleotide which anneals with the marker or anneals with a portion of a polynucleotide under stringent hybridization conditions. In another embodiment, the marker is phosphorylated Ser-273 on murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog. In still another embodiment, the metabolic disorder is selected from the group consisting of: glucose intolerance, insulin resistance, hypertension, dyslipidemia, obesity, type II diabetes, hyperglycemia, hyperinsulinemia, elevated systolic and diastolic blood pressure, hypertrigliceridemia, hypercholesterolemia, and body mass index greater than 30. In yet another embodiment, PPAR gamma comprises the amino acid sequence set forth in SEQ ID NO:1-7.

In still another aspect, methods are provided for assessing the efficacy of a compound that binds PPAR gamma for selectively promoting anti-metabolic disorder activity over classical PPAR gamma activation in a subject, the method comprising (a) detecting in a subject sample at a first point in time, the amount and/or activity of a marker, wherein the marker is a marker listed in Table 1 or 2, repeating step (a) the step during at least one subsequent point in time after administration of the compound; and comparing the amount and/or activity detected in steps a) and b), wherein a significantly higher amount

and/or activity of a marker listed in Table 1 in the first subject sample relative to at least one subsequent subject sample, indicates that the test compound selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation in the subject and/or wherein a significantly lower amount and/or activity of a marker listed in Table 2 in the first subject sample relative to at least one subsequent subject sample, indicates that the test compound selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation in the subject. In one embodiment, the subject has undergone treatment for a metabolic disorder, has completed treatment for a metabolic disorder, and/or is in remission from a metabolic disorder in between the first point in time and the subsequent point in time. In another embodiment, the cell type is selected from the group consisting of: preadipocytes, mature white adipocytes, brown adipocytes, monocytes, and macrophages. In still another embodiment, the first and/or at least one subsequent sample is selected from the group consisting of *ex vivo* and *in vivo* samples. In yet another embodiment, the first and/or at least one subsequent sample is obtained from an animal model of a metabolic disorder. In another embodiment, the first and/or at least one subsequent sample is selected from the group consisting of tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, and bone marrow. In still another embodiment, the first and/or at least one subsequent sample is a portion of a single sample obtained from the subject. In yet another embodiment, the first and/or at least one subsequent sample is a portion of pooled samples obtained from the subject. In another embodiment, the method further comprises performing steps a) and b) with rosiglitazone, for example, to use the treatment with rosiglitazone to compare with the results from the test compound. In still another embodiment, a significantly higher amount and/or activity comprises upregulating the amount and/or activity of the marker listed in Table 1 at least 25% relative to the second sample. In yet another embodiment, a significantly lower amount and/or activity comprises downregulating the amount and/or activity of the marker listed in Table 2 at least 25% relative to the second sample. In still another embodiment, the amount of the marker is compared, for example, wherein the amount of the marker is determined by determining the level of protein expression of the marker (*e.g.*, using a reagent which specifically binds with the protein such as an antibody, an antibody derivative, and an antibody fragment, such as an antibody that binds to PPAR-gamma and an antibody that binds to a peptide comprising a consensus cdk5 phosphorylated site). In yet another embodiment, the level of expression of the marker in the sample is assessed by detecting the presence in the sample of a

transcribed polynucleotide or portion thereof (*e.g.*, mRNA or a cDNA and/or further comprising amplifying the transcribed polynucleotide.) or is assessed by detecting the presence in the sample of a transcribed polynucleotide which anneals with the marker or anneals with a portion of a polynucleotide under stringent hybridization conditions. In another embodiment, the marker is phosphorylated Ser-273 on murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog. In still another embodiment, the metabolic disorder is selected from the group consisting of: glucose intolerance, insulin resistance, hypertension, dyslipidemia, obesity, type II diabetes, hyperglycemia, hyperinsulinemia, elevated systolic and diastolic blood pressure, hypertrigliceridemia, hypercholesterolemia, and body mass index greater than 30. In yet another embodiment, PPAR gamma comprises the amino acid sequence set forth in SEQ ID NO:1-7. In some embodiments, the methods for assessing the efficacy of a compound that binds PPAR gamma for selectively promoting anti-metabolic disorder activity over classical PPAR gamma activation in a subject can also be used to assess the efficacy of a compound that binds PPAR gamma for promoting anti-metabolic disorder over a control. Accordingly, such controls can include, for example, samples not exposed to the control and/or exposed to the compound at different doses, time intervals, and similar variables. Such methods enable clinicians to better stratify a given subject for monitoring and/or therapeutic intervention, either with drug therapy or with other modalities.

In yet another aspect, methods are provided for treating a subject afflicted with a metabolic disease comprising administering to the subject a compound that binds PPAR gamma and which selectively promotes anti-diabetic activity, thereby treating the subject afflicted with the metabolic disease. In one embodiment, the compound inhibits Ser-273 phosphorylation of murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog. In another embodiment, the compound is administered in a pharmaceutically acceptable formulation. In still another embodiment, the pharmaceutically acceptable formulation is an oral formulation. In yet another embodiment, the compound is a small molecule.

In another aspect, compounds are provided for treating a metabolic disorder (*e.g.*, diabetes and/or obesity) selectively promoting anti-metabolic disorder activity in a cell type, wherein the compound inhibits phosphorylation of Ser-273 on PPAR gamma. In one embodiment, the compound has less than 30% of the PPAR gamma agonist function of rosiglitazone. In another embodiment, the compound has an EC50 binding affinity for

PPAR gamma less than 1-200 nM. In still another embodiment, the compound upregulates expression of a marker listed in Table 1 at least 30% relative to rosiglitazone under identical conditions and/or downregulates expression of a marker listed in Table 2 at least 30% relative to rosiglitazone under identical conditions. In yet another embodiment, the metabolic disorder is selected from the group consisting of: glucose intolerance, insulin resistance, hypertension, dyslipidemia, obesity, type II diabetes, hyperglycemia, hyperinsulinemia, elevated systolic and diastolic blood pressure, hypertrigliceridemia, hypercholesterolemia, and body mass index greater than 30.

In still another aspect, isolated nucleic acid molecules are provided encoding a murine peroxisome proliferator activated receptor gamma 2 polypeptide having a non-phosphorylatable amino acid at position Ser-273 or a homolog thereof having a non-phosphorylatable amino acid at the corresponding serine residue position in the murine PPAR gamma 2 polypeptide, or a complement thereof. In one embodiment, the isolated isolated nucleic acid molecules encode a polypeptide having the amino acid sequence set forth in SEQ ID NO:2 or 3, and further encoding a non-phosphorylatable amino acid at position Ser-273, or a complement thereof. In another embodiment, the isolated nucleic acid molecules further encode a heterologous polypeptide.

In yet another aspect, vectors (*e.g.*, expression vectors) are provided comprising the nucleic acid molecules of the invention and host cells are also provided comprising the vectors of the invention.

In another aspect, methods are provided for producing a protein comprising culturing host cell of the invention in a suitable medium until the protein is produced. In one embodiment, the produced proteins are further isolated from the meidum or the host cell.

In still another aspect, isolated proteins are provided comprising a murine peroxisome proliferator activated receptor gamma 2 polypeptide having a non-phosphorylatable amino acid at position Ser-273 or a homolog thereof having a non-phosphorylatable amino acid at the corresponding serine residue position in the murine PPAR gamma 2 polypeptide. In one embodiment, the isolated protein comprises a polypeptide having the amino acid sequence set forth in SEQ ID NO:2 or 3, and further encoding a non-phosphorylatable amino acid at position Ser-273. In another embodiment, the isolated protein is operatively linked to a heterologous polypeptide.

In yet another aspect, methods are provided for making an isolated hybridoma which produces an antibody that specifically binds to murine PPAR gamma 2 phosphorylated at Ser-273 or a corresponding serine residue in a murine PPAR gamma 2 homolog or a fragment thereof, the method comprising immunizing a mammal using a composition comprising said murine PPAR gamma 2 phosphorylated at Ser-273 or a corresponding serine residue in a murine PPAR gamma 2 homolog or a fragment thereof, isolating splenocytes from the immunized mammal, fusing the isolated splenocytes with an immortalized cell line to form hybridomas, and screening individual hybridomas for production of an antibody which specifically binds with said polypeptide thereof to isolate the hybridoma.

In another aspect, antibodies produced by a hybridoma of the invention are provided.

In still another aspect, isolated antibodies or antigen binding portions thereof are provided that specifically bind to a polypeptide comprising the amino acid sequence of a murine PPAR gamma 2 phosphorylated at Ser-273 or a corresponding serine residue in a murine PPAR gamma 2 homolog or a fragment thereof. In one embodiment, the antibody or antigen binding portion thereof specifically binds the epitope having the amino acid sequence, KTTDK(pS)PFVIYDC. In another embodiment, the antibody or antigen binding portion thereof is a monoclonal, polyclonal, chimeric, or a humanized, antibody. In still another embodiment, the antibody or antigen binding portion thereof is detectably labeled. In yet another embodiment, the antibody or antigen binding portion thereof comprises an effector domain, an Fc domain, is a single-chain antibody, or is a Fab fragment.

In yet another aspect, kits are provided for assessing the ability of a compound to inhibit phosphorylation of PPAR gamma comprising one or more reagents for specifically detecting murine PPAR gamma 2 phosphorylated at Ser-273 or a corresponding serine residue in a murine PPAR gamma 2 homolog.

In another aspect, non-human animal models are provided comprising a mutated PPAR gamma 2 gene encoding a protein incapable of being phosphorylated at serine 273 or a corresponding serine residue in a murine PPAR gamma 2 homolog. In one embodiment, the mutation comprises a mutation of said serine to a non-phosphorylatable amino acid, such as alanine. In another embodiment, the non-human animal model is heterozygous or homozygous for the mutated PPAR gamma 2 gene encoding a protein incapable of being phosphorylated at serine 273 or a corresponding serine residue in a murine PPAR gamma 2

homolog. In another embodiment, the non-human animal model is a knock-in or a transgenic animal. In still another embodiment, the non-human animal model is a rodent and/or a mouse.

In still another aspect, methods are provided for identifying genes regulated in a subject, comprising expressing a mutated PPAR gamma gene encoding a protein incapable of being phosphorylated on Serine 273 or a corresponding serine residue in a murine PPAR gamma 2 homolog, determining the level of expression and/or activity of one or more candidate target genes of said mutated PPAR gamma gene, and identifying genes which exhibit significantly altered expression and/or activity relative to a control.

In yet another aspect, methods are provided for classifying a sample according to a predicted treatment outcome comprising comparing the level of expression of a marker or a plurality of markers in a biological sample and the level of expression of the marker or plurality of markers in a control sample, wherein the marker or plurality of markers are selected from the group consisting of the markers listed in Tables 1 and 2 (*e.g.*, phosphorylated Ser-273-PPAR gamma or homolog thereof) and the difference between the level of expression of the marker or plurality of markers in the biological sample and the control sample classifies the biological sample according to the predicted treatment outcome. In one embodiment, the predicted treatment outcome is selected from the treatment outcomes listed in Tables 1 and 2, such as increased insulin sensitivity. In another embodiment, the biological sample is obtained after a PPAR gamma ligand as been administered to the subject from which the biological sample was obtained.

In another aspect, methods are provided for identifying a subject likely to have a predicted treatment outcome comprising comparing the level of expression of a marker or a plurality of markers in a biological sample from the subject and the level of expression of the marker or plurality of markers in a control sample, wherein the marker or plurality of markers are selected from the group consisting of the markers listed in Tables 1 and 2 and the difference between the level of expression of the marker or plurality of markers in the biological samples from the subject and the control sample predicts the likelihood of a predicted treatment outcome in the subject. In one embodiment, the predicted treatment outcome is selected from the treatment outcomes listed in Tables 1 and 2, such as increased insulin sensitivity. In another embodiment, the biological sample is obtained after a PPAR gamma ligand as been administered to the subject.

It will also be understood that certain embodiments of the present invention can be used with more than one method, composition, kit, etc. described herein, according to knowledge available to the skilled artisan.

Brief Description of Figures

Figures 1A-1D depict results of cdk5-specific phosphorylation of serine 273 on murine PPAR gamma 2. Figure 1A shows conservation of phosphorylated serine residues of PPAR gamma polypeptides across numerous species. Figure 1B shows results of *in vitro* CDK kinase assays performed using cdk5/p35 or the indicated cdks/cyclins CDK5/p35 with either wild type (WT) or S273A mutated PPAR. Histone H1 or Rb were used as a positive controls. Figure 1C shows cdk5-mediated phosphorylation of PPAR gamma in HEK293 cells following co-expression of PPAR gamma with either wild type or kinase inactive mutant (KD) of CDK5; IP, immunoprecipitate; IB, immunoblot. Figure 1D shows that CDK5 phosphorylated PPAR gamma, but not PPAR alpha or PPAR delta isoforms.

Figures 2A-2C depict results of cdk5-specific phosphorylation of PPAR gamma upon exposure of cells to pro-inflammatory cytokines or free fatty acids (FFA). Figure 2A shows the results of treating differentiated 3T3-L1 cells with TNF-α, IL-6, or FFAs for the indicated times. Phosphorylation of PPAR gamma was detected using a phospho-S273 PPAR gamma antibody. Figure 2B shows the results of Western blot analyses of PPAR gamma phosphorylation in differentiated 3T3-L1 adipocytes expressing scrambled or CDK5 shRNA. Figure 2C shows the results of cells expressing scrambled or CDK5 shRNA stimulated with the indicated cytokines and analyzed with phospho-S273 PPAR gamma antibody. NT, no treatment.

Figures 3A-3E depict results of specific fat cell gene dysregulation by the cdk-5 mediated S273 phosphorylation of PPAR gamma. Figure 3A shows transcriptional activity of a PPAR-derived reporter gene in response to wild type or S273A mutant of PPAR gamma/RXRα in HEK-293 cells (± 10 μM rosiglitazone) (n=3). Figure 3B shows the results of PPAR gamma-null fibroblasts expressing retroviral wild type or S273A mutant PPAR gamma stained with Oil-Red-O 7 days after adipocyte differentiation. Expression of PPAR gamma was analyzed with an anti-PPAR gamma antibody. Figure 3C shows the results of gene expression in these cells by real-time quantitative PCR (qPCR) for expression of various genes (n=3). Figure 3D shows secreted adiponectin levels in cultured

medium (n=3). Figure 3E shows mRNA expression in tranplsanted fat pads as analyzed by real-time qPCR (n=5). Error bars are s.e.m.; * $p<0.05$; ** $p<0.01$).

Figures 4A-4C depict results of CDK5-mediated phosphorylation of PPAR gamma increase in fat tissues of high fat diet fed mice (HFD). Figure 4A shows results of white adipose tissue (epididymal) from mice on HFD for the indicated time analyzed with phospho-S273 PPAR gamma, PPAR gamma, phospho-Y15 CDK5, CDK5 and p35 antibodies. Figure 4B shows the results of selected metabolic parameters in mice fed chow or HFD. Results of body weights, glucose and insulin levels in C57BL/6J mice (n=6) on chow or HFD for 7- or 13- weeks after an overnight fast are shown (error bars are s.e.m.; ** $p<0.01$; *** $p<0.001$). Figure 4C shows the results of epididymal (Epi.) or inguinal (Ing.) fat tissue from 13 weeks HFD mice analyzed with phospho-S273 antibody.

Figures 5A-5F depict results of PPAR gamma ligands inhibiting phosphorylation of PPAR gamma. Figure 5A shows the results of TNF-α-induced phosphorylation of PPAR gamma in 3T3-L1 adipocytes expressing either wild type or Q286P mutant of PPAR gamma treated as indicated with rosiglitazone and/or GW9662. Figure 5B shows the results of an *in vitro* CDK5 kinase assay with CDK5/p35 and PPAR gamma incubated with either rosiglitazone or MRL24 and the indicated concentrations. Figure 5C shows transcriptional activity of a PPAR-derived reporter gene in response to rosiglitazone or MRL24 in HEK-293 cells (n=3; error bars are s.e.m.). Figure 5D shows the results of an *in vitro* kinase assay using CDK5/p35 and either Rb or PPAr gamma with rosiglitazone or MRL24. Figure 5E shows TNF-α-induced phosphorylation of PPAR gamma in HEK-293 cells with rosiglitazone or MRL24 at the indicated concentrations. Figure 5F shows the results of microarray analyses of differentiated PPAr gamma-null fibroblasts expressing wild type (NT, no treatment; rosiglitazone, or MRL24 treated) or S273A mutant PPAR gamma (n=3).

Figures 6A-6C depict results of atypical PPAR gamma ligands inhibiting CDK5-mediated phosphorylation of PPAR gamma . Figure 6A shows results of in vitro CDK5 kinase assays performed using purified CDK5/p35 and PPAR gamma with different PPAR gamma ligands (rosiglitazone, Mbx-102, BVT.13, nTZDpa or MRL24) at the indicated concentrations. Phosphorylation of PPAR gamma, total PPAR gamma and CDK5 were detected by Western blotting. Figure 6B shows structural diversity of numerous atypical PPAR gamma agonists. Figure 6C shows that numerous atypical PPAR gamma agonists have very weak agonist activity on a PPAR transcriptional response element.

Figure 7 depicts dysregulation of gene sets regulated by PPAR gamma phosphorylation in obese mice. mRNA expression of genes regualted by the phosphorylation of PPAr gamma in cells and transplanted fat pads in WAT of mice on either chow or HFD for 13 weeks (n=5; Error bars are s.e.m.; * $p<0.05$; ** $p<0.01$;*** $p<0.001$).

Figures 8A-8B depict differential HDX MS data for PPAR gamma-LBD ± rosiglitazone and MRL24. The HDX data shown correspond to four regions of interest: Helix 3 (IRIFQGCQF), the β-sheet region (ISEGQGFMTRE), Helix 12 (QEIYKDLY) and the Helix 2-2' link region containing the site of CDK5 phosphorylation (KTTDKSPFVIYDM). Figure 8A shows histograms indicating the percent reduction in HDX for each peptide region. Values are calculated relative to the measured %D value for apo PPAR gamma-LBD (n=4; error bars are s.e.m.). Figure 8B shows HDX data for the four peptides of interest plotted over the structures of PPAR gamma-LBD bound with rosiglitazone (left, PDB:2PRG) and MRL24 (right, PDB:2Q5P). Percent reduction in HDX relative to apo receptor is colored according to the key.

Figures 9A-9E depict results of anti-diabetic, atypical PPAR gamma agonists selectively inhibiting phosphorylation of PPAR gamma and reversing changes in gene expression *in vivo*. Figure 9A shows the results of glucose-tolerance tests in 16-week HFD mice treated with vehicle, rosiglitazone or MRL24 (n=10). Fasting insulin levels (Figure 9B) and body weight (Figure 9C) of these mice are shown. Figure 9D shows results of phosphorylation of PPAR gamma in WAT from these mice analyzed using a phospho-S273 antibody. Figure 9E shows real-time qPCR results of WAT from these mice analyzed for the expression of gene sets regulated by PPAR gamma phosphorylation (Error bars are s.e.m.; * $p<0.05$; ** $p<0.01$;*** $p<0.001$).

Figure 10 depicts results of increasing brown fat selective gene expression in white adipose tissues upon administration of no agonists, classical PPAR gamma agonists, or atypical PPAR gamma agonists in animals.

Figure 11 depicts results of respiration experiments performed on animals treated with or without atypical PPAR gamma agonists.

Figures 12A-12B depict a targeted knock-in animal construct generation (Figure 11A) and breeding (Figure 11B) strategy.

Figure 13 depicts a model of obesity-linked phsophorylation of PPAR gamma by CDK5.

Figures 14A-14C depict results of PPAR gamma phosphorylation levels of human subjects having type 2 diabetes treated with rosiglitazone at a dose of 4 mg/day for six months. Figure 14A shows the results of pPPAR gamma/PPAR gamma in percentage terms before and after the rosiglitazone treatment regimen. Figure 14B shows the correlation of insulin sensitivity in the subjects with the percentage change in the pPPAR gamma/PPAR gamma ratio due to the rosiglitazone treatment. Figure 14C provides quantitative data.

Brief Description of the Tables

Table 1 is a listing of exemplary genes, activities and assays whose expression and/or activity is increased relative to a control.

Table 2 is a listing of exemplary genes, activities, and assays whose expression and/or activity is decreased relative to a control.

Table 3 is a listing of exemplary primer sequences useful for quantifying expression levels of markers of the present invention.

Detailed Description of the Invention

In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.,* to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

The term "altered amount" of a biomarker or "altered level" of a biomarker refers to increased or decreased expression and/or activity of a biomarker of the present invention, at least in part, (*e.g.*, the markers set forth in Tables 1 and 2) in a sample as compared to that in a control sample. The term "altered amount" of a biomarker also includes an increased or decreased protein level of a biomarker in a sample as compared to the protein level of the biomarker in a normal, control sample.

The term "altered level of expression" of biomarkers of the present invention, at least in part, (*e.g.*, biomarkers set forth in Tables 1 and 2) refers to an expression level or copy number of a biomarker in a test sample such as a sample derived from a subject suffering from a metabolic disorder (*e.g.*, diabetes and/or obesity), that is greater or less than the standard error of the assay employed to assess expression or copy number, and

may be at least twice, at least twice three, at least twice four, at least twice five, or at least twice ten or more times the expression level or copy number of the biomarkers of the present invention, at least in part, (*e.g.*, biomarkers set forth in Tables 1 and 2) in a control sample (*e.g.*, a sample from a healthy subject not having the associated disease), or the average expression level or copy number of the biomarkers of the present invention, at least in part, (*e.g.*, biomarkers set forth in Tables 1 and 2) in several control samples. The altered level of expression is greater or less than the standard error of the assay employed to assess expression or copy number, and is at least twice, at least three, at least four, at least five, at least ten or more times the expression level or copy number of the biomarkers of the present invention, at least in part, (*e.g.*, biomarkers set forth in Tables 1 and 2) in a control sample (*e.g.*, a sample from a healthy subject not having the associated disease), or the average expression level or copy number of the biomarkers of the present invention, at least in part, (*e.g.*, biomarkers set forth in Tables 1 and 2) in several control samples.

The term "altered activity" of a biomarker refers to an activity of a biomarker which is increased or decreased in a disease state, *e.g.,* in a metabolic disorder (*e.g.*, diabetes and/or obesity) sample, as compared to the activity of the biomarker in a normal, control sample. Altered activity of a biomarker may be the result of, for example, altered expression of the biomarker, altered protein level of the biomarker, altered structure of the biomarker, or, *e.g.,* an altered interaction with other proteins involved in the same or different pathway as the biomarker. Biological activities, as described herein, may include subject responses as set forth in this application, as measured by gene expression changes and/or assays listed in Tables 1 and 2.

The term "altered structure" of a biomarker refers to the presence of mutations or mutations within the biomarker gene or maker protein, *e.g.,* mutations which affect expression or activity of the biomarker, as compared to the normal or wild-type gene or protein. For example, mutations include, but are not limited to inter- and intra-chromosomal rearrangement, substitutions, deletions, and insertion mutations. Mutations may be present in the coding or non-coding region of the biomarker.

"Binding compound" shall refer to a binding composition, such as a small molecule, an antibody, a peptide, a peptide or non-peptide ligand, a protein, an oligonucleotide, an oligonucleotide analog, such as a peptide nucleic acid, a lectin, or any other molecular entity that is capable of specifically binding to a target protein or molecule or stable complex formation with an analyte of interest, such as a complex of proteins.

"Binding moiety" means any molecule to which molecular tags can be directly or indirectly attached that is capable of specifically binding to an analyte. Binding moieties include, but are not limited to, antibodies, antibody binding compositions, peptides, proteins, nucleic acids and organic molecules having a molecular weight of up to about 1000 daltons and containing atoms selected from the group consisting of hydrogen, carbon, oxygen, nitrogen, sulfur and phosphorus.

A "biomarker" or "marker" is a gene, mRNA, or protein which may be altered, wherein said alteration is associated with a metabolic disorder (*e.g.*, diabetes and/or obesity). The alteration may be in amount, structure, and/or activity in a metabolic disorder (*e.g.*, diabetes and/or obesity) tissue or a metabolic disorder (*e.g.*, diabetes and/or obesity) cell, as compared to its amount, structure, and/or activity, in a normal or healthy tissue or cell (*e.g.,* a control), and is associated with a disease state, such as a metabolic disorder (*e.g.*, diabetes and/or obesity). For example, a biomarker of the present invention, at least in part, which is associated with a metabolic disorder (*e.g.*, diabetes and/or obesity) or predictive of responsiveness to anti-a metabolic disorder (*e.g.*, diabetes and/or obesity) therapeutics may have an altered nucleotide sequence, amino acid sequence, chromosomal translocation, intra-chromosomal inversion, copy number, expression level, protein level, protein activity, or methylation status, in a metabolic disorder (*e.g.*, diabetes and/or obesity) tissue or a metabolic disorder (*e.g.*, diabetes and/or obesity) cell as compared to a normal, healthy tissue or cell. Furthermore, a "biomarker" includes a molecule whose structure is altered, *e.g.,* mutated (contains an mutation), *e.g.,* differs from the wild type sequence at the nucleotide or amino acid level, *e.g.,* by substitution, deletion, or insertion, when present in a tissue or cell associated with a disease state, such as a metabolic disorder (*e.g.*, diabetes and/or obesity). In some embodiments, the biomarkers of the present invention, at least in part, are selected from the group of biomarkers listed in Tables 1 and 2.

"Complementary" refers to the broad concept of sequence complementarity between regions of two nucleic acid strands or between two regions of the same nucleic acid strand. It is known that an adenine residue of a first nucleic acid region is capable of forming specific hydrogen bonds ("base pairing") with a residue of a second nucleic acid region which is antiparallel to the first region if the residue is thymine or uracil. Similarly, it is known that a cytosine residue of a first nucleic acid strand is capable of base pairing with a residue of a second nucleic acid strand which is antiparallel to the first strand if the residue is guanine. A first region of a nucleic acid is complementary to a second region of the same

or a different nucleic acid if, when the two regions are arranged in an antiparallel fashion, at least one nucleotide residue of the first region is capable of base pairing with a residue of the second region. In certain embodiments, the first region comprises a first portion and the second region comprises a second portion, whereby, when the first and second portions are arranged in an antiparallel fashion, at least about 50%, at least about 75%, at least about 90%, or at least about 95% of the nucleotide residues of the first portion are capable of base pairing with nucleotide residues in the second portion. In other embodiments, all nucleotide residues of the first portion are capable of base pairing with nucleotide residues in the second portion.

The "copy number of a gene" or the "copy number of a biomarker" refers to the number of DNA sequences in a cell encoding a particular gene product. Generally, for a given gene, a mammal has two copies of each gene. The copy number can be increased, however, by gene amplification or duplication, or reduced by deletion.

As used herein, the term “diabetes” refers to a number of well-known conditions. Insulin resistance is defined as a state in which circulating insulin levels in excess of the normal response to a glucose load are required to maintain the euglycemic state (Ford E S, *et al.* JAMA. (2002) 287:356-9). Insulin resistance, and the response of a subject with insulin resistance to therapy, may be quantified by assessing the homeostasis model assessment to insulin resistance (HOMA-IR) score, a reliable indicator of insulin resistance (Katsuki A, *et al.* Diabetes Care 2001; 24:362-5). The estimate of insulin resistance by the homeostasis assessment model (HOMA)-IR score is calculated with the formula (Galvin P, *et al.* Diabet Med 1992;9:921-8): HOMA-IR=[fasting serum insulin (μU/mL)]x[fasting plasma glucose (mmol/L)/22.5]. Subjects with a predisposition for the development of impaired glucose tolerance (IGT) or type 2 diabetes are those having euglycemia with hyperinsulinemia are by definition, insulin resistant. A typical subject with insulin resistance is usually overweight or obese. The term “pre-diabetes” is the condition wherein an individual is pre-disposed to the development of type 2 diabetes. Pre-diabetes extends the definition of impaired glucose tolerance to include individuals with a fasting blood glucose within the high normal range 100 mg/dL (J. B. Meigs, *et al.* Diabetes 2003; 52:1475-1484) and fasting hyperinsulinemia (elevated plasma insulin concentration). The scientific and medical basis for identifying pre-diabetes as a serious health threat is laid out in a Position Statement entitled "The Prevention or Delay of Type 2 Diabetes" issued jointly by the American Diabetes Association and the National Institute of Diabetes and

Digestive and Kidney Diseases (Diabetes Care 2002; 25:742-749). Individuals likely to have insulin resistance are those who have two or more of the following attributes: 1) overweight or obese, 2) high blood pressure, 3) hyperlipidemia, 4) one or more 1.sup.st degree relative with a diagnosis of IGT or type 2 diabetes. Insulin resistance can be confirmed in these individuals by calculating HOMA-IR score. For the purpose of this invention, insulin resistance is defined as the clinical condition in which an individual has a HOMA-IR score >4.0 or a HOMA-IR score above the upper limit of normal as defined for the laboratory performing the glucose and insulin assays. Type 2 diabetes is defined as the condition in which a subject has a fasting blood glucose or serum glucose concentration greater than 125 mg/dl (6.94 mmol/L).

A biomarker is "fixed" to a substrate if it is covalently or non-covalently associated with the substrate such that the substrate can be rinsed with a fluid (*e.g.*, standard saline citrate, pH 7.4) without a substantial fraction of the biomarker dissociating from the substrate.

"Hazard ratio", as used herein, refers to a statistical method used to generate an estimate for relative risk. "Hazard ratio" is the ratio between the predicted hazard of one group versus another group. For example, subject populations treated with a PPAR gamma ligand versus without a PPAR gamma ligand can be assessed for whether or not the PPAR gamma ligand is effective in treating a metabolic disorder (*e.g.*, diabetes and/or obesity), particularly with regard to PPAR gamma phosphorylation status on serine 273.

The terms “homology” or “identity,” as used interchangeably herein, refer to sequence similarity between two polynucleotide sequences or between two polypeptide sequences, with identity being a more strict comparison. The phrases "percent identity or homology" and "% identity or homology" refer to the percentage of sequence similarity found in a comparison of two or more polynucleotide sequences or two or more polypeptide sequences. "Sequence similarity" refers to the percent similarity in base pair sequence (as determined by any suitable method) between two or more polynucleotide sequences. Two or more sequences can be anywhere from 0-100% similar, or any integer value there between. Identity or similarity can be determined by comparing a position in each sequence that may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same nucleotide base or amino acid, then the molecules are identical at that position. A degree of similarity or identity between polynucleotide sequences is a function of the number of identical or matching nucleotides at positions shared by the

polynucleotide sequences. A degree of identity of polypeptide sequences is a function of the number of identical amino acids at positions shared by the polypeptide sequences. A degree of homology or similarity of polypeptide sequences is a function of the number of amino acids at positions shared by the polypeptide sequences. The term "substantial homology," as used herein, refers to homology of at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or more.

"Likely to," as used herein, refers to an increased probability, that an item, object, thing or person will occur such as at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, or more (or any range inclusive). Thus, in one example, a subject that is likely to respond to treatment with the PPAR gamma ligands of the present invention, at least in part, has an increased probability of responding to treatment with the PPAR gamma ligand such that an anti-metabolic disorder activity is selectively promoted over classical PPAR gamma activation relative to a reference subject or group of subjects.

The terms "metabolic disorder" and "obesity related disorders" are used interchangeably herein and include a disorder, disease or condition which is caused or characterized by an abnormal metabolism (*i.e.*, the chemical changes in living cells by which energy is provided for vital processes and activities) in a subject. Metabolic disorders include diseases, disorders, or conditions associated with aberrant thermogenesis or aberrant adipose cell (*e.g.*, brown or white adipose cell) content or function. Metabolic disorders can be characterized by a misregulation (*e.g.*, downregulation or upregulation) of expression, structure, and/or expression of one or more biomarkers (including fragments thereof) and/or assays listed in Tables 1 and 2. Metabolic disorders can detrimentally affect cellular functions such as cellular proliferation, growth, differentiation, or migration, cellular regulation of homeostasis, inter- or intra-cellular communication; tissue function, such as liver function, muscle function, or adipocyte function; systemic responses in an organism, such as hormonal responses (*e.g.*, insulin response).

Examples of metabolic disorders include obesity, including insulin resistant obesity, diabetes, noninsulin dependent diabetes mellitus (NIDDM or Type H diabetes), insulin dependent diabetes mellitus (IDDM or Type I diabetes), type II diabetes, insulin resistance such as impaired glucose tolerance, glucose intolerance, atherosclerosis, atheromatous disease, heart disease, hypertension, stroke, Syndrome X, hyperphagia, endocrine

abnormalities, triglyceride storage disease, Bardet-Biedl syndrome, Lawrence-Moon syndrome, Prader-Labhart-Willi syndrome, Werner's syndrome, dysfunctions associated with lipid biosynthesis, lipid transport, triglyceride levels, plasma levels, and plasma cholesterol, dyslipidemias associated with hyperlipidemia, elevated free fatty acids, hypercholesterolemia, hypertriglyceridemia, elevated low density lipoprotein-(LDL)-cholesterol, elevated very low density lipoprotein-(VLDL)-cholesterol, elevated intermediate density lipoprotein-(IDL)-cholesterol, or reduced high density lipoprotein-(HDL)-cholesterol. A metabolic disorder (*e.g.*, diabetes and/or obesity) is "inhibited" if at least one symptom of the metabolic disorder (*e.g.*, diabetes and/or obesity) is alleviated, terminated, slowed, or prevented. As used herein, a metabolic disorder (*e.g.*, diabetes and/or obesity) is also "inhibited" if recurrence or metastasis of the metabolic disorder (*e.g.*, diabetes and/or obesity) is reduced, slowed, delayed, or prevented.

In addition, metabolic disorders are associated with one or more discrete phenotypes. For example, body mass index (BMI) of a subject is defined as the weight in kilograms divided by the square of the height in meters, such that BMI has units of kg/m^2. Overweight is defined as the condition wherein the individual has a BMI greater than or 25 kg/m^2 and less than 30 kg/m^2. In some embodiments, obesity is defined as the condition wherein the individual has a BMI equal to or greater than 30 kg/m^2. In another aspect, the term obesity is used to mean visceral obesity which can be defined in some embodiments as a waist-to-hip ratio of 1.0 in men and 0.8 in women, which, in another aspect defines the risk for insulin resistance and the development of pre-diabetes. Euglycemia is defined as the condition in which a subject has a fasting blood glucose concentration within the normal range, greater than 70 mg/dl (3.89 mmol/L) and less than 110 mg/dl (6.11 mmol/L). The word fasting has the usual meaning as a medical term. Impaired glucose tolerance (IGT), is defined as the condition in which a subject has a fasting blood glucose concentration or fasting serum glucose concentration greater than 110 mg/dl and less than 126 mg/dl (7.00 mmol/L), or a 2 hour postprandial blood glucose or serum glucose concentration greater than 140 mg/dl (7.78 mmol/L) and less than 200 mg/dl (11.11 mmol/L). The term impaired glucose tolerance is also intended to apply to the condition of impaired fasting glucose. Hyperinsulinemia is defined as the condition in which a subject with insulin resistance, with or without euglycemia, in which the fasting or postprandial serum or plasma insulin concentration is elevated above that of normal, lean individuals without insulin resistance, having a waist-to-hip ration <1.0 (for men) or <0.8 (for women). The terms "diabetes",

"prediabetes", and "insulin-sensitizing", "insulin resistance-improving" or "insulin resistance-lowering" (used interchangeably herein) have been described herein.

As used herein, "metabolic syndrome" refers to a condition present when more than one of these factors are present in a single individual. The factors include: central obesity (disproportionate fat tissue in and around the abdomen), atherogenic dyslipidemia (these include a family of blood fat disorders including, *e.g.*, high triglycerides, low HDL cholesterol, and high LDL cholesterol that can foster plaque buildups in the vascular system, including artery walls), high blood pressure (130/85 mmHg or higher), insulin resistance or glucose intolerance (the inability to properly use insulin or blood sugar), a chronic prothrombotic state (*e.g.*, characterized by high fibrinogen or plasminogen activator inhibitor [-1] levels in the blood), and a chronic proinflammatory state (*e.g.*, characterized by higher than normal levels of high-sensitivity C-reactive protein in the blood). In some embodiments, the "Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III)" may be used in the diagnosis of a metabolic disorder. Under the NCEP criteria, metabolic syndrome can be clinically identified by the presence of three or more of the following components in a single subject: (1) central obesity, as measured by waist circumference (women with a waist circumference greater than 35 inches; for men greater than 40 inches); (2) fasting blood triglycerides greater than or equal to 150 mg/dL; (3) blood HDL cholesterol (for women less than 50 mg/dL, for men less than 40 mg/dL); (4) blood pressure greater than or equal to 130/85 mmHg; and (5) fasting glucose greater than or equal to 110 mg/dL. Other features such as insulin resistance (*e.g.*, increased fasting blood insulin), prothrombotic state or proinflammatory state are not generally required for clinical diagnosis, though they are certainly also indicative of metabolic syndrome and follow-up studies on these attributes can be used to further confirm diagnosis of metabolic syndrome. For example, insulin resistance, even in the absence of the NCEP criteria, is often indicative of metabolic syndrome.

A biomarker "nucleic acid" is a nucleic acid (*e.g.*, DNA, mRNA, cDNA) encoded by or corresponding to a biomarker of the present invention, at least in part,. For example, such biomarker nucleic acid molecules include DNA (*e.g.*, genomic DNA and cDNA) comprising the entire or a partial sequence of any of the nucleic acid sequences set forth in Tables 1 and 2 or the complement or hybridizing fragment of such a sequence. The biomarker nucleic acid molecules also include RNA comprising the entire or a partial

sequence of any of the nucleic acid sequences set forth in Tables 1 and 2 or the complement of such a sequence, wherein all thymidine residues are replaced with uridine residues. A "biomarker protein" is a protein encoded by or corresponding to a biomarker of the present invention, at least in part,. A biomarker protein comprises the entire or a partial sequence of a protein encoded by any of the sequences set forth in Tables 1 and 2 or a fragment thereof. The terms "protein" and "polypeptide" are used interchangeably herein.

There is a known and definite correspondence between the amino acid sequence of a particular protein and the nucleotide sequences that can code for the protein, as defined by the genetic code (shown below). Likewise, there is a known and definite correspondence between the nucleotide sequence of a particular nucleic acid and the amino acid sequence encoded by that nucleic acid, as defined by the genetic code.

GENETIC CODE	
Alanine (Ala, A)	GCA, GCC, GCG, GCT
Arginine (Arg, R)	AGA, ACG, CGA, CGC, CGG, CGT
Asparagine (Asn, N)	AAC, AAT
Aspartic acid (Asp, D)	GAC, GAT
Cysteine (Cys, C)	TGC, TGT
Glutamic acid (Glu, E)	GAA, GAG
Glutamine (Gln, Q)	CAA, CAG
Glycine (Gly, G)	GGA, GGC, GGG, GGT
Histidine (His, H)	CAC, CAT
Isoleucine (Ile, I)	ATA, ATC, ATT
Leucine (Leu, L)	CTA, CTC, CTG, CTT, TTA, TTG
Lysine (Lys, K)	AAA, AAG
Methionine (Met, M)	ATG
Phenylalanine (Phe, F)	TTC, TTT
Proline (Pro, P)	CCA, CCC, CCG, CCT
Serine (Ser, S)	AGC, AGT, TCA, TCC, TCG, TCT
Threonine (Thr, T)	ACA, ACC, ACG, ACT
Tryptophan (Trp, W)	TGG
Tyrosine (Tyr, Y)	TAC, TAT
Valine (Val, V)	GTA, GTC, GTG, GTT
Termination signal (end)	TAA, TAG, TGA

An important and well known feature of the genetic code is its redundancy, whereby, for most of the amino acids used to make proteins, more than one coding nucleotide triplet may be employed (for example, illustrated above). Therefore, a number of different nucleotide sequences may code for a given amino acid sequence. Such nucleotide sequences are considered functionally equivalent since they result in the production of the same amino acid sequence in all organisms (although certain organisms may translate some sequences more efficiently than they do others). Moreover, occasionally, a methylated variant of a purine or pyrimidine may be found in a given nucleotide sequence. Such methylations do not affect the coding relationship between the trinucleotide codon and the corresponding amino acid. In addition, a skilled artisan will understand how to mutate nucleotides of a specific codon so as to specifically alter an encoded amino acid based on the relevant codon chart.

The "normal" copy number of a biomarker or "normal" level of expression of a biomarker is the level of expression, copy number of the biomarker, in a biological sample, *e.g.,* a sample containing tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, and bone marrow, from a subject, *e.g.,* a human, not afflicted with a metabolic disorder (*e.g.*, diabetes and/or obesity).

As used herein, "obesity" refers to a body mass index (BMI) of 30 $kg/^2m$ or more (National Institute of Health, Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults (1998)). However, the present invention, at least in part, is also intended to include a disease, disorder, or condition that is characterized by a body mass index (BMI) of 25 $kg/^2m$ or more, 26 $kg/^2m$ or more, 27 $kg/^2m$ or more , 28 $kg/^2m$ or more, 29 $kg/^2m$ or more, 29.5 $kg/^2m$ or more, or 29.9 $kg/^2m$ or more, all of which are typically referred to as overweight (National Institute of Health, Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults (1998)). The obesity described herein may be due to any cause, whether genetic or environmental. Examples of disorders that may result in obesity or be the cause of obesity include overeating and bulimia, polycystic ovarian disease, craniopharyngioma, the Prader-Willi Syndrome, Frohlich's syndrome, Type II diabetics, GH-deficient subjects, normal variant short stature, Turner's syndrome, and other pathological conditions showing reduced metabolic activity or a decrease in resting energy expenditure as a percentage of total fat-free mass, *e.g.*, children with acute lymphoblastic leukemia. "Prevention of obesity" refers to preventing obesity or an obesity related disorder from occurring if the

treatment is administered prior to the onset of the obese condition. Moreover, if treatment is commenced in subjects already suffering from or having symptoms of obesity or an obesity related disorder, such treatment is expected to prevent, or to prevent the progression of obesity or the obesity related disorder, and the medical sequelae of obesity, such as, *e.g.*, arteriosclerosis, Type II diabetes, polycystic ovarian disease, cardiovascular diseases, osteoarthritis, dermatological disorders, hypertension, insulin resistance, hypercholesterolemia, hypertriglyceridemia, and cholelithiasis. "Treatment of obesity" refers to reducing the BMI of the mammal to less than about 25.9, and maintaining that weight for a period of time, *e.g.*, for at least about 6 months. The treatment suitably results in an increase in metabolic activity.

An "overexpression" or "significantly higher level of expression, copy number, and/or activity" of biomarkers of the present invention, at least in part, (*e.g.*, biomarkers set forth in Tables 1 and 2) refers to an expression level, copy number, and/or activity in a test sample that is greater than the standard error of the assay employed to assess expression or copy number, and may be at least two, at least three, at least four, at least five, or at least ten or more times the expression level or copy number of the biomarkers of the present invention, at least in part, (*e.g.*, biomarkers set forth in Tables 1 and 2) in a control sample (*e.g.*, a sample from a healthy subject not afflicted with a metabolic disorder (*e.g.*, diabetes and/or obesity)), or the average expression level or copy number of the biomarkers of the present invention, at least in part, (*e.g.*, biomarkers set forth in Tables 1 and 2) in several control samples.

The term "probe" refers to any molecule which is capable of selectively binding to a specifically intended target molecule, for example a biomarker of the present invention, at least in part,. Probes can be either synthesized by one skilled in the art, or derived from appropriate biological preparations. For purposes of detection of the target molecule, probes may be specifically designed to be labeled, as described herein. Examples of molecules that can be utilized as probes include, but are not limited to, RNA, DNA, proteins, antibodies, and organic monomers.

As used herein, "PPAR gamma" refers to a member of the well known family, of the nuclear receptors. In addition, "Serine 273" or "phosphorylated Serine 273" of PPAR gamma refers to the amino acid numbering of the mouse PPAR gamma polypeptide from the N-terminus. Accordingly, a skilled artisan will readily understand that serine 273 of the mouse PAR gamma polypeptide is conserved across numerous species and that the

compositions and methods of the present invention apply equally well to the serine residues of isoforms, homologs, and orthologs in other species corresponding to said serine 273, including, for example, humans. For example, representative PPAR gamma species, as opposed to other members of the PPAR nuclear receptor superfamily (such as PPAR alpha and PPAR delta) are provided herein as follows:

Mouse PPAR gamma 1 (SEQ ID NO:1)

MVDTEMPFWPTNFGISSVDLSVMEDHSHSFDIKPFTTVDFSSISAPHYEDIPFTRADPMVAD
YKYDLKLQEYQSAIKVEPASPPYYSEKTQLYNRPHEEPSNSLMAIECRVCGDKASGFHYGVH
ACEGCKGFFRRTIRLKLIYDRCDLNCRIHKKSRNKCQYCRFQKCLAVGMSHNAIRFGRMPQA
EKEKLLAEISSDIDQLNPESADLRALAKHLYDSYIKSFPLTKAKARAILTGKTTDKSPFVIY
DMNSLMMGEDKIKFKHITPLQEQSKEVAIRIFQGCQFRSVEAVQEITEYAKNIPGFINLDLN
DQVTLLKYGVHEIIYTMLASLMNKDGVLISEGQGFMTREFLKNLRKPFGDFMEPKFEFAVKF
NALELDDSDLAIFIAVIILSGDRPGLLNVKPIEDIQDNLLQALELQLKLNHPESSQLFAKVL
QKMTDLRQIVTEHVQLLHVIKKTETDMSLHPLLQEIYKDLY

Mouse PPAR gamma 2 (SEQ ID NO:2)

MGETLGDSPVDPEHGAFADALPMSTSQEITMVDTEMPFWPTNFGISSVDLSVMEDHSHSFDI
KPFTTVDFSSISAPHYEDIPFTRADPMVADYKYDLKLQEYQSAIKVEPASPPYYSEKTQLYN
RPHEEPSNSLMAIECRVCGDKASGFHYGVHACEGCKGFFRRTIRLKLIYDRCDLNCRIHKKS
RNKCQYCRFQKCLAVGMSHNAIRFGRMPQAEKEKLLAEISSDIDQLNPESADLRALAKHLYD
SYIKSFPLTKAKARAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHITPLQEQSKEVAIRIF
QGCQFRSVEAVQEITEYAKNIPGFINLDLNDQVTLLKYGVHEIIYTMLASLMNKDGVLISEG
QGFMTREFLKNLRKPFGDFMEPKFEFAVKFNALELDDSDLAIFIAVIILSGDRPGLLNVKPI
EDIQDNLLQALELQLKLNHPESSQLFAKVLQKMTDLRQIVTEHVQLLHVIKKTETDMSLHPL
LQEIYKDLY

Human PPAR gamma (SEQ ID NO:3)

MGETLGDSPIDPESDSFTDTLSANISQEMTMVDTEMPFWPTNFGISSVDLSVMEDHSHSF
DIKPFTTVDFSSISTPHYEDIPFTRTDPVVADYKYDLKLQEYQSAIKVEPASPPYYSEKT
QLYNKPHEEPSNSLMAIECRVCGDKASGFHYGVHACEGCKGFFRRTIRLKLIYDRCDLNC
RIHKKSRNKCQYCRFQKCLAVGMSHNAIRFGRMPQAEKEKLLAEISSDIDQLNPESADLR
ALAKHLYDSYIKSFPLTKAKARAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHITPLQE
QSKEVAIRIFQGCQFRSVEAVQEITEYAKSIPGFVNLDLNDQVTLLKYGVHEIIYTMLAS
LMNKDGVLISEGQGFMTREFLKSLRKPFGDFMEPKFEFAVKFNALELDDSDLAIFIAVII
LSGDRPGLLNVKPIEDIQDNLLQALELQLKLNHPESSQLFAKLLQKMTDLRQIVTEHVQL
LQVIKKTETDMSLHPLLQEIYKDLY

Bovine PPAR gamma (SEQ ID NO:4)

MGETLGDALIDPESEPFAVTVSARTSQEITMVDTEMPFWPTNFGISSVDLSMMDDHSHAF
DIKPFTTVDFSSISTPHYEDIPFPRADPMVADYKYDLKLQEYQSAIKVEPVSPPYYSEKT
QLYSKPHEEPSNSLMAIECRVCGDKASGFHYGVHACEGCKGFFRRTIRLKLIYDRCDLNC
RIHKKSRNKCQYCRFQKCLAVGMSHNAIRFGRMPQAEKEKLLAEISSDIDQLNPESADLR
ALAKHLYDSYIKSFPLTKAKARAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHISPLQE
PSKEVAIRIFQGCQFRSVEAVQEITEYAKNIPGFVNLDLNDQVTLLKYGVHEIIYTMLAS
LMNKDGVLISEGQGFMTREFLKSLRKPFGDFMEPKFEFAVKFNALELDDSDLAIFIAVII
LSGDRPGLLNVKPIEDIQDNLLQALELQLKLNHPESSQLFAKLLQKMTDLRQIVTEHVQL
LQVIKKTETDMSLHPLLQEIYKDLY

Rabbit PPAR gamma (SEQ ID NO:5)

MVDTEMPFWPTNFGIGSVDLSVMDDHSHSFDIKPFTTVDFSSISAPHYEDLPFARADPMV
ADYKYDLKLQEYQSAIKVEPASPPYYSEKTQLYNKTHEEPSNSLMAIECRVCSDKASGFH
YGVHACEGCKGFFRRTIRLKLIYDRCDLNCRIHKKSRNKCQYCRFQKCLAVGMSHNAIRF
GRMPQAEKEKLLAEISSDIDQLNPESADLRALAKHLYDSYIKSFPLTKAKARAILTGKTT
DKSPFVIYDMNSLMMGEDKIKFKHITPLQEQSKEVAIRIFQGCQFRSVEAVQEITEYAKN
IPGFVSLDLNDQVTLLKYGVHEIIYTMLASLMNKDGVLISEGQGFMTREFLKSLRKPFGD

FMEPKFEFAVKFNALELDDSDLAIFIAVIILSGDRPGLLNVKPIEDIQDNLLQALELQLK
LNHPEASQLFAKLLQKMTDLRQIVTEHVQLLQVIKKTETDMSLHPLLQEIYKDLY

Pig PPAR gamma (SEQ ID NO:6)

MGETLGDSLIDPESDAFDTLSANISQEVTMVDTEMPFWPTNFGISSVDLSVMDDHSHSFD
IKPFTTVDFSSISTPHYEDIPFTRADPMVADYKYDLKLQDYQSAIKVEPVSPPYYSEKTQ
LYNKPHEEPSNSLMAIECRVCGDKASGFHYGVHACEGCKGFFRRTIRLKLIYDRCDLNCR
IHKKSRNKCQYCRFQKCLAVGMSHNAIRFGRMPQAEKEKLLAEISSDIDQLNPESADLRA
LAKHLYDSYIKSFPLTKAKARAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHITPLQEQ
SKEVAIRIFQGCQFRSVEAVQEITEYAKNIPGFVNLDLNDQVTLLKYGVHEIIYTMLASL
MNKDGVLISEGQGFMTREFLKSLRKPFGDFMEPKFEFAVKFNALELDDSDLAIFIAVIIL
SGDRPGLLNVKPIEDIQDNLLQALELQLKLNHPESSQLFAKLLQKMTDLRQIVTEHVQLL
QVIKKTETDMSLHPLLQEIYKDLY

Chicken PPAR gamma (SEQ ID NO:7)

MVDTEMPFWPVNFGISPVDLSAMDDHMHSFDIKPFTTVDFSSISSPHYEDIPLGRADQTSIDYKYDI
KLQDCQSAIKMEPPSPPYFSEKVQLYNKPHEESSNSLMAIECRVCGDKASGFHYGVHACEGCKGFFR
RTIRLKLIYDRCDLNCRIHKKSRNKCQYCRFQKCLAVGMSHNAIRFGRMPQAEKEKLLAEISSDIDQ
LNPESADLRALAKHLYDSYIKSFPLTKAKARAILTGKTTDKSPFVIYDMNSLRMGEDQIKCKHASPL
QEQNKEVAIRIFQRCQFRSVEAVQEITEFAKNIPGFVNLDLNDQVTLLKYGVHEIIYTLLASLMNKD
GVLISDGQGFMTREFLKSLRKPFCDFMEPKFEFAVKFNALELDDSDLAIFIAVIILSGDRPGLLNVK
PIEDIQDNLLQALELQLKLNHPESSQLFAKLLQKMTDLRQIVTEHVQLLQIIKKTETDMSLHPLLQE
IYKDLY

Mouse PPAR alpha (SEQ ID NO:8)

MVDTESPICPLSPLEADDLESPLSEEFLQEMGNIQEISQSIGEESSGSFGFADYQYLGSCPGSEGSV
ITDTLSPASSPSSVSCPVIPASTDESPGSALNIECRICGDKASGYHYGVHACEGCKGFFRRTIRLKL
VYDKCDRSCKIQKKNRNKCQYCRFHKCLSVGMSHNAIRFGRMPRSEKAKLKAEILTCEHDLKDSETA
DLKSLGKRIHEAYLKNFNMNKVKARVILAGKTSNNPPFVIHDMETLCMAEKTLVAKMVANGVEDKEA
EVRFFHCCQCMSVETVTELTEFAKAIPGFANLDLNDQVTLLKYGVYEAIFTMLSSLMNKDGMLIAYG
NGFITREFLKNLRKPFCDIMEPKFDFAMKFNALELDDSDISLFVAAIICCGDRPGLLNIGYIEKLQE
GIVHVLKLHLQSNHPDDTFLFPKLLQKMVDLRQLVTEHAQLVQVIKKTESDAALHPLLQEIYRDMY

Mouse PPAR delta (SEQ ID NO:9)

MEQPQEETPEAREEEKEEVAMGDGAPELNGGPEHTLPSSSCADLSQNSSPSSLLDQLQMGCDGASGG
SLNMECRVCGDKASGFHYGVHACEGCKGFFRRTIRMKLEYEKCDRICKIQKKNRNKCQYCRFQKCLA
LGMSHNAIRFGRMPEAEKRKLVAGLTASEGCQHNPQLADLKAFSKHIYNAYLKNFNMTKKKARSILT
GKSSHNAPFVIHDIETLWQAEKGLVWKQLVNGLPPYNEISVHVFYRCQSTTVETVRELTEFAKNIPN
FSSLFLNDQVTLLKYGVHEAIFAMLASIVNKDGLLVANGSGFVTHEFLRSLRKPFSDIIEPKFEFAV
KFNALELDDSDLALFIAAIILCGDRPGLMNVPQVEAIQDTILRALEFHLQVNHPDSQYLFPKLLQKM
ADLRQLVTEHAQMMQWLKKTESETLLHPLLQEIYKDMY

Mouse PPAR gamma 2 mRNA (SEQ ID NO:10; NM_011146.3)

1 caaaacacca gtgtgaatta cagcaaatct ctgttttatg ctgttatggg tgaaactctg
61 ggagattctc ctgttgaccc agagcatggt gccttcgctg atgcactgcc tatgagcact
121 tcacaagaaa ttaccatggt tgacacagag atgccattct ggcccaccaa cttcggaatc
181 agctctgtgg acctctccgt gatggaagac cactcgcatt cctttgacat caagcccttt
241 accacagttg atttctccag catttctgct ccacactatg aagacattcc attcacaaga
301 gctgacccaa tggttgctga ttacaaatat gacctgaagc tccaagaata ccaaagtgcg
361 atcaaagtag aacctgcatc tccaccttat tattctgaaa agacccagct ctacaacagg
421 cctcatgaag aaccttctaa ctccctcatg gccattgagt gccgagtctg tggggataaa
481 gcatcaggct tccactatgg agttcatgct tgtgaaggat gcaagggttt tttccgaaga
541 accatccgat tgaagcttat ttatgatagg tgtgatctta actgccggat ccacaaaaaa
601 agtagaaata aatgtcagta ctgtcggttt cagaagtgcc ttgctgtggg gatgtctcac
661 aatgccatca ggtttgggcg gatgccacag gccgagaagg agaagctgtt ggcggagatc
721 tccagtgata tcgaccagct gaacccagag tctgctgatc tgcgagccct ggcaaagcat
781 ttgtatgact catacataaa gtccttcccg ctgaccaaag ccaaggcgag ggcgatcttg
841 acaggaaaga caacggacaa atcaccattt gtcatctacg acatgaattc cttaatgatg
901 ggagaagata aaatcaagtt caaacatatc accccctgc aggagcagag caaagaggtg
961 gccatccgaa tttttcaagg gtgccagttt cgatccgtag aagccgtgca agagatcaca
1021 gagtatgcca aaaatatccc tggtttcatt aaccttgatt tgaatgacca agtgactctg

1081 ctcaagtatg gtgtccatga gatcatctac acgatgctgg cctccctgat gaataaagat
1141 ggagtcctca tctcagaggg ccaaggattc atgaccaggg agttcctcaa aagcctgcgg
1201 aagccctttg gtgactttat ggagcctaag tttgagtttg ctgtgaagtt caatgcactg
1261 gaattagatg acagtgactt ggctatattt atagctgtca ttattctcag tggagaccgc
1321 ccaggcttgc tgaacgtgaa gcccatcgag gacatccaag acaacctgct gcaggccctg
1381 gaactgcagc tcaagctgaa tcacccagag tcctctcagc tgttcgccaa ggtgctccag
1441 aagatgacag acctcaggca gatcgtcaca gagcacgtgc agctactgca tgtgatcaag
1501 aagacagaga cagacatgag ccttcacccc ctgctccagg agatctacaa ggacttgtat
1561 tagcaggaaa gtcccacccg ctgacaacgt gttccttcta ttgattgcac tattattttg
1621 agggaaaaaa atctgacacc taagaaattt actgtgaaaa agcatttaaa aacaaaaagt
1681 tttagaacat gatctatttt atgcatattg tttataaaga tacatttaca atttacttt
1741 aatattaaaa attaccacat tataaaatt

Mouse PPAR gamma 2 mRNA coding sequence (SEQ ID NO:11; NM_011146.3)

1 atgggtgaaa ctctgggaga ttctcctgtt gacccagagc atggtgcctt cgctgatgca
61 ctgcctatga gcacttcaca agaaattacc atggttgaca cagagatgcc attctggccc
121 accaacttcg gaatcagctc tgtggacctc tccgtgatgg aagaccactc gcattccttt
181 gacatcaagc cctttaccac agttgatttc tccagcattt ctgctccaca ctatgaagac
241 attccattca caagagctga cccaatggtt gctgattaca aatatgacct gaagctccaa
301 gaataccaaa gtgcgatcaa agtagaacct gcatctccac cttattattc tgaaaagacc
361 cagctctaca acaggcctca tgaagaacct tctaactccc tcatggccat tgagtgccga
421 gtctgtgggg ataaagcatc aggcttccac tatggagttc atgcttgtga aggatgcaag
481 ggtttttcc gaagaaccat ccgattgaag cttatttatg ataggtgtga tcttaactgc
541 cggatccaca aaaaaagtag aaataaatgt cagtactgtc ggtttcagaa gtgccttgct
601 gtggggatgt ctcacaatgc catcaggttt gggcggatgc cacaggccga gaaggagaag
661 ctgttggcgg agatctccag tgatatcgac cagctgaacc cagagtctgc tgatctgcga
721 gccctggcaa agcatttgta tgactcatac ataaagtcct tcccgctgac caaagccaag
781 gcgagggcga tcttgacagg aaagacaacg gacaaatcac catttgtcat ctacgacatg
841 aattccttaa tgatgggaga agataaaatc aagttcaaac atatcacccc cctgcaggag
901 cagagcaaag aggtggccat ccgaattttt caagggtgcc agtttcgatc cgtagaagcc
961 gtgcaagaga tcacagagta tgccaaaaat atccctggtt tcattaacct tgatttgaat
1021 gaccaagtga ctctgctcaa gtatggtgtc catgagatca tctacacgat gctggcctcc
1081 ctgatgaata aagatggagt cctcatctca gagggccaag gattcatgac cagggagttc
1141 ctcaaaagcc tgcggaagcc ctttggtgac tttatggagc ctaagtttga gtttgctgtg
1201 aagttcaatg cactggaatt agatgacagt gacttggcta tatttatagc tgtcattatt
1261 ctcagtggag accgcccagg cttgctgaac gtgaagccca tcgaggacat ccaagacaac
1321 ctgctgcagg ccctggaact gcagctcaag ctgaatcacc cagagtcctc tcagctgttc
1381 gccaaggtgc tccagaagat gacagacctc aggcagatcg tcacagagca cgtgcagcta
1441 ctgcatgtga tcaagaagac agagacagac atgagccttc acccccctgct ccaggagatc
1501 tacaaggact tgtattag

Human PPAR gamma 2 mRNA (SEQ ID NO:12; NM_015869.4)

1 ttcaagtctt tttcttttaa cggattgatc ttttgctaga tagagacaaa atatcagtgt
61 gaattacagc aaacccctat tccatgctgt tatgggtgaa actctgggag attctcctat
121 tgacccagaa agcgattcct tcactgatac actgtctgca aacatatcac aagaaatgac
181 catggttgac acagagatgc cattctggcc caccaacttt gggatcagct ccgtggatct
241 ctccgtaatg gaagaccact cccactcctt tgatatcaag cccttcacta ctgttgactt
301 ctccagcatt tctactccac attacgaaga cattccattc acaagaacag atccagtggt
361 tgcagattac aagtatgacc tgaaacttca agagtaccaa agtgcaatca aagtggagcc
421 tgcatctcca ccttattatt ctgagaagac tcagctctac aataagcctc atgaagagcc
481 ttccaactcc ctcatggcaa ttgaatgtcg tgtctgtgga gataaagctt ctggatttca
541 ctatggagtt catgcttgtg aaggatgcaa gggtttcttc cggagaacaa tcagattgaa
601 gcttatctat gacagatgtg atcttaactg tcggatccac aaaaaaagta gaaataaatg
661 tcagtactgt cggtttcaga aatgccttgc agtggggatg tctcataatg ccatcaggtt
721 tgggcggatg ccacaggccg agaaggagaa gctgttggcg gagatctcca gtgatatcga
781 ccagctgaat ccagagtccg ctgacctccg ggccctggca aaacatttgt atgactcata
841 cataaagtcc ttcccgctga ccaaagcaaa ggcgagggcg atcttgacag gaaagacaac
901 agacaaatca ccattcgtta tctatgacat gaattcctta atgatgggag aagataaaat
961 caagttcaaa cacatcaccc ccctgcagga gcagagcaaa gaggtggcca tccgcatctt
1021 tcagggctgc cagtttcgct ccgtggaggc tgtgcaggag atcacagagt atgccaaaag
1081 cattcctggt tttgtaaatc ttgacttgaa cgaccaagta actctcctca aatatggagt

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1141 ccacgagatc atttacacaa tgctggcctc cttgatgaat aaagatgggg ttctcatatc
1201 cgagggccaa ggcttcatga caagggagtt tctaaagagc ctgcgaaagc cttttggtga
1261 ctttatggag cccaagtttg agtttgctgt gaagttcaat gcactggaat tagatgacag
1321 cgacttggca atatttattg ctgtcattat tctcagtgga gaccgcccag gtttgctgaa
1381 tgtgaagccc attgaagaca ttcaagacaa cctgctacaa gccctggagc tccagctgaa
1441 gctgaaccac cctgagtcct cacagctgtt tgccaagctg ctccagaaaa tgacagacct
1501 cagacagatt gtcacggaac acgtgcagct actgcaggtg atcaagaaga cggagacaga
1561 catgagtctt cacccgctcc tgcaggagat ctacaaggac ttgtactagc agagagtcct
1621 gagccactgc caacatttcc cttcttccag ttgcactatt ctgagggaaa atctgacacc
1681 taagaaattt actgtgaaaa agcattttaa aaagaaaagg ttttagaata tgatctattt
1741 tatgcatatt gtttataaag acacatttac aatttacttt taatattaaa aattaccata
1801 ttatgaaatt gctgatagta
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Human PPAR gamma 2 mRNA coding sequence (SEQ ID NO:13; NM_015869.4)

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1 atgggtgaaa ctctgggaga ttctcctatt gacccagaaa gcgattcctt cactgataca
61 ctgtctgcaa acatatcaca agaaatgacc atggttgaca cagagatgcc attctggccc
121 accaactttg ggatcagctc cgtggatctc tccgtaatgg aagaccactc ccactccttt
181 gatatcaagc ccttcactac tgttgacttc tccagcattt ctactccaca ttacgaagac
241 attccattca caagaacaga tccagtggtt gcagattaca agtatgacct gaaacttcaa
301 gagtaccaaa gtgcaatcaa agtggagcct gcatctccac cttattattc tgagaagact
361 cagctctaca ataagcctca tgaagagcct tccaactccc tcatggcaat tgaatgtcgt
421 gtctgtggag ataaagcttc tggatttcac tatggagttc atgcttgtga aggatgcaag
481 ggtttcttcc ggagaacaat cagattgaag cttatctatg acagatgtga tcttaactgt
541 cggatccaca aaaaaagtag aaataaatgt cagtactgtc ggtttcagaa atgccttgca
601 gtggggatgt ctcataatgc catcaggttt gggcggatgc cacaggccga gaaggagaag
661 ctgttggcgg agatctccag tgatatcgac cagctgaatc cagagtccgc tgacctccgg
721 gccctggcaa aacatttgta tgactcatac ataaagtcct tcccgctgac caaagcaaag
781 gcgagggcga tcttgacagg aaagacaaca gacaaatcac cattcgttat ctatgacatg
841 aattccttaa tgatgggaga agataaaatc aagttcaaac acatcacccc cctgcaggag
901 cagagcaaag aggtggccat ccgcatcttt cagggctgcc agtttcgctc cgtggaggct
961 gtgcaggaga tcacagagta tgccaaaagc attcctggtt ttgtaaatct tgacttgaac
1021 gaccaagtaa ctctcctcaa atatggagtc cacgagatca tttacacaat gctggcctcc
1081 ttgatgaata aagatggggt tctcatatcc gagggccaag gcttcatgac aagggagttt
1141 ctaaagagcc tgcgaaagcc ttttggtgac tttatggagc ccaagtttga gtttgctgtg
1201 aagttcaatg cactggaatt agatgacagc gacttggcaa tatttattgc tgtcattatt
1261 ctcagtggag accgcccagg tttgctgaat gtgaagccca ttgaagacat tcaagacaac
1321 ctgctacaag ccctggagct ccagctgaag ctgaaccacc ctgagtcctc acagctgttt
1381 gccaagctgc tccagaaaat gacagacctc agacagattg tcacggaaca cgtgcagcta
1441 ctgcaggtga tcaagaagac ggagacagac atgagtcttc acccgctcct gcaggagatc
1501 tacaaggact tgtactag
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"PPAR gamma agonist," as used herein, refers to any compound that, by any mechanism, increases, or causes an increase in the activity of PPAR gamma or the heterodimer of PPAR gamma with the retinoid X receptor (RXR), either by direct binding to either PPAR gamma or RXR or indirectly through any other mechanism that affects the ability of PPAR gamma or the PPAR gamma-RXR heterodimer to influence gene expression.

A "classical PPAR gamma agonist" as used herein refers to a PPAR gamma ligand that promotes PPAR gamma-mediated gene expression, which is associated with promoting metabolic disorders. A classical PPAR gamma agonist promotes expression of "adipogenic" genes (see, for example, genes, activities and assays set forth in Table 2 below). Genes that are regulated by the PPAR gamma agonist, rosiglitazone, are an

example. Side effects associated with classical PPAR gamma agonists, include, for instance, weight gain, white adipocyte gain, fluid retention, peripheral edema, and pulmonary edema.

An "atypical PPAR gamma agonist" or "selective PPAR gamma agonist" as used herein refers to a PPAR gamma ligand that regulates expression of genes that are regulated predominantly and/or exclusively through cdk5-mediated phosphorylation of PPAR gamma. Examples include "anti-diabetic," "brown fat," and/or "increased respiration/oxygen consumption" genes (see, for example, Table 1). An example of an atypical PPAR gamma ligand is, for example, MRL24. In certain embodiments, atypical PPAR gamma agonists may promote anti-metabolic disorder activities without one or more of the side effects associated with classical PPAR gamma agonists, including, for example, weight gain, white adipocyte gain, fluid retention, peripheral edema, and pulmonary edema. Accordingly, exemplary atypical PPAR gamma ligands of the present invention, at least in part, may comprise compounds or pharmaceutical compositions having, for example, a blood sugar reducing effect, a blood lipid reducing effect, a blood insulin reducing effect, an insulin sensitivity enhancing effect, an insulin resistance improving effect, a body weight reducing effect, a central body girth (measured as waist:hip ratio) reducing effect, and/or, a body fat mass reducing effect.

PPAR gamma ligands include, but are not limited to, thiazolidinediones (*e.g.,* rosiglitazone, pioglitazone, MK-0533, MK 767 (KRP-297), MCC-555, netoglitazone, balaglitazone, rivoglitazone), non-thiazolidinediones (e,g, JTT-501, LSN862, DRF 4832, LM 4156, LY 510929, LY 519818, TY 51501, X 334), certain tyrosine-based derivatives (*e.g.*, GW1929, GW7845), phenylacetic acid-based derivatives, phenoxazine phenyl propanoic acid derivatives (*e.g.*, DRF 2725, DRF 2189), cinammic and dihydrocinammic acid-based derivatives (*e.g.*, tesaglitazar (AZ 242)), and 3-Phenyl-7-propylbenzisoxazoles (Adams A D, *et al.* Bioorg Med Chem Lett. (2003) 13:931-5), MRL-20, MRL-24, nTZDpa, SR145, SR147, Mbx-102, and BVT.13.

"Responsiveness," to "respond" to treatment, and other forms of this verb, as used herein, refer to the reaction of a subject to treatment with a PPAR gamma ligand. As an example, a subject responds to treatment with a PPAR gamma ligand if an anti-metabolic activity in the subject is retarded by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more. In another example, a subject responds to treatment with an atypical PPAR gamma ligand if the subject experiences a life expectancy extended by about 5%, 10%,

20%, 30%, 40%, 50% or more beyond the life expectancy predicted if no treatment is administered. In another example, a subject responds to treatment with a PPAR gamma ligand if the subject has an increased metabolic disorder-free survival, overall survival or increased time to progression of a metabolic disorder.

"Sample," "tissue sample," "subject sample," "subject cell or tissue sample" or "specimen" each refer to a collection of similar cells obtained from a tissue of a subject or subject. The source of the tissue sample may be solid tissue as from a fresh, frozen and/or preserved organ, tissue sample, biopsy, or aspirate; blood or any blood constituents; bodily fluids such as cerebral spinal fluid, amniotic fluid, peritoneal fluid or interstitial fluid; or cells from any time in gestation or development of the subject. The tissue sample may contain compounds that are not naturally intermixed with the tissue in nature such as preservatives, anticoagulants, buffers, fixatives, nutrients, antibiotics or the like.

The amount of a biomarker, *e.g.,* expression or copy number of biomarkers of the present invention, at least in part, (*e.g.*, biomarkers set forth in Tables 1 and 2), in a subject is "significantly" higher or lower than the normal amount of a biomarker, if the amount of the biomarker is greater or less, respectively, than the normal level by an amount greater than the standard error of the assay employed to assess amount, or at least two, three, four, five, ten or more times that amount. Alternately, the amount of the biomarker in the subject can be considered "significantly" higher or lower than the normal amount if the amount is at least about two, at least about three, at least about four, or at least about five times, higher or lower, respectively, than the normal amount of the biomarker.

As used herein, the terms "subject" and "patient" are used interchangeably. As used herein, the terms "subject" and "subjects" refer to an animal, *e.g.*, a mammal including a non-primate (*e.g.*, a cow, pig, horse, donkey, goat, camel, cat, dog, guinea pig, rat, mouse, sheep) and a primate (*e.g.*, a monkey, such as a cynomolgous monkey, gorilla, chimpanzee and a human).

As used herein, "time course" shall refer to the amount of time between an initial event and a subsequent event. For example, with respect to a subject's metabolic disorder (*e.g.*, diabetes and/or obesity), time course may relate to a subject's disease and may be measured by gauging significant events in the course of the disease, wherein the first event may be diagnosis and the subsequent event may be significant weight loss, insulin sensitivity, etc.

"Time to progression" or "TTP" refers to a time as measured from the start of the

treatment to progression of metabolic disorder (*e.g.*, diabetes and/or obesity) or censor. Censoring may come from a study end or from a change in treatment. Time to progression can also be represented as a probability as, for example, in a Kaplin-Meier plot where time to progression may represent the probability of being progression free over a particular time, that time being the time between the start of the treatment to progression or censor.

A "transcribed polynucleotide" is a polynucleotide (*e.g.,* an RNA, a cDNA, or an analog of one of an RNA or cDNA) which is complementary to or homologous with all or a portion of a mature RNA made by transcription of a biomarker of the present invention, at least in part, and normal post-transcriptional processing (*e.g.,* splicing), if any, of the transcript, and reverse transcription of the transcript.

"Treat," "treatment," and other forms of this word refer to the administration of a PPAR gamma ligand to impede growth of a metabolic disorder (*e.g.*, diabetes and/or obesity), to cause a metabolic disorder (*e.g.*, diabetes and/or obesity) to be ameliorated, to extend the expected survival time of the subject and/or time to progression of a metabolic disorder or the like.

An "underexpression" or "significantly lower level of expression, copy number, and/or activity" of biomarkers of the present invention, at least in part, (*e.g.*, biomarkers set forth in Tables 1 and 2) refers to an expression level or copy number in a test sample that is greater than the standard error of the assay employed to assess expression or copy number, for example, at least twice, at least three, at least four, at least five, or at least ten or more times less than the expression level, copy number, and/or activity of the biomarkers of the present invention, at least in part, (*e.g.*, biomarkers set forth in Tables 1 and 2) in a control sample (*e.g.*, a sample from a healthy subject not afflicted with a metabolic disorder (*e.g.*, diabetes and/or obesity)), or the average expression level, copy number, and/or activity of the biomarkers of the present invention, at least in part, (*e.g.*, biomarkers set forth in Tables 1 and 2) in several control samples.

"Unlikely to" refers to a decreased probability that an event, item, object, thing or person will occur with respect to a reference. Thus, a subject that is unlikely to respond to treatment with a PPAR gamma ligand has a decreased probability of responding to treatment with a PPAR gamma ligand relative to a reference subject or group of subjects (such as decreased by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, or more (or any range inclusive).

I. Nucleic Acids of the Invention

One aspect of the invention pertains to compositions and methods utilizing isolated nucleic acid molecules that encode PPAR gamma polypeptides or biologically active portions thereof that cannot be phosphorylated at serine 273 of the mouse as well as to corresponding isoforms, homologs, and orthologs in other species corresponding to said serine 273, including, for example, humans, that cannot be phosphorylated at corresponding serine residues (*i.e.*, hereinafter referred to as non-phosphorylatable PPAR gamma), as well as nucleic acid fragments sufficient for use as hybridization probes to identify non-phosphorylatable PPAR gamma polypeptides or biologically active portions thereof-encoding nucleic acid (*i.e.*, mRNA). As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (*i.e.*, cDNA or genomic DNA) and RNA molecules (*i.e.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded DNA. An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. An "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated non-phosphorylatable PPAR gamma nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived (*i.e.*, a brown adipocyte). Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention, at least in part,, *i.e.*, a nucleic acid molecule listed in Tables 1 and 2 or a nucleotide sequence which is at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, and at least about 95% or more (*e.g.*, about 96%, 96.5%, 97%, 97.5%, 98%, 98.5%, 99%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or more) homologous to a nucleotide sequence shown in Tables 1 and 2 or a portion thereof (*i.e.*, 400, 450, 500, or more nucleotides), can be isolated using standard molecular biology techniques and the sequence information provided herein.

For example, a human non-phosphorylatable PPAR gamma cDNA can be isolated from a human liver, heart, kidney, or brain cell line (from Stratagene, LaJolla, CA, or Clontech, Palo Alto, CA) using all or portion of the nucleotide sequence shown in Tables 1 and 2 as a hybridization probe and standard hybridization techniques (*i.e.*, as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). Moreover, a nucleic acid molecule encompassing all or a portion of a nucleotide sequence shown in Tables 1 and 2 or a nucleotide sequence which is at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, and at least about 95% or more homologous to the nucleotide sequence shown in a nucleotide sequence shown in Tables 1 and 2 can be isolated by the polymerase chain reaction and/or site-directed mutagenesis using oligonucleotide primers designed based upon the sequence of a nucleotide sequence shown in Tables 1 and 2 or the homologous nucleotide sequence. For example, mRNA can be isolated from liver cells, heart cells, kidney cells, brain cells, or brown adipocytes (*i.e.*, by the guanidinium-thiocyanate extraction procedure of Chirgwin *et al.* (1979) *Biochemistry* 18: 5294-5299) and cDNA can be prepared using reverse transcriptase (*i.e.*, Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD; or AMV reverse transcriptase, available from Seikagaku America, Inc., St. Petersburg, FL). Synthetic oligonucleotide primers for PCR amplification can be designed based upon the nucleotide sequence shown in a nucleotide sequence shown in Tables 1 and 2 or to the homologous nucleotide sequence. A nucleic acid of the invention can be amplified using cDNA or, alternatively, genomic DNA (including both germline and somatic genomic DNA), as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to a non-phosphorylatable PPAR gamma nucleotide sequence can be prepared by standard synthetic techniques, *i.e.*, using an automated DNA synthesizer.

Probes based on the non-phosphorylatable PPAR gamma nucleotide sequences can be used to detect transcripts or genomic sequences encoding the same or homologous proteins. In certain embodiments, the probe further comprises a label group attached thereto, *i.e.,* the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for

identifying cells or tissue which express a non-phosphorylatable PPAR gamma protein, such as by measuring a level of a PPAR gamma polypeptides or biologically active portions thereof that cannot be phosphorylated at serine 273-encoding nucleic acid in a sample of cells from a subject, *i.e.*, detecting non-phosphorylatable PPAR gamma mRNA levels.

Nucleic acid molecules encoding other non-phosphorylatable PPAR gamma family members and thus which have a nucleotide sequence which differs from the wild type PPAR gamma sequences of the sequences listed in Tables 1 and 2 are intended to be part of the invention. Moreover, nucleic acid molecules encoding non-phosphorylatable PPAR gamma proteins from different species, and thus which have a nucleotide sequence which differs from the non-phosphorylatable PPAR gamma sequences listed in Tables 1 and 2 are intended to be within the scope of the invention. For example, rat or monkey non-phosphorylatable PPAR gamma cDNA can be identified and/or synthesized based on known nucleotide sequences and engineered mutations of a human and/or mouse non-phosphorylatable PPAR gamma nucleic acid sequence.

In one embodiment, the nucleic acid molecule of the invention encodes a protein or portion thereof which includes an amino acid sequence which is sufficiently homologous to an amino acid sequence listed in Tables 1 and 2 such that the protein or portion thereof maintains one or more of the following biological activities described herein for mouse PPAR gamma polypeptide having a mutation of serine 273 to alanine (*e.g.*, the inability of the 273th amino acid to become phosphorylated). For example, any nucleic acid mutation that prevents encoding of serine or a phosphorylatable amino acid residue such as threonine or tyrosine, is contemplated. Any and all such mutations are readily known to a person having ordinary skill in the art based upon the degeneracy of the genetic code and codon algorithms in a species of interest.

In another embodiment, the protein is at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95% or more homologous to the entire amino acid sequence listed in Tables 1 and 2.

Portions of proteins encoded by the non-phosphorylatable PPAR gamma nucleic acid molecule of the invention are biologically active portions of the non-phosphorylatable PPAR gamma protein. As used herein, the term "biologically active portion of non-phosphorylatable PPAR gamma" is intended to include a portion, *e.g.*, a domain/motif, of non-phosphorylatable PPAR gamma that selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation according to one or more criteria as

described herein (for example, a consensus cdk5 phosphorylation motif and/or mutated PPAR gamma non-phosphorylatable at Ser-273).

Standard binding assays, *e.g.*, immunoprecipitations and yeast two-hybrid assays, as described herein, can be performed to determine the ability of a non-phosphorylatable PPAR gamma protein or a biologically active portion thereof to interact with a target of interest in assays that can be either PPAR gamma ligand-dependent or independent. To determine whether a non-phosphorylatable PPAR gamma family member of the present invention, at least in part, modulates a biomarker listed in Tables 1 and 2, *in vitro* transcriptional assays can be performed. To perform such an assay, the full length promoter/enhancer region of the gene of interest can be linked to a reporter gene such as chloramphenicol acetyltransferase (CAT) or luciferase and introduced into host cells (*e.g.*, liver cells such as Fao hepatoma cells, or COS cells). The same host cells can then be transfected a nucleic acid molecule encoding the non-phosphorylatable PPAR gamma molecule. The effect of the non-phosphorylatable PPAR gamma molecule can be measured by testing CAT or luciferase activity and comparing it to CAT or luciferase activity in cells which do not contain nucleic acid encoding the non-phosphorylatable PPAR gamma molecule. An increase or decrease in CAT or luciferase activity indicates a modulation of expression of the gene of interest. In another embodiment, because expression of the biomarkers listed in Tables 1 and 2 are known to be a critical component in the cascade of events leading to discrete biological pathways, this assay can also measure the ability of the non-phosphorylatable PPAR gamma molecule to modulate such pathways, including, for example, fatty acid uptake, induction of brown fat by histology, gene expression, uncoupled oxygen consumption, mitochondrial biogenesis, etc., energy expenditure *in vivo* by increased oxygen consumption, thermogenesis, no change in food intake, etc., serum fatty acid levels, serum adipokine (adiponectin for example) levels, white fat depots, fat mass, lean mass, fasting glucose, glucose tolerance, insulin tolerance, insulin sensitivity, insulin-dependent glucose uptake, suppression of obesity-induced inflammation by analyzing TNF-alpha secretion, MCP1 inflammation gene expression, etc. Such analyses are well known in the art.

The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence shown in Tables 1 and 2 (and portions thereof) due to degeneracy of the genetic code and thus encode non-phosphorylatable PPAR gamma protein in a number of ways readily known to a person having ordinary skill in the art. For example, the skilled

artisan will further appreciate that changes can be introduced by mutation into the nucleotide sequence of Tables 1 and 2 thereby leading to changes in the amino acid sequence of the encoded non-phosphorylatable PPAR gamma protein, without altering the functional ability of the non-phosphorylatable PPAR gamma protein having the S273A mutation described in the examples. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues can be made in the sequence of. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of non-phosphorylatable PPAR gamma (*e.g.*, the sequence listed in Tables 1 and 2) without altering the activity of non-phosphorylatable PPAR gamma, whereas an "essential" amino acid residue is required for non-phosphorylatable PPAR gamma activity. Other amino acid residues, however, (*e.g.*, those that are not conserved or only semi-conserved between mouse and human) may not be essential for activity and thus are likely to be amenable to alteration without altering non-phosphorylatable PPAR gamma activity. Furthermore, amino acid residues that are essential for non-phosphorylatable PPAR gamma functions related to thermogenesis and/or adipogenesis, but not essential for non-phosphorylatable PPAR gamma functions related to gluconeogenesis, are likely to be amenable to alteration.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding non-phosphorylatable PPAR gamma proteins that contain changes in amino acid residues that are not essential for non-phosphorylatable PPAR gamma activity. Such non-phosphorylatable PPAR gamma proteins differ in amino acid sequence from Tables 1 and 2 yet retain at least one of the non-phosphorylatable PPAR gamma activities described herein.

"Sequence identity or homology", as used herein, refers to the sequence similarity between two polypeptide molecules or between two nucleic acid molecules. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, *e.g.*, if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous or sequence identical at that position. The percent of homology or sequence identity between two sequences is a function of the number of matching or homologous identical positions shared by the two sequences divided by the number of positions compared x 100. For example, if 6 of 10, of the positions in two sequences are the same then the two sequences are 60% homologous or have 60% sequence identity. By way of example, the DNA sequences ATTGCC and TATGGC share 50%

homology or sequence identity. Generally, a comparison is made when two sequences are aligned to give maximum homology. Unless otherwise specified "loop out regions", *e.g.*, those arising from, from deletions or insertions in one of the sequences are counted as mismatches.

The comparison of sequences and determination of percent homology between two sequences can be accomplished using a mathematical algorithm. The alignment can be performed using the Clustal Method. Multiple alignment parameters include GAP Penalty =10, Gap Length Penalty = 10. For DNA alignments, the pairwise alignment parameters can be Htuple=2, Gap penalty=5, Window=4, and Diagonal saved=4. For protein alignments, the pairwise alignment parameters can be Ktuple=1, Gap penalty=3, Window=5, and Diagonals Saved=5.

In a certain embodiments, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available online), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available online), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0) (available online), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

An isolated nucleic acid molecule encoding a non-phosphorylatable PPAR gamma protein homologous to the protein listed in Tables 1 and 2 can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of Tables 1 and 2 or a homologous nucleotide sequence such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into Tables 1 and 2 or the homologous nucleotide sequence by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of

amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in non-phosphorylatable PPAR gamma is replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a non-phosphorylatable PPAR gamma coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for a non-phosphorylatable PPAR gamma activity described herein to identify mutants that retain non-phosphorylatable PPAR gamma activity. Following mutagenesis of the sequence selected from Tables 1 and 2, the encoded protein can be expressed recombinantly (as described herein) and the activity of the protein can be determined using, for example, assays described herein.

Non-phosphorylatable PPAR gamma levels may be assessed by any of a wide variety of well known methods for detecting expression of a transcribed molecule or protein. Non-limiting examples of such methods include immunological methods for detection of proteins, protein purification methods, protein function or activity assays, nucleic acid hybridization methods, nucleic acid reverse transcription methods, and nucleic acid amplification methods.

In some embodiments, non-phosphorylatable PPAR gamma levels are ascertained by measuring gene transcript (*e.g.*, mRNA), by a measure of the quantity of translated protein, or by a measure of gene product activity. Expression levels can be monitored in a variety of ways, including by detecting mRNA levels, protein levels, or protein activity, any of which can be measured using standard techniques. Detection can involve quantification of the level of gene expression (*e.g.,* genomic DNA, cDNA, mRNA, protein, or enzyme activity), or, alternatively, can be a qualitative assessment of the level of gene expression, in particular in comparison with a control level. The type of level being detected will be clear from the context.

In a particular embodiment, the non-phosphorylatable PPAR gamma mRNA expression level can be determined both by *in situ* and by *in vitro* formats in a biological

sample using methods known in the art. The term "biological sample" is intended to include tissues, cells, biological fluids and isolates thereof, isolated from a subject, as well as tissues, cells and fluids present within a subject. Many expression detection methods use isolated RNA. For *in vitro* methods, any RNA isolation technique that does not select against the isolation of mRNA can be utilized for the purification of RNA from cells (see, *e.g.*, Ausubel *et al.*, ed., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York 1987-1999). Additionally, large numbers of tissue samples can readily be processed using techniques well known to those of skill in the art, such as, for example, the single-step RNA isolation process of Chomczynski (1989, U.S. Patent No. 4,843,155).

The isolated mRNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, polymerase chain reaction analyses and probe arrays. One diagnostic method for the detection of mRNA levels involves contacting the isolated mRNA with a nucleic acid molecule (probe) that can hybridize to the mRNA encoded by the gene being detected. The nucleic acid probe can be, for example, a full-length cDNA, or a portion thereof, such as an oligonucleotide of at least 7, 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to a mRNA or genomic DNA encoding non-phosphorylatable PPAR gamma. Other suitable probes for use in the diagnostic assays of the invention are described herein. Hybridization of an mRNA with the probe indicates that non-phosphorylatable PPAR gamma is being expressed.

In one format, the mRNA is immobilized on a solid surface and contacted with a probe, for example by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In an alternative format, the probe(s) are immobilized on a solid surface and the mRNA is contacted with the probe(s), for example, in a gene chip array, *e.g.,* an Affymetrix™ gene chip array. A skilled artisan can readily adapt known mRNA detection methods for use in detecting the level of the non-phosphorylatable PPAR gamma mRNA expression levels.

An alternative method for determining the non-phosphorylatable PPAR gamma mRNA expression level in a sample involves the process of nucleic acid amplification, *e.g.*, by rtPCR (the experimental embodiment set forth in Mullis, 1987, U.S. Patent No. 4,683,202), ligase chain reaction (Barany, 1991, *Proc. Natl. Acad. Sci. USA,* 88:189-193), self sustained sequence replication (Guatelli *et al.*, 1990, *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional amplification system (Kwoh *et al.*, 1989, *Proc. Natl. Acad.*

Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi *et al.*, 1988, *Bio/Technology* 6:1197), rolling circle replication (Lizardi *et al.*, U.S. Patent No. 5,854,033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well-known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers. As used herein, amplification primers are defined as being a pair of nucleic acid molecules that can anneal to 5' or 3' regions of a gene (plus and minus strands, respectively, or vice-versa) and contain a short region in between. In general, amplification primers are from about 10 to 30 nucleotides in length and flank a region from about 50 to 200 nucleotides in length. Under appropriate conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule comprising the nucleotide sequence flanked by the primers.

For *in situ* methods, mRNA does not need to be isolated from the cells prior to detection. In such methods, a cell or tissue sample is prepared/processed using known histological methods. The sample is then immobilized on a support, typically a glass slide, and then contacted with a probe that can hybridize to the non-phosphorylatable PPAR gamma mRNA.

As an alternative to making determinations based on the absolute non-phosphorylatable PPAR gamma expression level, determinations may be based on the normalized non-phosphorylatable PPAR gamma expression level. Expression levels are normalized by correcting the absolute non-phosphorylatable PPAR gamma expression level by comparing its expression to the expression of a non-non-phosphorylatable PPAR gamma gene, *e.g.*, a housekeeping gene that is constitutively expressed. Suitable genes for normalization include housekeeping genes such as the actin gene, or epithelial cell-specific genes. This normalization allows the comparison of the expression level in one sample, *e.g.*, a subject sample, to another sample, *e.g.*, a normal sample, or between samples from different sources.

The level or activity of a non-phosphorylatable PPAR gamma protein can also be detected and/or quantified by detecting or quantifying the expressed polypeptide. The non-phosphorylatable PPAR gamma polypeptide can be detected and quantified by any of a number of means well known to those of skill in the art. These may include analytic biochemical methods such as electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion

chromatography, and the like, or various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, Western blotting, and the like. A skilled artisan can readily adapt known protein/antibody detection methods for use in determining whether cells express non-phosphorylatable PPAR gamma.

In addition to the nucleic acid molecules encoding non-phosphorylatable PPAR gamma proteins described above, another aspect of the invention pertains to isolated nucleic acid molecules which are antisense thereto. An "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *i.e.*, complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire non-phosphorylatable PPAR gamma coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding non-phosphorylatable PPAR gamma. The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues. In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding non-phosphorylatable PPAR gamma. The term "noncoding region" refers to 5' and 3' sequences which flank the coding region that are not translated into amino acids (*i.e.*, also referred to as 5' and 3' untranslated regions).

II. Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to the use of vectors and expression vectors containing a nucleic acid encoding non-phosphorylatable PPAR gamma (or a portion thereof). As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-

episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions. Adenoviral vectors comprising a non-phosphorylatable PPAR gamma nucleic acid molecule are used in certain embodiments.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (*e.g.*, non-phosphorylatable PPAR gamma proteins, mutant forms of non-phosphorylatable PPAR gamma, fusion proteins, etc.).

The recombinant expression vectors of the invention can be designed for expression of non-phosphorylatable PPAR gamma in prokaryotic or eukaryotic cells. For example, non-phosphorylatable PPAR gamma can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. In one embodiment, the coding sequence of the non-phosphorylatable PPAR gamma is cloned into a pGEX expression vector to create a vector encoding a fusion protein comprising, from the N-terminus to the C-terminus, GST-thrombin cleavage site-non-phosphorylatable PPAR gamma. The fusion protein can be purified by affinity chromatography using glutathione-agarose resin. Recombinant non-phosphorylatable PPAR gamma unfused to GST can be recovered by cleavage of the fusion protein with thrombin.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase

transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those utilized in *E. coli* (Wada *et al.* (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the non-phosphorylatable PPAR gamma expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerivisae* include pYepSec1 (Baldari, *et al.*, (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA).

Alternatively, non-phosphorylatable PPAR gamma can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, Sf 9 cells) include the pAc series (Smith *et al.* (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to non-phosphorylatable PPAR gamma mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced.

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either

mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, non-phosphorylatable PPAR gamma protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Fao hepatoma cells, primary hepatocytes, Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (*Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable biomarker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable biomarker can be introduced into a host cell on the same vector as that encoding non-phosphorylatable PPAR gamma or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable biomarker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) non-phosphorylatable PPAR gamma protein. Accordingly, the invention further provides methods for producing non-phosphorylatable PPAR gamma protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding non-phosphorylatable PPAR gamma has been introduced) in a suitable medium

until non-phosphorylatable PPAR gamma is produced. In another embodiment, the method further comprises isolating non-phosphorylatable PPAR gamma from the medium or the host cell.

The host cells of the invention can also be used to produce nonhuman transgenic animals (*e.g.*, knock-in animals). The nonhuman transgenic animals can be used in screening assays designed to identify agents or compounds, *e.g.*, drugs, pharmaceuticals, etc., which are capable of ameliorating detrimental symptoms of selected disorders such as glucose homeostasis disorders, weight disorders or disorders associated with insufficient insulin activity. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which non-phosphorylatable PPAR gamma-coding sequences (for example, murine PPAR gamma encoding alanine or other non-phosphorylatable amino acid at position 273 rather than serine) have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous non-phosphorylatable PPAR gamma sequences have been introduced into their genome or homologous recombinant animals in which endogenous non-phosphorylatable PPAR gamma sequences have been altered. Such animals are useful for studying the function and/or activity of non-phosphorylatable PPAR gamma and for identifying and/or evaluating modulators of non-phosphorylatable PPAR gamma activity using the methods and criteria described herein. As used herein, a "transgenic animal" is a nonhuman animal, a mammal, a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include nonhuman primates, sheep, dogs, cows, goats, chickens, amphibians, etc. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a nonhuman animal, a mammal, a mouse, in which an endogenous non-phosphorylatable PPAR gamma gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing non-phosphorylatable PPAR gamma-encoding nucleic acid into the male pronuclei of a fertilized oocyte, *e.g.*, by microinjection, retroviral infection, and allowing the oocyte to

develop in a pseudopregnant female foster animal. The human non-phosphorylatable PPAR gamma cDNA sequence can be introduced as a transgene into the genome of a nonhuman animal. Alternatively, a nonhuman homologue of the human non-phosphorylatable PPAR gamma gene, such as a mouse non-phosphorylatable PPAR gamma gene, can be used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to the non-phosphorylatable PPAR gamma transgene to direct expression of non-phosphorylatable PPAR gamma protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder *et al.*, U.S. Patent No. 4,873,191 by Wagner *et al.* and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of the non-phosphorylatable PPAR gamma transgene in its genome and/or expression of non-phosphorylatable PPAR gamma mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding non-phosphorylatable PPAR gamma can further be bred to other transgenic animals carrying other transgenes.

To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a non-phosphorylatable PPAR gamma gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, replace, the wild-type, phosphorylatable PPAR gamma gene. The non-phosphorylatable PPAR gamma gene can be a human gene (*e.g.*, from a human genomic clone isolated from a human genomic library screened with the cDNA of a sequence listed in Tables 1 and 2), and also a nonhuman homologue of a human non-phosphorylatable PPAR gamma gene. For example, a mouse non-phosphorylatable PPAR gamma gene can be used to construct a homologous recombination vector suitable for altering an endogenous phosphorylatable PPAR gamma gene in the mouse genome. In certain embodiments, the vector is designed such that, upon homologous recombination, the endogenous phosphorylatable PPAR gamma gene is functionally disrupted (*i.e.*, no longer encodes a phosphorylatable protein; also referred to as a "knock in" vector). In the homologous recombination vector, the altered portion of the

non-phosphorylatable PPAR gamma gene is flanked at its 5' and 3' ends by additional nucleic acid of the non-phosphorylatable PPAR gamma gene to allow for homologous recombination to occur between the exogenous non-phosphorylatable PPAR gamma gene carried by the vector and an endogenous non-phosphorylatable PPAR gamma gene in an embryonic stem cell. The additional flanking non-phosphorylatable PPAR gamma nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see *e.g.*, Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced non-phosphorylatable PPAR gamma gene has homologously recombined with the endogenous non-phosphorylatable PPAR gamma gene are selected (see *e.g.*, Li, E. *et al.* (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec *et al.*; WO 91/01140 by Smithies *et al.*; WO 92/0968 by Zijlstra *et al.*; and WO 93/04169 by Berns *et al.*

In another embodiment, transgenic nonhuman animals can be produced which contain selected systems which allow for regulated expression of the transgene. One example of such a system is the *cre/loxP* recombinase system of bacteriophage P1. For a description of the *cre/loxP* recombinase system, see, *e.g.*, Lakso *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.* (1991) *Science* 251:1351-1355. If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the *Cre* recombinase and a selected protein are required. Such animals can be provided through the construction of "double"

transgenic animals, *e.g.*, by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the nonhuman transgenic animals described herein can also be produced according to the methods described in Wilmut, I. *et al.* (1997) *Nature* 385:810-813 and PCT International Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, *e.g.*, a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter G_0 phase. The quiescent cell can then be fused, *e.g.*, through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyst and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal will be a clone of the animal from which the cell, *e.g.*, the somatic cell, is isolated.

Transgenic animals, such as S273A PPAR gamma mutant knock-in animals, have numerous uses, as further described herein.

In one embodiment, for example, knock-in animals homozygous for the S273A mutation of PPAR gamma can be used to test the efficacy of potential anti-metabolic disorder therapeutics. MRL-24 and similar compounds which block the phosphorylation of PPAR gamma at serine 273 by cdk5 (phopho-blockers) are believed to improve metabolic disorders without having the side-effects of classical agonists, such as TZDs. For example, PPAR gamma is active in both lean and obese individuals; however in these states, PPAR gamma exhibits slightly different transcription of target genes. It is believed that this difference in transcriptional activity is due to the inhibitory phosphorylation of PPAR gamma at Ser-273. Mice with both copies of PPAR gamma mutated at Ser-273 to alanine or other non-phosphorylatable amino acid residue should retain normal glucose tolerance when faced with the challenge of a high-fat diet. The glucose lowering effect of MRL-24 does not require PPAR gamma agonism, an effect that is likely responsible for weight gain and fluid retention observed upon administration of classical PPAR gamma agonists. In general, it is believed that such classical PPAR gamma agonists (*e.g.*, rosiglitizone) will have the positive glucose lowering only in WT mice but not in S273A mice. However the side effects, such as weight gain and fluid retention are believed to still occur in both strains. Hence, an ideal compound would have no effect in the knock-in mice making it a good model for testing off-target effects of the drug.

In another embodiment, cells lines taken from these knock-in mice can be used for *in vitro* testing of potential compounds. Specific chemical entities are believed to only alter target genes in wild-type cells, not in knock-in cells.

In still another embodiment, bone marrow transplantation from a wild type animal to S273A knock-in animals and *vice versa* can be used to analyze activity of such mutant polypeptides in immune system cells (*e.g.*, macrophages and monocytes) in a wild type host background and *vice versa*. One example of this use would be to determine whether risk of atherosclerosis due to CD36-dependent decreased lipid uptake is mediated by immune system cells.

III. Isolated Non-Phosphorylatable PPAR gamma Proteins and Anti-Non-Phosphorylatable PPAR Gamma Antibodies

Another aspect of the invention pertains to the use of isolated non-phosphorylatable PPAR gamma proteins, and biologically active portions thereof, as well as peptide fragments suitable for use as immunogens to raise anti-non-phosphorylatable PPAR gamma antibodies. An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of non-phosphorylatable PPAR gamma protein in which the protein is separated from cellular components of the cells in which it is naturally or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of non-phosphorylatable PPAR gamma protein having less than about 30% (by dry weight) of non-non-phosphorylatable PPAR gamma protein (also referred to herein as a "contaminating protein"), less than about 20% of non-non-phosphorylatable PPAR gamma protein, less than about 10% of non-non-phosphorylatable PPAR gamma protein, and less than about 5% non-non-phosphorylatable PPAR gamma protein. When the non-phosphorylatable PPAR gamma protein or biologically active portion thereof is recombinantly produced, it is also substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, less than about 10%, and less than about 5% of the volume of the protein preparation. The language "substantially free of chemical precursors or other chemicals" includes preparations of non-phosphorylatable PPAR gamma protein in which the protein is separated from chemical precursors or other chemicals which are involved in the synthesis

of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of non-phosphorylatable PPAR gamma protein having less than about 30% (by dry weight) of chemical precursors or non-non-phosphorylatable PPAR gamma chemicals, less than about 20% chemical precursors or non-non-phosphorylatable PPAR gamma chemicals, less than about 10% chemical precursors or non-non-phosphorylatable PPAR gamma chemicals, and less than about 5% chemical precursors or non-non-phosphorylatable PPAR gamma chemicals. In certain embodiments, isolated proteins or biologically active portions thereof lack contaminating proteins from the same animal from which the non-phosphorylatable PPAR gamma protein is derived. Typically, such proteins are produced by recombinant expression of, for example, a human non-phosphorylatable PPAR gamma protein in a nonhuman cell.

An exemplary isolated non-phosphorylatable PPAR gamma protein or a portion thereof of the invention selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation according to one or more criteria as described herein.

The portion of the protein may be a biologically active portion as described herein. In another embodiment, the non-phosphorylatable PPAR gamma protein has an amino acid sequence shown in Tables 1 and 2, respectively, or an amino acid sequence which is at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, and at least about 95%, 95.5%, 96%, 96.5%, 97%, 97.5%, 98%, 98.5%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or more homologous to the amino acid sequence shown in Tables 1 and 2. In yet another embodiment, the non-phosphorylatable PPAR gamma protein has an amino acid sequence which is encoded by a nucleotide sequence which hybridizes, *e.g.*, hybridizes under stringent conditions, to the nucleotide sequence listed in Tables 1 and 2 or a nucleotide sequence which is at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, and at least about 95% or more homologous to the nucleotide sequence shown in Tables 1 and 2. The non-phosphorylatable PPAR gamma proteins of the present invention, at least in part, also possess at least one of the non-phosphorylatable PPAR gamma biological activities described herein. For example, a non-phosphorylatable PPAR gamma protein of the present invention, at least in part, includes an amino acid sequence encoded by a nucleotide sequence which hybridizes, *e.g.*, hybridizes under stringent conditions, to the nucleotide sequence listed in Tables 1 and 2 and which selectively promotes anti-metabolic disorder

activity over classical PPAR gamma activation according to one or more criteria as described herein.

As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage. See Immunology--A Synthesis (2.sup.nd Edition, E. S. Golub and D. R. Gren, Eds., Sinauer Associates, Sunderland, Mass. (1991)), which is incorporated herein by reference. The term "amino acid" or "amino acid residue," as used herein, refers to naturally occurring L amino acids or to D amino acids. The commonly used one- and three-letter abbreviations for amino acids are used herein (Bruce Alberts *et al.*, Molecular Biology of the Cell, Garland Publishing, Inc., New York (4th ed. 2002)). Stereoisomers (*e.g.*, D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as alpha,alpha-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention, at least in part,. Examples of unconventional amino acids include: 4-hydroxyproline, .gamma.-carboxyglutamate, .epsilon.-N,N,N-trimethyllysine, .epsilon.-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, .sigma.-N-methylarginine, and other similar amino acids and imino acids (*e.g.*, 4-hydroxyproline). In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxy-terminal direction, in accordance with standard usage and convention.

In other embodiments, the non-phosphorylatable PPAR gamma protein is substantially homologous to the amino acid sequence listed in Tables 1 and 2 and retains the functional activity of the protein listed in Tables 1 and 2 yet differs in amino acid sequence due to natural allelic variation or mutagenesis, as described in detail in subsection I above. Accordingly, in another embodiment, the non-phosphorylatable PPAR gamma protein is a protein which comprises an amino acid sequence which is at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, and at least about 95% or more homologous to the amino acid sequence listed in Tables 1 and 2.

Biologically active portions of the non-phosphorylatable PPAR gamma protein include peptides comprising amino acid sequences derived from the amino acid sequence of the non-phosphorylatable PPAR gamma protein, *e.g.*, the amino acid sequence shown in Tables 1 and 2 or the amino acid sequence of a protein homologous to the non-phosphorylatable PPAR gamma protein, which include fewer amino acids than the full length non-phosphorylatable PPAR gamma protein or the full length protein which is

homologous to the non-phosphorylatable PPAR gamma protein, and exhibit at least one activity of the non-phosphorylatable PPAR gamma protein. Typically, biologically active portions (peptides, *e.g.*, peptides which are, for example, 5, 10, 15, 20, 30, 35, 36, 37, 38, 39, 40, 50, 100 or more amino acids in length) comprise a domain or motif, *e.g.*, a consensus cdk5 phosphorylation motif and/or mutated PPAR gamma non-phosphorylatable at Ser-273. Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the activities described herein. The biologically active portions of the non-phosphorylatable PPAR gamma protein include one or more selected domains/motifs or portions thereof having biological activity.

Non-phosphorylatable PPAR gamma proteins are produced by recombinant DNA techniques. For example, a nucleic acid molecule encoding the protein is cloned into an expression vector (as described above), the expression vector is introduced into a host cell (as described above) and the non-phosphorylatable PPAR gamma protein is expressed in the host cell. The non-phosphorylatable PPAR gamma protein can then be isolated from the cells by an appropriate purification scheme using standard protein purification techniques. Alternative to recombinant expression, a non-phosphorylatable PPAR gamma protein, polypeptide, or peptide can be synthesized chemically using standard peptide synthesis techniques. Moreover, native non-phosphorylatable PPAR gamma protein can be isolated from cells (*e.g.*, brown adipocytes), for example using an anti-non-phosphorylatable PPAR gamma antibody (described further below).

The invention also provides non-phosphorylatable PPAR gamma chimeric or fusion proteins. As used herein, a non-phosphorylatable PPAR gamma "chimeric protein" or "fusion protein" comprises a non-phosphorylatable PPAR gamma polypeptide operatively linked to a non-non-phosphorylatable PPAR gamma polypeptide. A "non-phosphorylatable PPAR gamma polypeptide" refers to a polypeptide having an amino acid sequence corresponding to non-phosphorylatable PPAR gamma, whereas a "non-non-phosphorylatable PPAR gamma polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the non-phosphorylatable PPAR gamma protein, *e.g.*, a protein which is different from the non-phosphorylatable PPAR gamma protein and which is derived from the same or a different organism. Within the fusion protein, the term "operatively linked" is intended to indicate that the non-phosphorylatable PPAR gamma polypeptide and the non-non-phosphorylatable

PPAR gamma polypeptide are fused in-frame to each other. The non-non-phosphorylatable PPAR gamma polypeptide can be fused to the N-terminus or C-terminus of the non-phosphorylatable PPAR gamma polypeptide. For example, in one embodiment the fusion protein is a GST-non-phosphorylatable PPAR gamma fusion protein in which the non-phosphorylatable PPAR gamma sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant non-phosphorylatable PPAR gamma. In another embodiment, the fusion protein is a non-phosphorylatable PPAR gamma protein containing a heterologous signal sequence at its N-terminus. In certain host cells (*e.g.*, mammalian host cells), expression and/or secretion of non-phosphorylatable PPAR gamma can be increased through use of a heterologous signal sequence.

A non-phosphorylatable PPAR gamma chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). A non-phosphorylatable PPAR gamma-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the non-phosphorylatable PPAR gamma protein.

The present invention, at least in part, also pertains to homologues of the non-phosphorylatable PPAR gamma proteins which function as either a non-phosphorylatable PPAR gamma agonist (mimetic) or a non-phosphorylatable PPAR gamma antagonist. In a certain embodiments, the non-phosphorylatable PPAR gamma agonists and antagonists stimulate or inhibit, respectively, a subset of the biological activities of the naturally occurring form of the non-phosphorylatable PPAR gamma protein. Thus, specific

biological effects can be elicited by treatment with a homologue of limited function. In one embodiment, treatment of a subject with a homologue having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the naturally occurring form of the non-phosphorylatable PPAR gamma protein.

Homologues of the non-phosphorylatable PPAR gamma protein can be generated by mutagenesis, *e.g.*, discrete point mutation or truncation of the non-phosphorylatable PPAR gamma protein. As used herein, the term "homologue" refers to a variant form of the non-phosphorylatable PPAR gamma protein which acts as an agonist or antagonist of the activity of the non-phosphorylatable PPAR gamma protein. An agonist of the non-phosphorylatable PPAR gamma protein can retain substantially the same, or a subset, of the biological activities of the non-phosphorylatable PPAR gamma protein.

In an alternative embodiment, homologues of the non-phosphorylatable PPAR gamma protein can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, of the non-phosphorylatable PPAR gamma protein for non-phosphorylatable PPAR gamma protein agonist or antagonist activity. In one embodiment, a variegated library of non-phosphorylatable PPAR gamma variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of non-phosphorylatable PPAR gamma variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential non-phosphorylatable PPAR gamma sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display) containing the set of non-phosphorylatable PPAR gamma sequences therein. There are a variety of methods which can be used to produce libraries of potential non-phosphorylatable PPAR gamma homologues from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential non-phosphorylatable PPAR gamma sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, *e.g.*, Narang, S.A. (1983) *Tetrahedron* 39:3; Itakura *et al.* (1984) *Annu. Rev. Biochem.* 53:323; Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.* (1983) *Nucleic Acid Res.* 11:477.

In addition, libraries of fragments of the non-phosphorylatable PPAR gamma protein coding can be used to generate a variegated population of non-phosphorylatable PPAR gamma fragments for screening and subsequent selection of homologues of a non-phosphorylatable PPAR gamma protein. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a non-phosphorylatable PPAR gamma coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal fragments of various sizes of the non-phosphorylatable PPAR gamma protein.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of non-phosphorylatable PPAR gamma homologues. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a new technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify non-phosphorylatable PPAR gamma homologues (Arkin and Youvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delagrave *et al.* (1993) *Protein Engineering* 6(3):327-331).

In one embodiment, an isolated non-phosphorylatable PPAR gamma protein, or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind non-phosphorylatable PPAR gamma using standard techniques for polyclonal and monoclonal antibody preparation. For example, a peptide having the sequence, KTTDK(pS)PFVIYDC, with or without serine phrosphorylation, can be used for this purpose. Alternatively, the full-length non-phosphorylatable PPAR gamma protein can be

used or, alternatively, antigenic peptide fragments of non-phosphorylatable PPAR gamma can be used as immunogens. A non-phosphorylatable PPAR gamma immunogen typically is used to prepare antibodies by immunizing a suitable subject, (*e.g.*, rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed non-phosphorylatable PPAR gamma protein or a chemically synthesized non-phosphorylatable PPAR gamma peptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic non-phosphorylatable PPAR gamma preparation induces a polyclonal anti-non-phosphorylatable PPAR gamma antibody response.

Accordingly, another aspect of the invention pertains to the use of anti-non-phosphorylatable PPAR gamma antibodies. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, such as non-phosphorylatable PPAR gamma. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and $F(ab')_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind non-phosphorylatable PPAR gamma. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of non-phosphorylatable PPAR gamma. A monoclonal antibody composition thus typically displays a single binding affinity for a particular non-phosphorylatable PPAR gamma protein with which it immunoreacts.

Polyclonal anti-non-phosphorylatable PPAR gamma antibodies can be prepared as described above by immunizing a suitable subject with a non-phosphorylatable PPAR gamma immunogen. The anti-non-phosphorylatable PPAR gamma antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized non-phosphorylatable PPAR gamma. If desired, the antibody molecules directed against non-phosphorylatable PPAR gamma can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, *i.e.*, when the anti-non-phosphorylatable PPAR

gamma antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also, Brown *et al.* (1981) *J. Immunol.* 127:539-46; Brown *et al.* (1980) *J. Biol. Chem.* 255:4980-83; Yeh *et al.* (1976) *Proc. Natl. Acad. Sci. USA* 76:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer)* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol. Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.,* 54:387-402; M. L. Gefter *et al.* (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a non-phosphorylatable PPAR gamma immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds non-phosphorylatable PPAR gamma.

Any of the many well-known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-non-phosphorylatable PPAR gamma monoclonal antibody (see, *i.e.*, G. Galfre *et al.* (1977) *Nature* 266:550-52; Gefter *et al. Somatic Cell Genet.*, cited *supra*; Lerner, *Yale J. Biol. Med.*, cited *supra*; Kenneth, *Monoclonal Antibodies*, cited *supra*). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention, at least in part, with an immortalized mouse cell line. In some embodiments, immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, *i.e.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG").

Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind non-phosphorylatable PPAR gamma, *i.e.*, using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-non-phosphorylatable PPAR gamma antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with non-phosphorylatable PPAR gamma to thereby isolate immunoglobulin library members that bind non-phosphorylatable PPAR gamma. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* PCT International Publication No. WO 92/18619; Dower *et al.* PCT International Publication No. WO 91/17271; Winter *et al.* PCT International Publication WO 92/20791; Markland *et al.* PCT International Publication No. WO 92/15679; Breitling *et al.* PCT International Publication WO 93/01288; McCafferty *et al.* PCT International Publication No. WO 92/01047; Garrard *et al.* PCT International Publication No. WO 92/09690; Ladner *et al.* PCT International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Bio/Technology* 9:1369-1372; Hay *et al.* (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J.* 12:725-734; Hawkins *et al.* (1992) *J. Mol. Biol.* 226:889-896; Clackson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3576-3580; Garrard *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19:4133-4137; Barbas *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7978-7982; and McCafferty *et al. Nature* (1990) 348:552-554.

Additionally, recombinant anti-non-phosphorylatable PPAR gamma antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using

methods described in Robinson *et al.* International Application No. PCT/US86/02269; Akira, *et al.* European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.* European Patent Application 173,494; Neuberger *et al.* PCT International Publication No. WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:214-218; Nishimura *et al.* (1987) *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *BioTechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

An anti-non-phosphorylatable PPAR gamma antibody (*e.g.*, monoclonal antibody) can be used to isolate non-phosphorylatable PPAR gamma by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-non-phosphorylatable PPAR gamma antibody can facilitate the purification of natural non-phosphorylatable PPAR gamma from cells and of recombinantly produced non-phosphorylatable PPAR gamma expressed in host cells. Moreover, an anti-non-phosphorylatable PPAR gamma antibody can be used to detect non-phosphorylatable PPAR gamma protein (*e.g.*, in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the non-phosphorylatable PPAR gamma protein. Anti-non-phosphorylatable PPAR gamma antibodies can be used to monitor protein levels in a cell or tissue, *e.g.,* adipose cells or tissue, as part of a clinical testing procedure, *e.g.*, in order to monitor a safe dosage of an uncoupling agent. Detection can be facilitated by coupling (*e.g.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of

bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

In vivo techniques for detection of non-phosphorylatable PPAR gamma protein include introducing into a subject a labeled antibody directed against the protein. For example, the antibody can be labeled with a radioactive biomarker whose presence and location in a subject can be detected by standard imaging techniques.

IV. Methods and Compounds of the Invention

The methods of the invention relate to the identification and use of PPAR gamma ligands that selectively promote anti-metabolic disorder activity over classical PPAR gamma activation. Such ligands are referred to herein as "atypical."

The term "selectively promotes" is intended to encompass any differential regulation of PPAR gamma activity that promotes, activates, stimulates, enhances, or results in promotion of genes regulated predominantly and/or exclusively through cdk5-mediated phosphorylation of PPAR gamma, such as "anti-diabetic" and/or "brown fat" and/or "increased respiration/oxygen consumption" genes (*e.g.*, those selectively promoted by the atypical PPAR gamma ligand, MRL24), in contrast to promoting metabolic disorders, such as "adipogenic" genes (*e.g.*, those regulated by the classical PPAR gamma agonist, rosiglitazone). In another aspect, the invention is related to methods for identifying such genes regulated predominantly and/or exclusively through cdk5-mediated phosphorylation of PPAR gamma, such as "anti-diabetic" and/or "brown fat" and/or "increased respiration/oxygen consumption" genes, such as those selectively promoted by the atypical PPAR gamma ligand, MRL24, by assaying gene expression profiles of samples, for example, in the presence or absence of test compounds that selectively inhibit PPAR gamma phosphorylation or in the presence or absence (wild type PPR gamma) of non-phosphorylatable PPAR gamma. In another aspect, the invention relates to methods for treating metabolic disorders, *e.g.*, diabetes and/or obesity, in a subject comprising administering to the subject an agent that selectively regulates genes predominantly and/or exclusively through cdk5-mediated phosphorylation of PPAR gamma, such as "anti-diabetic" and/or "brown fat" and/or "increased respiration/oxygen consumption" genes, such as those selectively promoted by the atypical PPAR gamma ligand, MRL24.

As used herein, the term "agent" and "therapeutic agent" is defined broadly as anything that cells from a subject with obesity or an obesity-related disorder may be exposed to in a therapeutic protocol.

A. PPAR gamma Ligand Screening Assays

The invention provides methods for identifying compounds or agents which can selectively promote anti-metabolic disorder activity over classical PPAR gamma activation, such as atypical PPAR gamma ligands. These methods are also referred to herein as drug screening assays and typically include the step of screening a candidate/test compound or agent for the ability to interact with (*e.g.*, bind to) a PPAR gamma protein, to modulate the interaction of a PPAR gamma protein and a target molecule, and/or to modulate PPAR gamma nucleic acid expression and/or PPAR gamma protein activity. Candidate/test compounds or agents which have one or more of these abilities can be used as drugs to treat disorders characterized by aberrant, abnormal, and/or unwanted PPAR gamma nucleic acid expression and/or PPAR gamma protein activity. Candidate/test compounds include, for example, small organic and inorganic molecules (*e.g.*, molecules obtained from combinatorial and natural product libraries).

The test compounds of the present invention, at least in part, can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409),

plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

In one embodiment, the invention provides assays for screening candidate/test compounds which interact with (*e.g.*, bind to) PPAR gamma protein. Typically, the assays are cell-based assays. The cell, for example, can be of mammalian origin, *e.g.*, a liver cell, a skeletal muscle cell, immune cell, such as a monocyte or macrophage, or a fat cell, such as a preadipocyte, white adipocyte, and/or brown adipocyte, In other embodiments, the cell can endogenously harbor non-phosphorylatable PPAR gamma encoding nucleic acid sequences, such as in the case of knock-in animals described herein.

In other embodiments, the assays are cell-free assays which include the steps of combining a PPAR gamma protein or a biologically active portion thereof, and a candidate/test compound, *e.g.*, under conditions which allow for interaction of (*e.g.*, binding of) the candidate/test compound to the PPAR gamma protein or portion thereof to form a complex, and detecting the formation of a complex, in which the ability of the candidate compound to interact with (*e.g.*, bind to) the PPAR gamma polypeptide or fragment thereof is indicated by the presence of the candidate compound in the complex. Formation of complexes between the PPAR gamma protein and the candidate compound can be quantitated, for example, using standard immunoassays. Such analyses would identify test compounds as PPAR gamma ligands.

In another embodiment, the invention provides screening assays to identify candidate/test compounds which modulate (*e.g.*, stimulate or inhibit) the interaction (and most likely PPAR gamma activity as well) between a PPAR gamma protein and a molecule (target molecule) with which the PPAR gamma protein normally interacts. Examples of such target molecules include proteins in the signaling pathway promoting anti-metabolic disorder activity by PPAR gamma over classical PPAR gamma activation Typically, the assays are cell-free assays which include the steps of combining a PPAR gamma protein or a biologically active portion thereof, a PPAR gamma target molecule and a candidate/test compound, *e.g.*, under conditions wherein but for the presence of the candidate compound, along with known modulators and/or cofactors, such as cdk5 and/or p35/p25 that bind to or modulate the target molecule, and detecting the formation of a complex which includes the PPAR gamma protein and the target molecule or detecting the interaction/reaction of the

PPAR gamma protein and the target molecule. Detection of complex formation can include direct quantitation of the complex by, for example, measuring inductive effects of the PPAR gamma protein. A statistically significant change, such as a decrease, in the interaction of the PPAR gamma and target molecule (*e.g.*, in the formation of a complex between the PPAR gamma and the target molecule) in the presence of a candidate compound (relative to what is detected in the absence of the candidate compound) is indicative of a modulation (*e.g.*, stimulation or inhibition) of the interaction between the PPAR gamma protein and the target molecule. Modulation of the formation of complexes between the PPAR gamma protein and the target molecule can be quantitated using, for example, an immunoassay.

To perform the above drug screening assays, it is desirable to immobilize either PPAR gamma or its target molecule to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Interaction (*e.g.*, binding of) of PPAR gamma to a target molecule, in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion polypeptide can be provided which adds a domain that allows the polypeptide to be bound to a matrix. For example, glutathione-S-transferase/ PPAR gamma fusion polypeptides can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtitre plates, which are then combined with the cell lysates (*e.g.*, ^{35}S-labeled) and the candidate compound, and the mixture incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly, or in the supernatant after the complexes are dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of PPAR gamma-binding polypeptide found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing polypeptides on matrices can also be used in the exemplary drug screening assays of the invention. For example, either PPAR gamma or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated PPAR gamma molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (*e.g.*, biotinylation kit, Pierce

Chemicals, Rockford, Ill.), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with PPAR gamma, such as phospher-Ser-273-specific anti-PPAR gamma antibodies, but which do not interfere with binding of the polypeptide to its target molecule can be derivatized to the wells of the plate, and PPAR gamma trapped in the wells by antibody conjugation. As described above, preparations of a PPAR gamma-binding polypeptide and a candidate compound are incubated in the PPAR gamma-presenting wells of the plate, and the amount of complex trapped in the well can be quantitated. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the PPAR gamma target molecule, or which are reactive with PPAR gamma polypeptide and compete with the target molecule; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the target molecule.

In yet another embodiment, the invention provides a method for identifying a compound or agent (*e.g.*, a screening assay) capable of selectively promoting anti-metabolic disorder activity over classical PPAR gamma activation. Such compounds would also be useful for the treatment of a metabolic disorder, as described further herein. Methods for assaying the ability of the compound or agent to modulate the expression of the PPAR gamma nucleic acid or activity of the PPAR gamma protein can be cell-free or cell-based assays.

Accordingly, the samples can comprise *in vitro*, ex vivo, and/or *in vivo* samples. Such samples can also be selected from any number of cell types relevant for metabolic disorder analyses, including, for example, preadipocytes, white adipocytes, brown adipocytes, monocytes, and macrophages.

In addition, the cell-free and/or cell-based assays described herein can be used according to methods well-known to the skilled artisan for determining the effect of a compound or agent to differentially regulate PPAR gamma activity that promotes, activates, stimulates, enhances, or results in promotion of genes regulated predominantly and/or exclusively through cdk5-mediated phosphorylation of PPAR gamma, such as "anti-diabetic" and/or "brown fat" and/or "increased respiration/oxygen consumption" genes, such as those selectively promoted by the atypical PPAR gamma ligand, MRL24, in contrast to promoting metabolic disorders, such as "adipogenic" genes especially those regulated by the classical PPAR gamma agonist, rosiglitazone. This can involve identifying

genes regulated predominantly and/or exclusively through cdk5-mediated phosphorylation of PPAR gamma, such as "anti-diabetic" and/or "brown fat" and/or "increased respiration/oxygen consumption" genes, according to methods well known in the art. Such genes can be analyzed in their endogenous cellular contexts or assayed according to any of a number of well-known gene reporter systems, such as using transcriptional assays operatively-linked to PPAR gamma responsive promoters, PPAR gamma transactivation assays (*e.g.*, GAL4-PPAR gamma on a upstream activation site (UAS) promoter), etc.

Candidate compounds which produce a statistically significant change in PPAR gamma-dependent responses (either stimulation or inhibition) can be identified. Such statistically significant changes can be measured according to a number of criteria and/or relative to a number of controls. For example, significant modulation of gene expression, assay change, etc. can be assessed if the output under analysis is greater than or less than 1.1-, 1.2-, 1.3-, 1.4-, 1.5-, 1.6-, 1.7-, 1.8-, 1.9-, 2.0-, 2.1-, 2.2-, 2.3-, 2.4-, 2.5-, 2.6-, 2.7-, 2.8-, 2.9-, 3.0-, 3.1-, 3.2-, 3.3-, 3.4-, 3.5-, 3.6-, 3.7-, 3.8-, 3.9-, 4.0-, 4.1-, 4.2-, 4.3-, 4.4-, 4.5-, 4.6-, 4.7-, 4.8-, 4.9-, 5.0-, 5.5-, 6.0, 6.5-, 7.0-, 7.5-, 8.0-, 8.5-, 9.0- 9.5-, 10-, 11-, 12-, 13-, 14-, 15-, 16-, 17-, 18-, 19-, 20-fold or more different (including any range inclusive), relative to a control.

Generally, determining expression profiles can be performed using arrays involving the following steps: (a) obtaining an RNA sample from a subject and preparing labeled nucleic acids therefrom (the "target nucleic acids" or "targets"); (b) contacting the target nucleic acids with the array under conditions sufficient for target nucleic acids to bind with corresponding probes on the array, *e.g.*, by hybridization or specific binding; (c) optionally removing unbound targets from the array; (d) detecting bound targets, and (e) analyzing the results. As used herein, "nucleic acid probes" or "probes" are nucleic acids attached to the array, whereas "target nucleic acids" are nucleic acids that are hybridized to the array. Each of these steps and methods and variations for carrying them out are well known to a skilled artisan.

In certain embodiments, it is sufficient to determine the expression of a relatively small number of genes (*e.g.*, one or only a few genes), as opposed to hundreds or thousands of genes. Although microarrays can be used in these embodiments, various other methods of detection of gene expression are available. Although certain similarities with array-based detection, such as RNA isolation, labeling, etc. may be common, the following methods can offer advantages in terms of ease-of-use and time constraints.

In one embodiment, RNA obtained form a sample is reverse transcribed into a first cDNA strand and subjected to PCR, *e.g.*, RT-PCR. House keeping genes, or other genes whose expression does not vary can be used as internal controls and controls across experiments. Following the PCR reaction, the amplified products can be separated by electrophoresis and detected. By using quantitative PCR, the level of amplified product will correlate with the level of RNA that was present in the sample. The amplified samples can also be separated on a agarose or polyacrylamide gel, transferred onto a filter, and the filter hybridized with a probe specific for the gene of interest. Numerous samples can be analyzed simultaneously by conducting parallel PCR amplification, *e.g.*, by multiplex PCR.

Quantitative PCR techniques can also be used based on numerous technologies, such as TaqMan or probes labeled at the 5' and 3' ends with a reporter and quencher fluorescent dye, respectively (FQ probe), which anneals between the two PCR primers. Only specific product will be detected when the probe is bound between the primers. As PCR amplification proceeds, the 5'-nuclease activity of Taq polymerase initially cleaves the reporter dye from the probe. The signal generated when the reporter dye is physically separated from the quencher dye is detected by measuring the signal with an attached CCD camera. Each signal generated equals one probe cleaved which corresponds to amplification of one target strand. PCR reactions may be set up using the PE Applied Biosystem TaqMan PCR Core Reagent Kit according to the instructions supplied. This technique is further described, *e.g.*, in U.S. Pat. No. 6,326,462.

In other embodiments, dot blot, "sandwich" hybridization, deep sequencing, SAGE, Northern blotting, in situ hybridization, and other similar methods can be used, especially as further described, for example, in Sambrook *et al.*, "Molecular Cloning: A Laboratory Manual" (New York, Cold Spring Harbor Laboratory, 1989).

In other methods, the level of expression of a gene can be detected by measuring the level of protein encoded by the gene. This can be done, *e.g.*, by immunoprecipitation, ELISA, or immunohistochemistry using an agent, *e.g.*, an antibody, that specifically detects the protein encoded by the gene. Other techniques include Western blot analysis. Immunoassays are commonly used to quantitate the levels of proteins in cell samples, and many other immunoassay techniques are known in the art. The invention is not limited to a particular assay procedure, and therefore is intended to include both homogeneous and heterogeneous procedures. Exemplary immunoassays which can be conducted according to the invention include fluorescence polarization immunoassay (FPIA), fluorescence

immunoassay (FIA), enzyme immunoassay (EIA), nephelometric inhibition immunoassay (NIA), enzyme linked immunosorbent assay (ELISA), and radioimmunoassay (RIA), ELISA, etc. alone or in combination or alternatively with NMR, MALDI-TOF, LC-MS/MS. An indicator moiety, or label group, can be attached to the subject antibodies and is selected so as to meet the needs of various uses of the method which are often dictated by the availability of assay equipment and compatible immunoassay procedures. General techniques to be used in performing the various immunoassays noted above are known to those of ordinary skill in the art.

In the case of polypeptides which are secreted from cells, such as adipokines, such as adiponectin, or obesity-induced inflammation proteins, such as TNF-alpha, the level of expression of these polypeptides can be measured in biological fluids.

Complementing such gene expression analyses, assays that phenotypically report markers of metabolic disorder activity can also be performed. For example, assays reporting the induction of anti-metabolic disorder activity such as anti-obesity, anti-diabetes, brown fat induction (*e.g.*, in white fat cells and/or de novo from preadipocytes), etc. can be assessed according to expression of brown fat specific genes, including UCP-1, cidea, and PGC-1a, as well as genes involved in mitochondrial biogenesis and uncoupled oxygen consumption. In other embodiments, phenotypic assays for brown fat induction can also be assessed, such as analyzing histology, uncoupled oxygen consumption, uncoupled respiration by mitochondria, fatty acid beta oxidation, thermogenesis (heat production), respiration measurements, weight changes, fluid retention, mitochondrial biogenesis, energy expenditure, adipogenesis, serum fatty acid levels, serum adipokine levels, white and/or brown fat depots, fat and/or lean mass (*e.g.*, as assayed by dual energy X-ray absorptiometry (DEXA) scanning), glucose tolerance tests, insulin tolerance tests, and modulation of obesity-induced inflammation.

In yet another aspect of the invention, the PPAR gamma proteins can be used as "bait proteins" in two-hybrid, three-hybrid, etc. assays (see, *e.g.*, U.S. Pat. No. 5,283,317; Zervos *et al.* (1993) Cell 72:223-232; Madura *et al.* (1993) J. Biol. Chem. 268:12046-12054; Bartel *et al.* (1993) Biotechniques 14:920-924; Iwabuchi *et al.* (1993) Oncogene 8:1693-1696; and Brent WO 94/10300), to identify other proteins, which bind to or interact with PPAR gamma ("PPAR gamma-binding proteins" or " PPAR gamma-bp") and selectively modulate PPAR gamma activity that promotes anti-metabolic activities over

classical PPAR gamma activation. Such interactions can comprise ligand-dependent or ligand-independent methods.

In all of these methods, the use of proper controls can be important. In one embodiment, atypical PPAR gamma modulators of the present invention, at least in part, can be identified in a method as described herein such that the assay output (*e.g.*, the genes and assays listed in Tables 1 and 2) is compared relative to a cell in the absence of the candidate compound. In one embodiment, comparison can be made relative to a cell contacted with a known PPAR gamma agonist, such as the robust classical PPAR gamma agonist, rosiglitazone. In another embodiment, comparison can be made relative to a cell comprising encoding and/or expressing a non-phosphorylatable PPAR gamma polypeptide (*e.g.*, a S273A mutant). In still another embodiment, comparison can be made relative to ratios of gene expression an analyses, for example, the ratio of expression of a gene listed in Table 1 relative to that of a gene listed in Table 2 or *vice versa*.

B. Compounds

PPAR gamma ligands known in the art and/or identified using the methods described herein, as well as endpoints analyzed using the methods described herein, can be described according to a number of criteria. For example, the compound or agents can have less than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50% or less of the classical PPAR agonist activity (or any range inclusive) relative to another PPAR gamma agonist, such as the robust classical PPAR gamma agonist, rosiglitazone. Besides rosiglitazone, other classical PPAR gamma agonists, such as pioglitazone, can be used. In some embodiments, the classical PPAR agonist activity can be measured according to methods described herein, including, for example, analyzing gene expression of biomarkers listed in Tables 1 and 2. For example, significant modulation of gene expression, assay change, etc. can be assessed if the output under analysis is greater than or less than 1.1-, 1.2-, 1.3-, 1.4-, 1.5-, 1.6-, 1.7-, 1.8-, 1.9-, 2.0-, 2.1-, 2.2-, 2.3-, 2.4-, 2.5-, 2.6-, 2.7-, 2.8-, 2.9-, 3.0-, 3.1-, 3.2-, 3.3-, 3.4-, 3.5-, 3.6-, 3.7-, 3.8-, 3.9-, 4.0-, 4.1-, 4.2-, 4.3-, 4.4-, 4.5-, 4.6-, 4.7-, 4.8-, 4.9-, 5.0-, 5.5-, 6.0, 6.5-, 7.0-, 7.5-, 8.0-, 8.5-, 9.0- 9.5-, 10-, 11-, 12-, 13-, 14-, 15-, 16-, 17-, 18-, 19-, 20-fold or more, different (including any range inclusive) relative to a control.

In another embodiment, the compound or agent can have an EC50 binding affinity for PPAR gamma at a dose of 100 μM, 90 μM, 80 μM, 70 μM, 60 μM, 50 μM, 40 μM, 30 μM, 20 μM, 10 μM, 9 μM, 8 μM, 7 μM, 6 μM, 5 μM, 4.5 μM, 4 μM, 3.5 μM, 3 μM, 2.5 μM, 2 μM, 1.5 μM, 1 μM, 900 nM, 850 nM, 800 nM, 750 nM, 700 nM, 650 nM, 600 nM, 550 nM, 500 nM, 450 nM, 400 nM, 350 nM, 300 nM, 250 nM, 200 nM, 150 nM, 100 nM, 95 nM, 90 nM, 85 nM, 80 nM, 75 nM, 70 nM, 65 nM, 60 nM, 55 nM, 50 nM, 45 nM, 40 nM, 35 nM, 30 nM, 25 nM, 20 nM, 15 nM, 10 nM, 5 nM, 4 nM, 3 nM, 2 nM, 1 nM or less (or any range inclusive). An EC_{50} value may be determined and used for comparative purposes. This value is the concentration of drug needed to inhibit a relevant PPAR gamma function by 50% relative to the control.

In still another embodiment, the compound or agent can stabilize certain domains of the PPAR gamma polypeptide, for example, as measured using HDX techniques. Without being bound by theory, it is believed that helix 12 is only stabilized by classical PPAR agonists (*i.e.*, strong/full), whereas atypical PPAR agonists (*i.e.*, partial/intermediate) agonists, such as compounds and agents obtained using the screening methods described herein, do not exhibit statistically significant stabilization patterns of helix 12. In one embodiment, by contrast, the atypical agonists stabilize the beta sheet. In another embodiment, the atypical agonists stabilize helix 3. Assays to determine protein domain stabilization are well known in the art and described, for example, in Bruning *et al.* (2007) Structure 15:1258-1271, which is incorporated in its entirety herein by this reference. In some embodiments, the compound or agent can stabilize the beta sheet and/or helix 3 by at least 100%, 95%, 90%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20% (or any range inclusive) relative to control (*e.g.*, a known atypical PPAR gamma agonist such as MRL24 and/or MRL-20).

In yet another embodiment, the compound or agent can belong to a structural class of compounds, such as a family of small molecules. Examples of classical PPAR gamma ligands that act as strong agonists include thizolidinediones (TZD) and thiazolidine derivatives known as thiazolidinediones, *e.g.*, proglitazone (also known as AD-4833 and U-72107E), troglitazone (also known as CS-045) (Sankyo) and C1-991 (Parke-Davis), BRL 49653, ciglitazone, englitazone and chemical derivatives thereof. These compounds are conventionally known for the treatment of diabetes. See *e.g.*, U.S. Pat. Nos. 4,812,570; 4,775,687; 4,725,610; 4,582,839; and 4,572,912 for exemplary sources of such compounds. U.S. Pat. No. 5,521,201 and European Patent Applications 0008203, 0139421, 0155845,

0177353, 0193256, 0207581 and 0208420, and Chem. Pharm. Bull 30 (10) 3580-3600 relate to thiazolidinedione derivatives, and describe commercial sources/synthetic schemes for a variety of TZD and TZD-like analogs, which may be useful in carrying out the methods of the present invention, at least in part. By contrast, examples of atypical PPAR gamma ligands that selectively promote anti-metabolic activities over classical PPAR gamma activation include MRL-20, MRL-24, nTZDpa, SR145, SR147, Mbx-102, and BVT.13, as described, for example, in Bruning *et al.* (2007) Structure 15:1258-1271, which is incorporated in its entirety herein by this reference

C. Methods Of Treatment

Atypical PPAR gamma modulators of the present invention, at least in part, can be used to treat, for example, metabolic disorders described herein, including weight disorders, *e.g.*, obesity, and disorders associated with insufficient insulin activity, *e.g.*, diabetes. In some embodiments, such PPAR gamma modulators can also be used to determine the efficacy, toxicity, or side effects of treatment with such an agent. These methods of treatment generally include the steps of administering atypical PPAR gamma modulators in a pharmaceutical composition, as described in subsection V below, to a subject in need of such treatment, *e.g.*, a subject with a disorder described herein.

The term "administering" is intended to include routes of administration which allow the agent to perform its intended function of increasing non-phosphorylatable PPAR gamma expression and/or activity. Examples of routes of administration which can be used include injection (subcutaneous, intravenous, parenterally, intraperitoneally, intrathecal, etc.), oral, inhalation, and transdermal. The injection can be bolus injections or can be continuous infusion. Depending on the route of administration, the agent can be coated with or disposed in a selected material to protect it from natural conditions which may detrimentally affect its ability to perform its intended function. The agent may be administered alone, or in conjunction with a pharmaceutically acceptable carrier. Further the agent may be coadministered with a pharmaceutically acceptable carrier. The agent also may be administered as a prodrug, which is converted to its active form *in vivo*.

The term "effective amount" of an agent that induces non-phosphorylatable PPAR gamma expression and/or activity is that amount necessary or sufficient to promote non-phosphorylatable PPAR gamma expression and/or activity in the subject or population of

subjects. The effective amount can vary depending on such factors as the type of therapeutic agent(s) employed, the size of the subject, or the severity of the disorder.

It will be appreciated that individual dosages may be varied depending upon the requirements of the subject in the judgment of the attending clinician, the severity of the condition being treated and the particular compound being employed. In determining the therapeutically effective amount or dose, a number of additional factors may be considered by the attending clinician, including, but not limited to: the pharmacodynamic characteristics of the particular agent and its mode and route of administration; the desired time course of treatment; the species of mammal; its size, age, and general health; the specific disease involved; the degree of or involvement or the severity of the disease; the response of the individual subject; the particular compound administered; the mode of administration; the bioavailability characteristics of the preparation administered; the dose regimen selected; the kind of concurrent treatment; and other relevant circumstances.

Treatment can be initiated with smaller dosages which are less than the effective dose of the compound. Thereafter, in one embodiment, the dosage should be increased by small increments until the optimum effect under the circumstances is reached. For convenience, the total daily dosage may be divided and administered in portions during the day if desired.

The effectiveness of any particular agent to treat metabolic disorders can be monitored by comparing two or more samples obtained from a subject undergoing anti-metabolic disorder treatment. In general, a first sample is obtained from the subject prior to beginning therapy and one or more samples during treatment. In such a use, a baseline of expression of cells from subjects with metabolic disorders prior to therapy is determined and then changes in the baseline state of expression of cells from subjects with metabolic disorders is monitored during the course of therapy. Alternatively, two or more successive samples obtained during treatment can be used without the need of a pre-treatment baseline sample. In such a use, the first sample obtained from the subject is used as a baseline for determining whether the expression of cells from subjects with metabolic disorders is increasing or decreasing.

Another aspect of the invention relates to a method for identifying a compound or agent (*e.g.*, peptides, peptidomimetics, small molecules or other drugs) capable of inducing the expression and/or activity of non-phosphorylatable PPAR gamma.

V. Pharmaceutical Compositions

In another aspect, the present invention, at least in part, provides pharmaceutically acceptable compositions which comprise a therapeutically-effective amount of an agent that selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. As described in detail below, the pharmaceutical compositions of the present invention, at least in part, may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; (4) intravaginally or intrarectally, for example, as a pessary, cream or foam; or (5) aerosol, for example, as an aqueous aerosol, liposomal preparation or solid particles containing the compound.

The phrase "therapeutically-effective amount" as used herein means that amount of an agent that selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation, which is effective for producing some desired therapeutic effect, *e.g.*, weight loss, at a reasonable benefit/risk ratio.

The phrase "pharmaceutically acceptable" is employed herein to refer to those agents, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject chemical from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl

cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

The term "pharmaceutically-acceptable salts" refers to the relatively non-toxic, inorganic and organic acid addition salts of the agents that induce non-phosphorylatable PPAR gamma expression and/or activity encompassed by the invention. These salts can be prepared in situ during the final isolation and purification of the agents, or by separately reacting a purified agents agent in its free base form with a suitable organic or inorganic acid, and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, phosphate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, napthylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate salts and the like (See, for example, Berge *et al.* (1977) "Pharmaceutical Salts", *J. Pharm. Sci.* 66:1-19).

In other cases, the agents useful in the methods of the present invention, at least in part, may contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable bases. The term "pharmaceutically-acceptable salts" in these instances refers to the relatively non-toxic, inorganic and organic base addition salts of agents that induce non-phosphorylatable PPAR gamma expression and/or activity. These salts can likewise be prepared in situ during the final isolation and purification of the agents, or by separately reacting the purified agents agent in its free acid form with a suitable base, such as the hydroxide, carbonate or bicarbonate of a pharmaceutically-acceptable metal cation, with ammonia, or with a pharmaceutically-acceptable organic primary, secondary or tertiary amine. Representative alkali or alkaline earth salts include the lithium, sodium, potassium, calcium, magnesium, and aluminum salts and the like. Representative organic amines useful for the formation of

base addition salts include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like (see, for example, Berge *et al.*, *supra*).

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations useful in the methods of the present invention, at least in part, include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal, aerosol and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient, which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 1 per cent to about ninety-nine percent of active ingredient, for example, from about 5 per cent to about 70 per cent, from about 10 per cent to about 30 per cent.

Methods of preparing these formulations or compositions include the step of bringing into association an agent that induces non-phosphorylatable PPAR gamma expression and/or activity with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a agents agent with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Particularly advantageous formulations contemplated for the compounds and compositions of the present invention, at least in part, include oral formulations.

Formulations suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a agents agent as an active ingredient. A compound may also be administered as a bolus, electuary or paste.

In solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically-acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, acetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered peptide or peptidomimetic moistened with an inert liquid diluent.

Tablets, and other solid dosage forms, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and

other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions, which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions, which can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active agent may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Formulations for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more agents with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active agent.

Formulations which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

Dosage forms for the topical or transdermal administration of an agent that induces non-phosphorylatable PPAR gamma expression and/or activity include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active component may be mixed under sterile conditions with a pharmaceutically-acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to a agents agent, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to an agent that induces non-phosphorylatable PPAR gamma expression and/or activity, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

The agent that induces non-phosphorylatable PPAR gamma expression and/or activity can be alternatively administered by aerosol. This is accomplished by preparing an aqueous aerosol, liposomal preparation or solid particles containing the compound. A nonaqueous (*e.g.*, fluorocarbon propellant) suspension could be used. Sonic nebulizers minimize exposing the agent to shear, which can result in degradation of the compound.

Ordinarily, an aqueous aerosol is made by formulating an aqueous solution or suspension of the agent together with conventional pharmaceutically acceptable carriers and stabilizers. The carriers and stabilizers vary with the requirements of the particular compound, but typically include nonionic surfactants (Tweens, Pluronics, or polyethylene glycol), innocuous proteins like serum albumin, sorbitan esters, oleic acid, lecithin, amino acids such as glycine, buffers, salts, sugars or sugar alcohols. Aerosols generally are prepared from isotonic solutions.

Transdermal patches have the added advantage of providing controlled delivery of a agents agent to the body. Such dosage forms can be made by dissolving or dispersing the agent in the proper medium. Absorption enhancers can also be used to increase the flux of

the peptidomimetic across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the peptidomimetic in a polymer matrix or gel.

Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention.

Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more agents in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution, which, in turn, may depend upon crystal size and crystalline form.

Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of an agent that induces non-phosphorylatable PPAR gamma expression and/or activity in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions, which are compatible with body tissue.

When the agents of the present invention, at least in part, are administered as pharmaceuticals, to humans and animals, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (*e.g.*, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be determined by the methods of the present invention, at least in part, so as to obtain an amount of the active ingredient, which is effective to achieve the desired therapeutic response for a particular subject, composition, and mode of administration, without being toxic to the subject.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Pat. No. 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.* (1994) Proc. Natl. Acad. Sci. USA 91:3054 3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

Table 1: Increased Gene Expression Levels, Activities and/or Assays

Genes

Gene Symbol	Gene Name	GenBank Gene Accession Number	GenBank Protein Accession Number	Gene ID
adipsin	complement factor D	*e.g.*, NM_013459.2 and NM_001928.2	*e.g.*, NP_038487.1 and NP_001919.2	*e.g.*, 11537 and 1675

fatty acid transporter cd36	fatty acid transporter/cd36	*e.g.*, NM_007643.3 and NM_000072.3 and NM_001001547.2 and NM_001001548.2 and NM_001127443.1 and NM_001127444.1	*e.g.*, NP_031669.2 and NP_000063.2 and NP_001001547.1 and NP_001001548.1 and NP_001120915.1 and NP_001120916.1	*e.g.*, 12491 and 948
adiponectin	adiponectin	*e.g.*, NM_009605.4 and NM_004797.2	*e.g.*, NP_0033735.3 and NP_004788.1	*e.g.*, 11450 and 9370
UCP-1	uncoupling protein 1	*e.g.*, NM_009463.3 and NM_021833.4	*e.g.*, NP_033489.1 and NP_068605.1	*e.g.*, 22227 and 7350
cidea	cell death-inducing DFFA-like effector a	*e.g.*, NM_007702.2 and NM_001279.3 and NM_198289.2	*e.g.*, NP_031728.1 and NP_001270.1 and NP_938031.1	*e.g.*, 12683 and 1149
PGC1a	Peroxisome porliferative activated receptor, gamma, coactivator 1 alpha	*e.g.*, NM_008904.2 and NM_013261.3	*e.g.*, NP_032930.1 and NP_037393.1	*e.g.*, 19017 and 10891
Elovl3	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	*e.g.*, NM_007703.2 and NM_152310.1	*e.g.*, NP_031729.1 and NP_689523.1	*e.g.*, 12686 and 83401
C/EBPbeta	CCAAT/enhancer binding protein beta	*e.g.*, NM_009883.3 and NM_005194.2	*e.g.*, NP_034013.1 and NP_005185.2	*e.g.*, 12608 and 1051
Cox7a1	cyotchrome c oxidase subunit VIIa polypeptide 1	*e.g.*, NM_009944.3 and NM_001864.2	*e.g.*, NP_034074.1 and NP_001855.1	*e.g.*, 12865 and 1346
Otopetrin	Otopetrin 1	*e.g.*, NM_172709.3 and NM_177998.1	*e.g.*, NP_766297.2 and NP_819056.1	*e.g.*, 21906 and 133060
Type II deiodinase	Deiodinase, iodothyronine, type II	*e.g.*, NM_010050.2 and NM_000793.4 and NM_001007023.2 and NM_013989.3	*e.g.*, NP_034180.1 and NP_000784.2 and NP_001007024.1 and NP_054644.1	*e.g.*, 13371 and 1734
cytochrome C	cytochrome c	*e.g.*, NM_009989.2 and NM_018947.4	*e.g.*, NP_034119.1 and NP_061820.1	*e.g.*, 13067 and 54205
cox4i1	cytochrome c oxidase subunit IV isoform 1	*e.g.*, NM_009941.2 and NM_001861.2	*e.g.*, NP_034071.1 and NP_001852.1	*e.g.*, 12857 and 1327
coxIII	mitochondrially encoded cytochrome c oxidase III	*e.g.*, NC_005089.1 and ENST00000362079	*e.g.*, NP_904334.1 and ENSP00000354982	*e.g.*, 17705 and 4514
cox5b	cytochrome c oxidase subunit Vb	*e.g.*, NM_009942.2 and NM_001862.2	*e.g.*, NP_034072.2 and NP_001853.2	*e.g.*, 12859and 1329
cox8b	cytochrome c oxidase subunit 8B, mitochondrial precursor	*e.g.*, NM_007751.3	*e.g.*, NP_031777.1	*e.g.*, 12869 and 404544
glut4	solute carrier family 2 (facilitated glucose transporter), member 4	*e.g.*, NM_009204.2 and NM_001042.2	*e.g.*, NP_033230.2 and NP_001033.1	*e.g.*, 20528 and 6517

atpase b2	ATPase, H+ transportying, lysosomal 56/58kDa, V1 subunit B2	*e.g.*, NM_057213.2 and NM_001693.3	*e.g.*, NP_476561.1 and NP_001684.2	*e.g.*, 117596 and 526
coxII	mitochondrially encoded cytochrome c oxidase II	*e.g.*, NC_005089.1 and ENST00000361739	*e.g.*, NP_904331 and ENSP00000354876	*e.g.*, 17709 and 4513
atp5o	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	*e.g.*, NM_138597.2 and NM_001697.2	*e.g.*, NP_613063.1 and NP_001688.1	*e.g.*, 28080 and 539
ndufb5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16kDa	*e.g.*, NM_025316.2 and NM_002492.2	*e.g.*, NP_079592.2 and NP_002483.1	*e.g.*, 66046 and 4711
Rarres2	retinoic acid receptor responder (tazarotene induced) 2	*e.g.*, NM_027852.2 and NM_002889.3	*e.g.*, NP_082128.1 and NP_002880.1	*e.g.*, 71660 and 5919
Car3	carbonic anhydrase 3	*e.g.*, NM_007606.3 and NM_005181.3	*e.g.*, NP_031632.2 and NP_005172.1	*e.g.*, 12350 and 761
Peg10	paternally expressed 10	*e.g.*, NM_001040611.1 and NM_001040152.1 and NM_001172437.1 and NM_001172438.1 and NM_015068.3	*e.g.*, NP_001035701.1 and NP_001035242.1 and NP_001165908.1 and NP_001165909.1 and NP_055883.2	*e.g.*, 170676 and 23089
Cidec	Cidec cell death-inducing DFFA-like effector c	*e.g.*, NM_178373.3 and NM_022094.2	*e.g.*, NP_848460.1 and NP_071377.2	*e.g.*, 14311 and 63924
Cd24a	CD24a antigen	*e.g.*, NM_009846.2 and NM_013230.2	*e.g.*, NP_033976.1 and NP_037362.1	*e.g.*, 12484 and 100133941
Nr1d2	nuclear receptor subfamily 1, group D, member 2	*e.g.*, NM_011584.4 and NM_001145425.1 and NM_005126.4	*e.g.*, NP_035714.3 and NP_001138897.1 and NP_005117.3	*e.g.*, 353187 and 9975
Ddx17	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	*e.g.*, NM_001040187.1 and NM_001098504.1 and NM_001098505.1 and NM_006386.4 and NM_030881.3	*e.g.*, NP_001035277.1 and NP_001091974.1 and NP_001091975.1 and NP_006377.2 and NP_112020.1	*e.g.*, 67040 and 10521
Aplp2	amyloid beta (A4) precursor-like protein 2	*e.g.*, NM_001102455.1 and NM_001142276.1 and NM_001142277.1 and NM_001142278.1 and NM_001642.2	*e.g.*, NP_001095925.1 and NP_001135748.1 and NP_001135749.1 and NP_001135750.1 and NP_001633.1	*e.g.*, 11804 and 334
Nr3c1	nuclear receptor subfamily 3, group C, member 1	*e.g.*, NM_008173.3 and NM_000176.2 and NM_001018074.1 and NM_001018075.1 and NM_001018076.1 and NM_001018077.1 and NM_001020825.1 and NM_001024094.1	*e.g.*, NP_032199.3 and NP_000167.1 and NP_001018084.1 and NP_001018085.1 and NP_001018086.1 and NP_001018087.1 and NP_001018661.1 and NP_001019265.1	*e.g.*, 14815 and 2908
Rybp	RING1 and YY1 binding protein	*e.g.*, NM_019743.3 and NM_012234.4	*e.g.*, NP_062717.2 and NP_036366.3	*e.g.*, 56353 and 23429
Txnip	thioredoxin interacting protein	*e.g.*, NM_001009935.2 and NM_006472.3	*e.g.*, NP_001009935.1 and NP_006463.3	*e.g.*, 56338 and 10628

Assays

improved stabilization of Helix 3
stabilization of beta sheet
Energy expenditure *in vivo* (including increase O2 consumption and heat production in combination with no change in food intake)
Mitochondrial biogenesis
Uncoupled oxygen consumption
Induction of brown fat program in white fat cells, including histological analysis
Fatty acid uptake into cells (monocytes, adipocytes)

Table 2: Decreased Gene Expression Levels, Activities, and/or Assays

Genes

Gene Symbol	Gene Name	GenBank Gene Accession Number	Gen Bank Protein Accession Number	GeneID
Phosphorylated PPAR gamma at Ser-273 or corresponding serine in a homolog	peroxisome proliferator activated receptor gamma	*e.g.*, NM_011146.3 and NM_015869.4	*e.g.*, NP_035276.2 and NP_056953.2	*e.g.*, 19016 and 5468
aP2	adipocyte fatty-acid-binding protein 4	*e.g.*, NM_024406.1 and NM_001442.2	*e.g.*, NP_077717.1 and NP_001433.1	*e.g.*, 11770 and 2167
C/EBPalpha	CCAAT/enhancer binding protein alpha	*e.g.*, NM_007678.3 and NM_004364.3	*e.g.*, NP_031704.2 and NP_004355.2	*e.g.*, 12606 and 1050
add1	adipocyte determination and differentiation factor 1	*e.g.*, NM_011480.3 and NM_001005291.2 and NM_004176.4	*e.g.*, NP_035610.1 and NP_00100529.1 and NP_004167.3	*e.g.*, 20787and 6720
fatty acid synthase (FAS)	fatty acid synthase	*e.g.*, NM_007988.3 and NM_004104.4	*e.g.*, NP_032014.3 and NP_004095.4	*e.g.*, 14104 and 2194
leptin	leptin	*e.g.*, NM_008493.3 and NM_000230.2	*e.g.*, NP_032519.1 and NP_000221.1	*e.g.*, 16846 and 3952
LPL	lipoprotein lipase	*e.g.*, NM_008509.2 and NM_000237.2	*e.g.*, NP_032535.2 and NP_000228.1	*e.g.*, 16956 and 4023
ACC1	acetyl-coenzyme A carboxylase 1	*e.g.*, NM_133360.2 and NM_198834.1 and NM_198836.1 and NM_198837.1 and NM_198838.1 and NM_198839.1	*e.g.*, NP_579938.2 and NP_942131.1 and NP_942133.1 and NP_942134.1 and NP_942135.1 and NP_942136.1	*e.g.*, 107476 and 31

Cyp2f2	cytochrome P450, family 2, subfamily f, polypeptide 2	*e.g.*, NM_007817.2 and NM_000774.3	*e.g.*, NP_031843.2 and NP_000765.2	*e.g.*, 13107 and 1572
Selenbp1	selenium binding protein 1	*e.g.*, NM_009150.3 and NM_003944.2	*e.g.*, NP_033176.2 and NP_003935.2	*e.g.*, 20341 and 8991
Acyl	acyl-Coenzyme A dehydrogenase, very long chain	*e.g.*, NM_017366.2 and NM_000018.2 and NM_001033859.1	*e.g.*, NP_059062.1 and NP_000009.1 and NP_001029031.1	*e.g.*, 11370 and 37
Nr1d1	nuclear receptor subfamily 1, group D, member 1	*e.g.*, NM_145434.3 and NM_021724.2	*e.g.*, NP_663409.2 and NP_068370.1	*e.g.*, 217166 and 9572

Assays

Glucose / insulin tolerance tests
Fasting glucose
White fat depots including fat mass and lean mass assayed by DEXA scan
Serum adipokine levels
Serum fatty acid levels
adipogenesis
Phosphorylation of Ser-273-PPAR gamma (assayed by Western blotting, Immunoprecipitation followed by western blotting, ELISA, NMR, MALDI-TOF mass spectorometry, LC-MS/MS)
insulin sensitivity in fat cells (insulin-dependent glucose uptake)
transcriptional (reporter gene) assays on PPARgamma responsive promoters
transactivation assay of PPAR gamma (GAL4-PPARgamma) on UAS promoter
derivation of cell lines from PPAR S273A knock-in mouse
use of existing PPARgamma null-cell lines stably expressing wild-type and mutant
Suppression of obesity-induced inflammation (TNF-alpha secretion, gene expression of inflammation genes such as MCP1)

Table 3: Primer Sequences

Gene	Forward primer	Reverse primer
aP2	AAGGTGAAGAGCATCATAACCCT	TCACGCCTTTCATAACACATTCC
C/EBPα	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAACTCCAGCAC
LPL	GGGAGTTTGGCTCCAGAGTTT	TGTGTCTTCAGGGGTCCTTAG
Fasn	GCTGGCATTCGTGATGGAGTCGT	AGGCCACCAGTGATGATGTAACTCT
CD36	AAGCTATTGCGACATGATT	GATCCGAACACAGCGTAGAT
Glut4	GTGACTGGAACACTGGTCCTA	CCAGCCACGTTGCATTGTAG
Adiponectin	TGTTCCTCTTAATCCTGCCCA	CCAACCTGCACAAGTTCCCTT
Adipsin	CATGCTCGGCCCTACATGG	CACAGAGTCGTCATCCGTCAC
Resistin	AAGAACCTTTCATTTCCCCTCCT	GTCCAGCAATTTAAGCCAATGTT
Angiotensinogen	TCTCCTTTACCACAACAAGAGCA	CTTCTCATTCACAGGGGAGGT
TNF-α	CCCTCACACTCAGATGATCTTCT	GCTACGACGTGGGCTACAG
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
PAI-1	TTCAGCCCTTGCTTGCCTC	ACACTTTTACTCCGAAGTCGGT
Leptin	GAGACCCCTGTGTCCGGTTC	CTGCGTGTGTGAAATGTCATTG
PPARγ	GCATGGTGCCTTCGCTGA	TGGCATCTCTGTGTCAACCATG
TBP	ACCCTTCACCAATGACTCCTATG	TGACTGCAGCAAATCGCTTGG
Cyp2f2	GTCGGTGTTCACGGTGTACC	AAAGTTCCGCAGGATTTGGAC
Ranes2	GCCTGGCCTGCATTAAAATGG	CTTGCTTCAGAATTGGGCAGT
Selenbp1	ATGGCTACAAAATGCACAAAGTG	CCTGTGTTCCGGTAAATGCAG
Car3	TGACAGGTCTATGCTGAGGGG	CAGCGTATTTTACTCCGTCCAC
Pygl0	TGCTTGCACAGAGCTACAGTC	AGTTTGGGATAGGGGCTGCT
Cldn	ATGGACTACGCCATGAAGTCT	CGGTGCTAACACGACAGGG
Col4a	GTTGCACCGTTTCCCGGTAA	CCCCTCTGGTGGTAGCGTTA
Acyl	CAGCCAAGGCAATTTCAGAGC	CTCGACGTTTGATTAACTGGTCT
Nr1d2	TGAACGCAGGAGGTGTGATTG	GAGGACTGGAAGCTATTCTCAGA
Dax17	TCTTCAGCCAACAATCCCAATC	GGCTCTATCGGTTTCACTACG
Aqp2	GTGGTGGAAGACCGTGACTAC	TCGGGGGAACTTTAACATCGT
Nr3c1	AGCTCCCCCTGGTAGAGAC	GGTGAAGACGCAGAAACCTTG
Rybp	CGACCAGGCCAAAAAGACAAG	CACATCGCAGATGCTGCATT
Tcap	TCTTTTGAGGTGGTCTTCAACG	GCTTTGACTCGGGTAACTTCACA
Nr1d1	TACATTGGCTCTAGTGGCTCC	CAGTAGGTGATGGTGGGAAGTA

Exemplification

This invention is further illustrated by the following examples, which should not be construed as limiting.

Example 1: Materials And Methods For Examples 1-10

a. Cell Culture

3T3-L1 and HEK-293 cells were obtained from ATCC. PPAR gamma-null mouse embryonic fibroblasts (MEFs) (Reaven *et al., Diabetes* 37: 1020-1024 (1988)) were cultured in Dulbecco's modified Eagle's medium and 10% fetal bovine serum. FLAG-PPAR gamma and FLAG-PPAR gamma S273A were subcloned into pMSCV-puro restroviral vector (Stratagene). For retrovirus production, Phoenix packaging cells were transfected with 10 μg retroviral vectors (Kinsella *et al.*, *Hum. Gene Ther.* 7: 1405-1413 (1996)). After 48 hours, the viral supernatant was collected and filtered. Following infection of the cells with the retrovirus, cells expressing the ectopic protein were selected by incubation with 2 μg/ml puromycin. Adipocyte differentiationon 3T3-L1 or MEFs was induced by treating cells with 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 850 mM insulin for 48h and cells were switched to the maintenance medium containing 850 nM insulin. Lipid accumulation in the cells was detected by Oil Red O staining. The amount of secreted adiponectin into cell medium was analyzed by ELISA (Millipore). All chemicals for cell culture were obtained from Sigma unless otherwise indicated.

b. DNA constructs and shRNA of CDK5

HA-WT CDK5, HA-KD CDK5 and Myc-p35 were obtained from Addgene. Murine PPARα or PPARδ were subcloned into FLAG-pcDNA3.1 (Invitrogen). The sequence used for lentivial shRNA expression vector (pLKO.1; Open Biosystems) targeting CDK5 was 5'-TGTAAGAGAATAAAGCGTGAA-3'. For lentivirus production, HEK-293T cells (ATCC) were transfected with 10 μg lentiviral vectors (Kinsella *et al.*, *Hum. Gene Ther.* 7: 1405-1413 (1996)). Following infection of the cells with the lentivirus, cells were selected by incubation with 2 μg/ml puromycin.

c. *In vitro* kinase assay

Kinase assays were performed using an active cdk5/p35 protein and recombinant PPAR gamma protein as a substrate in kinase buffer in the presence of ATP. The reaction was stopped by adding sample buffer. Samples were then subjected to SDS-PAGE, and

detected by CDK substrate antibody. In particular, active cdk5/p35, cdk1/cdc2, cdk2/cyclin A, cdk2/cyclin E or cdk4/cyclin D1 kinases were purchased from Millipore or Cell Signaling Technology. *In vitro* CDK kinase assay was performed according to the manufacturer's instructions (Cell Signaling Technology). Briefly, 1 μg of immuno-purified WT or S273A mutant of PPAR gamma were incubated with active CDK kinase in kinase assay buffer (25 mM Tris-HCl pH 7.5, 5 mM beta-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) containing 20 μM ATP for 15 min at 30°C. Positive control for assay, purified histone H1 (Millipore) or Rb (Cell Signaling Technology) were used. Several PPAR gamma ligands were pre-incubated with substrates for 30 min, and the assay was performed. Phosphorylation of substrates after SDS–PAGE was analyzed with anti-CDK substrate antibody (Cell Signaling Technology).

d. Preparation of cell or tissue lysates, immunoprecipitation and immunoblotting

HEK-293 cells expressing CDK5 or PPAR gamma were collected after transfection. Total cell lysates were incubated with FLAG M2 agarose (Sigma) at 4°C. Immunoprecipitates or total cell lysates were analyzed with anti-CDK substrate, FLAG or HA (Roche) antibodies. Differentiated 3T3-L1 adipocytes were treated with TNF-α (50 ng/ml), IL-6 (50 ng/ml), IL-1β (50 ng/ml) or FFAs (400 μM palmitic acid and oleic acid mixtures) for the indicated times, and cell lysates were analyzed with phospho-specific or PPAR gamma antibodies. 3T3-L1 adipocytes were pre-treated with various PPAR gamma ligands, and incubated with TNF-α. For tissue lysates, WAT from mice was homogenized in RIPA buffer (50 mM Tris pH7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS with protease and phosphatase inhibitors). For western blotting, a rabbit polyclonal phospho-specific antibody against PPAR gamma Ser273 was produced by New England Peptides with a synthetic phosphopeptide corresponding to residues surrounding Ser273 of PPART gamma (Ac-KTTDKpSPFVIYDC-amide). Total tissue lysates were analyzed with anti-PPAR gamma, phospho-CDK5 (Y15), CDK5 and p35 antibodies (Santa Cruz). Phosphorylation of PPAR gamma on Ser112 or Ser273 was detected by phospho-specific antibodies (Ser112, Millipore, MAB3632).

e. Reporter gene assay

In some examples, HEK-293 cells were seeded in 12-well plates at 1.5×10^5 cells/well. HEK-293 cells were transfected with pDR-1 (3X, based on the UCP1 PPAR gamma recognition site)-luciferase reporter plasmid, PPAR gamma, RXRa and pRL-

renillin using Lipofectamine 2000 (Invitrogen). Following an overnight transfection, the cells were treated with rosiglitazone or MRL24 for 24 hours. The cells were harvested and reporter gene assays were carried out using the Dual-Luciferase kit (Promega). Luciferase activity was normalized to renillia activity.

f. Gene expression analysis

Total RNA was isolated from cells or tissues using Trizol reagents (Invitrogen). The RNA was reverse-transcribed using ABI reverse transcipton kit. Quantitative PCR reactions were performed with SYBR green fluorescent dye using an ABI9300 PCR machine. Relative mRNA expression was determiend by the ΔΔ-Ct method normalized to TATA-binding protein (TBP) levels. The sequences of primers used in this study are found in Table 3.

g. Generation of fat pads in nude mice

PPAR gamma-null fibroblasts (1×10^7) stably expressing WT or S273A mutant of PPAR gamma were implanted subcutaneously into 7-8 week-old male NCR nude mice (Taconic) according to the previous methods (n=5 mice per group) (Walkey *et al.*, *J. Biol. Chem.* 283: 24290-24294 (2008)). Six weeks after injection, the fat pads were isolated for the analysis of gene expression.

h. Microarray analysis

Total RNA was isolated from PPAR gamma-null fibroblasts expressing WT or S273A mutant of PPAR gamma or WT cells treated with 1μM rosiglitazone or MRL24 for 24 hours. Array hybridization and scanning were performed by the Dana-Farber Cancer Institute Microarray Core Facility using Affymetrix GeneChip Mouse Genome 430 2.0 arrays according to established methods (Lockhart *et al., Nat. Biotechnol.* 14: 1675-1680 (1996)). The array data were analyzed using the DNA-Chip Analyzer (dChip) sofware (Li *et al.*, *Proc. Natl. Acad. Sci. U S A* 98: 31-36 (2001)) The statistical significance of differences in gene expression was assessed by an unpaired *t*-test ($p<0.05$). To create refined gene sets regulated by cdk5 phopshorylation of PPAR gamma, *p*-value as well as fold-change of gene expression in WT versus S273A mutant cells was first calculated, and -log *p*-value versus $\log_2$ fold-change was plotted. From this list of genes, 53 genes were selected which were changed in magnitude (4 fold difference) and statistical significance

($p<0.05$). The selected genes were validated in cells or transplanted fat pads by qPCR, the resulting gene set, consisting of 17 genes, was analyzed in WAT of mice using qPCR.

i. Animals

All animal experiments were performed according to procedures approved by Beth Israel Deaconess Medical Center's Institutional Animal Care and Use Committee. 4 to 5-weekold male C57BL/6J mice were obtained from the Jackson Laboratory. Mice were fed a regular diet (10% kcal fat, D12450B, Research Diets Inc.) or a high fat diet (60% kcal fat, D12492, Research Diets Inc.) as indicated time periods. For glucose tolerant tests, mice were intraperitoneally (i.p.) injected daily 10 mg/kg rosiglitazone or MRL24 for 6 days, and fasted overnight prior to i.p. injection of 2 g/kg D-glucose. Glucose was measured in tail vain blood at intervals after glucose injection using a Truetrack glucometer. Serum insulin concentrations were determined by ELISA (Crystal Chem).

Example 2: cdk5 Specifically Phosphorylates Serine 273 Of Mouse PPAR Gamma

Pro-inflammatory cytokines are secreted from both fat cells and immune cells residing in adipose tissue, specifically when animals or humans become obese (Hotamisligil *et al.*, *Science* 259: 87-91 (1993)). Since PPAR gamma is a dominant regulator of adipogenesis and gene expression in fat cells, the structure of the molecule was analyzed and it was determined that the primary amino acid sequence of PPAR gamma contained a consensus site for phosphorylation by the protein kinase cdk5 at serine 273 of PPARγ2 (Figure 1A). This protein kinase, despite being a member of the cdk gene family, is not regulated by cyclins and is instead activated by p35/25 which are targets of numerous cytokines and pro-inflammatory signals (Dhavan *et al.*, *Nat. Rev. Mol. Cell. Biol.* 2: 749-759 (2001)). This cdk5 site is conserved in all sequenced mammalian PPAR gammas, but is not found in other members of the PPAR gene family. The cdk5 site in murine PPAR gamma can be phosphorylated when incubated *in vitro* with cdk5 and its activating cofactor p35. In fact, PPAR gamma is phosphorylated as efficiently under these conditions as is histone H1, a known substrate of the cdk5/p35 complex (Figure 1B). Mutation of serine 273 to alanine completely blocks phosphorylation of PPAR gamma, indicating that there were no other cryptic sites for this protein kinase on PPAR gamma (Figure 1B). Other

members of the cdk protein family did not phosphorylate PPAR gamma. Cdk5 also phosphorylated PPAR gamma in cells, as shown by co-transfection of this kinase with the wild-type (WT) and mutant PPAR gamma (Figure 1C). This phosphorylation in cells was detected with an antibody against a peptide phosphorylated at a consensus cdk5 site. A version of cdk5 with a mutation inactivating the kinase activity (KD) did not modify PPAR gamma. Moreover, these results were independent of phosphorylation of serine 112. Finally, cdk5 did not modify murine PPAR alpha or PPAR delta in cells (Figure 1D).

Example 3: Phosphorylation Of PPAR Gamma At Ser 273 Regulates Adipogenesis

Obesity is characterized by elevated circulating levels of pro-inflammatory cytokines and free fatty acids. As noted above, cdk5 is known to be activated by cytokines (*e.g.*, TNF-alpha) (Dhavan *et al.*, *Nat. Rev. Mol. Cell. Biol.* 2: 749-759 (2001); Utreras. *et al., J. Biol. Chem.* 284: 2275-2284 (2009)). Figure 2A shows that treatment of 3T3-L1 adipocytes with TNF-α or IL-6 causes phosphorylation of PPAR gamma at the cdk5 site, as shown by a specific antibody made against a phosphopeptide derived from the cdk5 site at serine 273. While there have been no reports of activation of cdk5 by free fatty acids (FFAs), these are known to be elevated in obesity (Reaven *et al., Diabetes* 37: 1020-1024 (1988)). Figure 2A also shows that the phosphorylation of PPAR gamma at serine 273 occurs upon treatment of the fat cells with high levels of FFAs. The cytokine and FFA induced phosphorylations are indeed occurring through cdk5 and not a different kinase as shown in Figures 2B and 2C, where these modifications are ablated by treatment of cells with an shRNA which is directed against murine cdk5.

A study was performed to determine how the cdk5 modification of PPAR gamma altered the ability of this receptor to affect adipogenesis, and gene expression within differentiated fat cells. The wild type and S273A mutant alleles of PPAR gamma were expressed at equal RNA and protein levels in fibroblasts that completely lack PPAR gamma (Figures 3A and 3B; Rosen *et al., Genes & Dev.* 16: 22-26 (2002)). These initial experiments took advantage of the considerable basal level of cdk5-mediated phosphorylation in cultured cells (visible in the exposure shown in Figure 2B), without addition of any other ligands. Serum has been shown to activate cdk5 in cultured cells (Musa *et al., J. Androl.* 21: 392-402 (2000)). Treatment of cells with cytokines and FFAs, which induce a more robust activation of cdk5, leads to a dedifferentiation of adipocytes, as has been shown earlier (Torti. *et al., Science* 229: 867-869 (1985)), and so could not be

used in these experiments. The mutant and wild type PPAR gamma alleles both drove PPAR gamma transcriptional activity and adipogenesis with equal efficiency (Figures 3A and 3B). While most classical fat cell genes, like aP2 and C/EBPα were expressed to exactly equal levels, certain genes were sensitive to mutation in the PPAR gamma cdk5 site (Figure 3C). These include the key fatty acid transporter, cd36, and the adipokines, adiponectin, adipsin and leptin. Mutation of the cdk5 site also caused an increase in the secretion of adiponectin into the culture medium (Figure 3D).

To examine the ability of this cdk5-mediated phosphorylation to alter fat cell gene expression in an *in vivo* context, wild type and mutant cells were transplanted under the skin of nude mice (Figure 8A; Green *et al.*, *J. Cell. Physiol.* 101: 169-171 (1979)). After 6 weeks, both cell types formed fat pads that could be isolated and analyzed for gene expression. Again, adiponectin and adipsin were both markedly dysregulated, being elevated in the cells expressing mutant vs. wt PPAR gamma (Figure 10B)._ This implies that the cdk5-mediated phophorylation of PPAR gamma reduced the expression of both of these adipokines. While both cd36 and leptin were expressed at slightly higher levels in the mutant vs. wt cells, this did not reach statistical significance in these transplant experiments.

Example 4: Phosphorylation Of PPAR Gamma At Ser 273 Occurs In Obesity

It is notable that several genes whose expression is dysregulated by the cdk5 mutation in PPAR gamma , such as adiponectin, adipsin and leptin, are known to be inappropriately regulated in many forms of animal and human obesity. In fact, the reduced levels of adiponectin in obesity has been shown to be at least partially responsible for the insulin-resistance that usually accompanies obesity (Hu *et al.*, *J. Biol. Chem.* 271: 10697-10703 (1996)). This fact, along with the activation of cdk5 by cytokines and high fatty acid levels, directed experiments to test whether cdk5 is activated in the adipose tissues of obese mice and whether PPAR gamma is phosphorylated by cdk5 specifically in the context of obesity. Mice were placed on a standard chow or a high fat, high sugar diet containing 60% kcal from fat, and adipose tissues were harvested at several points after initiation of this protocol. Mice developed a trend toward elevated insulin, indicative of insulin resistance, at 7 weeks. Overt hyperinsulinemia, was apparent at 13 weeks after initiation of this diet (Figure 4B). As shown in Figure 4A, there was no activation of cdk5 (detectable by a specific phophorylation at tyrosine 15 of murine cdk5) after 3 weeks of this high fate diet.

However, activated cdk5 was easily observed after 7 weeks of the high fat diet. This time point also showed increased appearance of the cleaved p25 protein, the more stable form of this activating subunit for cdk4 (Figure 4A; Dhavan *et al.*, *Nat. Rev. Mol. Cell. Biol.* 2: 749-759 (2001)). There was a detectable basal level of phosphorylation on serine 273 in chow fed animals, and there was no increase in this modification after 3 weeks on the high fat diet. However, by 7 weeks on this diet, epidydimal fat tissues showed a clear increase in this phosphorylation of PPAR gamma compared to chow fed controls. This difference in phosphorylation between chow and high fat-fed animals was obvious after 13 weeks on this diet. Cdk5-mediated modification was also observed in two different white fat depots: inguinal fat, which is a type of subcutaneous fat, and epididymal fat, which is considered a visceral depot (Cinti, S., *Prostaglandins Leukot Essent Fatty Acids* 73: 9-15 (2005)). Figure 4C illustrates that this increase in phosphorylation by cdk5 occurs in both white fat depots, with greater intensity of phosphorylation at S273 in the epididymal fat.

Example 5: PPAR Gamma Ligands Can Inhibit Phosphorylation Of PPAR Gamma At Ser 273

Anti-diabetic drugs of the thiazolidinedione (TZD) class, such as rosiglitazone, are known to bind to and activate PPAR gamma, improving insulin sensitivity of mice and humans (Willson *et al.*, *Annu. Rev. Biochem.* 70: 341-367 (2001)). To ask whether TZDs and other anti-diabetic PPAR gamma ligands alter the cdk5-mediated phosphorylation of this receptor, fat cells expressing wild type PPAR gamma were treated with TNF alpha, rosiglitazone, or a combination of the two agents. Figure 5A shows that rosiglitazone inhibited this modification at approximately 1 μM, similar to the dose required for other PPAR gamma mediated activities in adipose cells (Lehmann *et al., J. Biol. Chem.* 270: 12953-12956 (1995)). GW 9662, a PPAR gamma antagonist (Leesnitzer. *et al., Biochemistry* 41: 6640-6650 (2002)), completely blocked this effect of rosiglitazone. The effect of these compounds on the cdk5-mediated phosphorylation of a mutant form of PPAR gamma (Q286P; Sarraf *et al. Mol. Cell* 3:799-804 (1999)), a naturally occurring mutant of the receptor that can no longer directly bind any known ligands was also examined. Rosiglitazone could not interfere with the cdk5 phosphorylation in this case, implying that direct binding of the ligand was required for this inhibition.

The most obvious interpretation of this data would be that the PPAR gamma agonist turned off the expression of the protein kinase or turned on the expression of a relevant

protein phosphatase working on the receptor. However, a study was performed to determine whether rosiglitazone and other PPAR gamma ligands work directly to inhibit cdk5 phosphorylation *in vitro*. To address this, purified PPAR gamma and cdk5/p35 were mixed with or without rosiglitazone under appropriate conditions to achieve modification *in vitro*. Suprisingly, Figure 5B shows that rosiglitazone blocked this cdk5-mediated phosphorylation *in vitro*, with a half-maximally effective dose of about 30 nM, near the K_d of this compound for PPAR gamma binding (Lehmann *et al. J. Biol. Chem.* 270:12953-12956 (1995)). Importantly, this inhibition is not caused by a general inhibition of cdk5 activity, since incubation with rosiglitazone does not inhibit the ability of cdk5 to phosphorylate the Rb protein (Figure 5D).

Example 6: Atypical PPAR Gamma Agonists Selectively Inhibit Phosphorylation Of PPAR Gamma At Ser 273 Over Classical Activation Of PPAR Gamma Activity

The fact that the PPAR gamma ligand rosiglitazone has the ability to block cdk5-mediated phosphorylation of PPAR gamma independent of both DNA binding and direct transcriptional agonism indicated an intriguing explanation for how some of PPAR gamma ligands with poor agonist properties can have substantial anti-diabetic activity. MRL24 is a non-TZD compound that has been reported to bind avidly to PPAR gamma and have excellent anti-diabetic activity in mice (Acton *et al., Bioorg. Med. Chem. Lett.* 15: 357-362 (2005)). However, this compound is a poor agonist toward PPAR gamma in transcription assays, and consequently has virtually no ability to promote adipogenesis, a classical effect of a PPAR gamma agonist. As reported previously (Acton *et al., Bioorg. Med. Chem. Lett.* 15: 357-362 (2005) and as shown in Figure 5D, MRL24 has very weak agonist activity on a PPAR transcriptional response element, compared to rosiglitazone. On the other hand, MRL24 was very effective at blocking the cdk5-mediated phosphorylation of PPAR gamma (Figure 5B). For example, 30 nM of MRL24 inhibited the modification of PPAR gamma as well as 300 nM of rosiglitazone, both *in vitro* and in cells (Figures 5B and 5E). Again, this effect was not a result of a general inhibition of cdk5 itself because this kinase retained full ability to phosphorylate Rb in the presence of *MRL24 in vitro*. MRL24 had only a small fraction of the agonist activity of rosiglitazone at every dose studied (Figure 5D). In fact, 30 nM MRL24, which blocked the TNF-α-mediated phosphorylation of PPAR gamma essentially completely (Figure 5E), had essentially undetectable agonist activity on PPAR gamma in the same cells (Figure 5D). In addition, the ability of a number of

additional anti-diabetic PPAR gamma ligands with poor agonist properties to inhibit cdk5-mediated phosphorylation was assessed (Gregoire *et al., Mol. Endocrinol.* 23: 975-988 (2009); Berger *et al., Mol. Endocrinol.* 17: 662-676 (2003); Ostberg *et al., J.Biol. Chem.* 279: 41124-41130 (2004)). As shown in Figure 6, all were effective at inhibiting the cdk5-mediated phosphorylation of PPAR gamma.

Example 7: Atypical PPAR Gamma Agonists Selectively Improves Insulin Sensitivity Over Classical Activation Of PPAR Gamma Activity

To take a systematic view of changes in gene expression caused in the same cells by both the cdk5 mutation in PPAR gamma and these PPAR gamma ligands, Affimetrix analyses of gene expression with RNA from cells expressing the wild type and mutant PPAR gammas, plus wild type cells treated with rosiglitazone or MRL24 were tested. As shown in Figure 5F, unsupervised clustering of all these data resulted in several notable clusters of gene expression. When the data derived from cells containing the cdk5 mutation in PPAR gamma (Mut) were compared to that from the wild type controls, the genes with differential expression segregate into four major groups. One group, labeled "a", were genes whose expression was decreased by this mutation, thus implying that these genes are activated by cdk5 phosphorylation of PPAR gamma. This same group of genes was also suppressed by both rosiglitazone and MRL24, though neither ligand functioned as dramatically in this suppression as did the non-phophorylatable mutant of PPAR gamma. This is understandable in that it is unlikely that treatment with these ligands could drive the dephosphorylation of PPAR gamma comparable to the mutant protein. The very small cluster labeled "b" contained *Ahnak* nucleoprotein and proteolipid protein (*plp*) 1, which are both critical for myelination (Hakak *et al., Proc. Natl. Acad. Sci. U S A* 98: 4746-4751 (2001); Salim *et al., Glia* 57: 535-549 (2009)), and sorting nexin 5 (*Snx5*), which is important for vesicle trafficking (Merino-Trigo *et al., J. Cell. Sci.* 117: 6413-6424 (2004)). A larger cluster, labeled "c", represented genes that were increased in the mutant cells, representing those that were decreased by the cdk5-mediated phosphorylation of PPAR gamma. Several of the genes known to be dysregulated in obesity, including *adiponectin* and *adipsin*, were present in this cluster. Rosiglitazone also increased essentially all of these genes, but Figure 5F indicates that this occured as part of a very large gene set increased by rosiglitzone action. Indeed, the genes induced most dramatically by rosiglitazone (labeled "d") did not correspond to the cdk5 mutation-induced gene set and

were largely the clasic genes of adipogenesis, like *aP2* and lipoprotein lipase (*Lpl*). In sharp contrast, the gene cluster strongly increased by MRL 24 was much smaller than that induced by rosiglitazone, and corresponded remarkably well to the gene set induced by the mutation in the cdk5 site of PPAR gamma. These data indicate that both rosiglitazone and MRL24 control most of the same genes that are affected by the cdk5 phsphorylation of PPAR gamma, but that there is a much closer correspondence between the genes induced by MRL24 and those induced by the genetic inhibition of the cdk5-mediated PPAR gamma phosphorylation. Taken together, the data presented in Figures 3-5 indicate that the cdk5 modification of PPAR gamma is a major source of gene dysregulation and pathology of adipose tissues in obesity.

To further analyze the link, a refined gene set regulated by the cdk5 modification of PPAR gamma was created, using the concept of principal component analysis of gene expression data. For this, data was utilized from cell culture and from the transplantation of these cells *in vivo*. The expression of these genes was then examined in the adipose tissue of mice on a chow or high fat diet for 13 weeks. Figure 7 shows that the great majority of these genes had dysregulated expression in an obesity-dependent manner. In total, of the 17 genes found to be most significantly reduced by cdk5 phosphorylation of PPAR gamma, at least 12 of these genes were quantitatively altered in obesity in the direction predicted. Genes well-known to be quantitatively dysregulated in obesity like *adiponectin* and *adipsin* were in this group. Also in this group were several genes not known to be associated with obesity, such as retinoic acid receptor responder 2 (*Rarres2*), selenium binding protein 1 (*Slelnbp1*), and carbonic anhydrase 3 (*Car3*).

To develop a structural understanding of how PPAR gamma ligands affect the cdk5-mediated phosphorylation of PPAR gamma, hydrogen/deuterium exchange (HDX) techniques linked to mass spectrometry were used 9 Maier *et al.*, *Methods Enzymol.* 402: 312-360 (2005)).

As shown in Figure 8a, rosiglitazone dramatically reduced H/D exchange in helix 12 (H12), the helix in the ligand-binding domain comprising part of the AF-2 surface of the receptor that is responsible for classical agonism (Nolte *et al., Nature* 395: 137-143 (1998); Bruning *et al., Structure* 15: 1258-1271 (2007)). By contrast, MRL24 had no statistically significant effect on the dynamics of this helix (Bruning *et al., Structure* 15: 1258-1271 (2007)). On the other hand, MRL24 had a more marked impact on H/D exchange kinetics across H3 (amino acids 309-315), the β-sheet at amino acids 369-379, and the cdk5 site itself at serine

273 in PPAR gamma. Rosiglitazone also affected the exchange across H3, and the beta sheet region, but did not alter the H/D exchange across serine 273 to any significant extent. When these HDX data are mapped onto the known co-crystal structures of PPAR gamma with each of these ligands bound, it is clear that both PPAR gamma ligands reduced the dynamic nature of this receptor in regions near the cdk5 site. However, MRL24 reduced the dynamic nature of these regions to a greater extent than rosiglitazone (Figure 8b). It is thus believed that this reduced dynamic nature in the H3, β-sheet, and cdk5 site itself, induced by the PPAR gamma ligands (especially MRL24), "freezes" this region in a configuration less favorable to, or even incompatible with, the cdk5 phosphorylation.

In addition, it was further investigated whether the PPAR gamma ligands can alter the cdk5-mediated phosphorylation that occurs *in vivo*, and whether these agents regulate the genes controlled by cdk5 action on PPAR gamma. As shown in Figures 9A through 9C, treatment with either rosiglitazone or MRL24 at 10 mg/kg for 7 days dramatically improved glucose tolerance of high-fat fed mice and reduced fasting insulin levels without inducing changes in body weight. Importantly, these anti-diabetic doses of both of these compounds reduced the cdk5-mediated phosphorylation of PPAR gamma in the adipose tissue of every mouse treated with these agents (Figure 9D). Furthermore 12 of the 17 genes most significantly controlled by cdk5 action on PPAR gamma (as described above) were altered by the action of one or both agents (Figure 9E). These data indicate that these anti-diabetic PPAR gamma ligands inhibited cdk5 phosphorylation of PPAR gamma *in vivo* and reversed changes in gene expression due to that modification.

Example 8: Atypical PPAR Gamma Agonists Selectively Brown White Fat And Increases Whole Body Energy Expenditure

Figure 10 shows that atypical PPAR gamma agonist selectively induces expression of brown fat selective genes, UCP1 and PGC-1alpha in white fat isolated from mice, with similar potency as typical agonist, rosiglitazone. Inguinal fat depots were isolated from mice treated with saline (control), rosiglitazone at 10 mg/kg or MRL24 at 3 mg/kg for 10 days. Total RNA was isolated from these samples. Subsequently, expression of UCP1 and PGC-1alpha was quantified by real-time PCR. mRNA level of UCP1 and PGC-1alpha was normalized with that of 18S.

Figure 11 shows that atypical PPAR gamma agonists also selectively increase whole body oxygen consumption *in vivo*. Oxygen consumption was measured in mice treated

with sailine (control), rosiglitazone or MRL24 at 10 mg/kg for 10 days using Comprehensive Lab Animal Monitoring System (CLAMS).

Example 9: Generating And Characterizing A Knock-In Mouse Line Encoding PPAR Gamma Non-Phosphorylatable At Serine 273

PPAR gamma serine 273 resides within the 5th exon of PPAR gamma (reference sequence obtained from Ensembl PPAR gamma ENSMUSG00000000440). A BAC (Bacterial artificial chromosome) clone RP23-232H15 containing the PPAR gamma genomic locus was purchased from Invitrogen. An 11kb DNA fragment centered around the 5th exon of PPAR gamma was retrieved from the BAC into a cloning plasmid containing a diphtheria toxin negative selection marker. A fragment containing the S273A mutation and a novel site for restriction endonuclease cleavage was introduced into this parent plasmid. Lastly, the DNA sequence encoding the loxP-Neo-loxP positive selection cassette was introduced into the targeting plasmid (Figure 12A). This plasmid was linearized and electroporated into ES cells. ES cells were cultured, screened for correct targeting, and implanted into mice. Progeny will be bred to Zp3-Cre expressing mice to delete the Neomycin cassette from within the PPAR gamma gene. These mice will then be bred to homozygosity in the absence of the Cre gene (Figure 12B).

Homozygous S273A mutant PPAR gamma knock-in mice may be used, at least in part, to test the efficacy of potential therapeutics. MRL-24 and similar compounds which block the phosphorylation of PPAR gamma at serine 273 by cdk5 (phopho-blockers) are believed to improve the metabolic syndrome without having the side-effects of classical agonists, such as TZDs.

For example, PPAR gamma is active in both lean and obese individuals; however in these states, PPAR gamma exhibits slightly different transcription of target genes. Without being bound by theory, it is believed that this difference in transcriptional activity is due to the inhibitory phosphorylation of PPAR gamma at Ser-273. Mice with both copies of PPAR gamma mutated at Ser-273 to alanine or other non-phosphorylatable amino acid residues are believed to retain normal glucose tolerance when faced with the challenge of a high-fat diet.

The glucose lowering effect of MRL-24 does not require PPAR gamma agonism, an effect that is likely responsible for weight gain and fluid retention observed upon administration of classical PPAR gamma agonists. Without being bound by theory, it is

believed that such classical PPAR gamma agonists (*e.g.*, rosiglitizone) will have the positive glucose lowering only in WT mice but not in S273A mice. However the side effects, such as weight gain and fluid retention will still occur in both strains. Hence, an ideal compound would have no effect in the knock-in mice making it a good model for testing off-target effects of the drug.

Cells lines taken from these knock in mice can be used for *in vitro* testing of potential compounds. Specific chemical entities are believed to only alter target genes in wild-type cells, not in knock-in cells.

S273A PPAR gamma mutant knock-in animals can also be used in bone marrow transplantation assays. Bone marrow transplantation from a wild type animal to S273A PPAR gamma mutant knock-in animals and *vice versa* can be used to analyze activity of such mutant PPAR gamma polypeptides in immune system cells (*e.g.*, macrophages and monocytes) in a wild type host background and *vice versa*, for example, to determine whether risk of atherosclerosis due to CD36-dependent decreased lipid uptake is mediated by immune system cells.

Based on all of the data provided herein, Figure 13 provides a model of how, without being bound by theory, phosphorylation of PPAR gamma at Ser-273 is believed to selectively mediate anti-metabolic activities.

Example 10: Ser-273 Phosphorylation Status of PPAR Gamma Is Predictive Of Increased Glucose Sensitivity

Nine human subjects having type 2 diabetes were treated with rosiglitazone at a dose of 4 mg/day for six months. CLAMP analyses were performed before and after the rosiglitazone treatment regimen. Figure 14 provides data indicating the high correlation between decreased levels of phosphorylated Ser-273 produced through the rosiglitazone administration regimen and improved insulin sensitivity. Those subjects who did not exhibit significant decreases in phosphorylated Ser-273 levels were metabolically non-responsive to rosiglitazone (*i.e.*, did not exhibit improved insulin sensitivity.

Incorporation by Reference

The contents of all references, patent applications, patents, and published patent applications, as well as the Figures and the Sequence Listing, cited throughout this application are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed:

1. A method of identifying a compound which inhibits Ser-273 phosphorylation of murine peroxisome proliferator activated receptor gamma (PPAR gamma) 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog comprising:

a) contacting a sample comprising said murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog with a test compound; and

b) determining the ability of the test compound to inhibit said Ser-273 phosphorylation of murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog,

thereby identifying a compound which selectively inhibits said Ser-273 phosphorylation of murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog.

2. The method of claim 1, wherein the sample is selected from the group consisting of *in vitro*, *ex vivo*, and *in vivo* samples.

3. The method of claim 1, wherein inhibition of said Ser-273 phosphorylation of murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog is determined by analyzing the amount of Serine 273 phophorylated PPAR gamma relative to total PPAR gamma and comparing the ratio to a control.

4. The method of claim 3, wherein the control is the ratio from treatment with rosiglitazone under standard conditions.

5. The method of claim 1, further comprising a step of determining whether the test compound directly binds said murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog.

6. A method of identifying a compound that binds PPAR gamma and which selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation in a cell type, the method comprising,

a) determining whether the compound binds PPAR gamma; and

b) comparing the amount and/or activity of a marker in a first sample of the cell type maintained in the presence of the test compound, wherein the marker is selected from the

group of markers listed in Table 1 or 2, to the amount and/or activity of the marker in a second sample which is a control,

wherein a significantly higher amount and/or activity of a marker listed in Table 1 in the first sample relative to the second sample indicates that the test compound selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation in the cell type and/or

wherein a significantly lower amount and/or activity of a marker listed in Table 2 in the first sample relative to the second sample, indicates that the test compound selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation in the cell type.

7. The method of claim 6, wherein the cell type is selected from the group consisting of: preadipocytes, mature white adipocytes, brown adipocytes, monocytes, and macrophages.

8. The method of claim 6, wherein the first and/or second sample is selected from the group consisting of *in vitro*, *ex vivo*, and *in vivo* samples.

9. The method of claim 6, wherein the first and/or second sample is obtained from an animal model of a metabolic disorder.

10. The method of claim 6, wherein the first and/or second sample is selected from the group consisting of tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, and bone marrow.

11. The method of claim 6, wherein the first and second samples are portions of a single sample obtained from a subject.

12. The method of claim 6, wherein the first and second samples are portions of pooled samples obtained from a subject.

13. The method of claim 6, wherein the second sample comprises cells of the same cell type as the first sample maintained in the absence of the test compound.

14. The method of claim 6, wherein the second sample comprises cells of the same cell type as the first sample treated with rosiglitazone.

15. The method of claim 6, wherein a significantly higher amount and/or activity comprises upregulating the amount and/or activity of the marker listed in Table 1 at least 25% relative to the second sample.

16. The method of claim 6, wherein a significantly lower amount and/or expression comprises downregulating the amount and/or activity of the marker listed in Table 2 at least 25% relative to the second sample.

17. The method of claim 6, wherein the amount of the marker is compared.

18. The method of claim 17, wherein the amount of the marker is determined by determining the level of protein expression of the marker.

19. The method of claim 18, wherein the presence of the protein is detected using a reagent which specifically binds with the protein.

20. The method of claim 19, wherein the reagent is selected from the group consisting of an antibody, an antibody derivative, and an antibody fragment.

21. The method of claim 19, wherein the reagent comprises an antibody that binds to PPAR-gamma and an antibody that binds to a peptide comprising a consensus cdk5 phosphorylated site.

22. The method of claim 17, wherein the level of expression of the marker in the sample is assessed by detecting the presence in the sample of a transcribed polynucleotide or portion thereof.

23. The method of claim 22, wherein the transcribed polynucleotide is an mRNA or a cDNA.

24. The method of claim 22, wherein the step of detecting further comprises amplifying the transcribed polynucleotide.

25. The method of claim 17, wherein the level of expression of the marker in the sample is assessed by detecting the presence in the sample of a transcribed polynucleotide which

anneals with the marker or anneals with a portion of a polynucleotide under stringent hybridization conditions.

26. The method of claim 6, wherein the marker is phosphorylated Ser-273 on murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog.

27. The method of claim 6, wherein the metabolic disorder is selected from the group consisting of: glucose intolerance, insulin resistance, hypertension, dyslipidemia, obesity, type II diabetes, hyperglycemia, hyperinsulinemia, elevated systolic and diastolic blood pressure, hypertrigliceridemia, hypercholesterolemia, and body mass index greater than 30.

28. The method of claim 6, wherein PPAR gamma comprises the amino acid sequence set forth in SEQ ID NO:1-7.

29. A method for assessing the efficacy of a compound that binds PPAR gamma for selectively promoting anti-metabolic disorder activity over classical PPAR gamma activation in a subject, the method comprising:

a) detecting in a subject sample at a first point in time, the amount and/or activity of a marker, wherein the marker is a marker listed in Table 1 or 2;

b) repeating step a) during at least one subsequent point in time after administration of the compound; and

c) comparing the amount and/or activity detected in steps a) and b),

wherein a significantly higher amount and/or activity of a marker listed in Table 1 in the first subject sample relative to at least one subsequent subject sample, indicates that the test compound selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation in the subject and/or

wherein a significantly lower amount and/or activity of a marker listed in Table 2 in the first subject sample relative to at least one subsequent subject sample, indicates that the test compound selectively promotes anti-metabolic disorder activity over classical PPAR gamma activation in the subject.

30. The method of claim 29, wherein between the first point in time and the subsequent point in time, the subject has undergone treatment for a metabolic disorder, has completed treatment for a metabolic disorder, and/or is in remission from a metabolic disorder.

31. The method of claim 29, wherein the cell type is selected from the group consisting of: preadipocytes, mature white adipocytes, brown adipocytes, monocytes, and macrophages.

32. The method of claim 29, wherein the first and/or at least one subsequent sample is selected from the group consisting of *ex vivo* and *in vivo* samples.

33. The method of claim 29, wherein the first and/or at least one subsequent sample is obtained from an animal model of a metabolic disorder.

34. The method of claim 29, wherein the first and/or at least one subsequent sample is selected from the group consisting of tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, and bone marrow.

35. The method of claim 29, wherein the first and/or at least one subsequent sample is a portion of a single sample obtained from the subject.

36. The method of claim 29, wherein the first and/or at least one subsequent sample is a portion of pooled samples obtained from the subject.

37. The method of claim 29, further comprising steps a) and b) with rosiglitazone.

38. The method of claim 37, wherein the results from treatment with rosiglitazone are compared with the results from the test compound.

39. The method of claim 29, wherein a significantly higher amount and/or activity comprises upregulating the amount and/or activity of the marker listed in Table 1 at least 25% relative to the second sample.

40. The method of claim 29, wherein a significantly lower amount and/or activity comprises downregulating the amount and/or activity of the marker listed in Table 2 at least 25% relative to the second sample.

41. The method of claim 29, wherein the amount of the marker is compared.

42. The method of claim 41, wherein the amount of the marker is determined by determining the level of protein expression of the marker.

43. The method of claim 42, wherein the presence of the protein is detected using a reagent which specifically binds with the protein.

44. The method of claim 43, wherein the reagent is selected from the group consisting of an antibody, an antibody derivative, and an antibody fragment.

45. The method of claim 43, wherein the reagent comprises an antibody that binds to PPAR-gamma and an antibody that binds to a peptide comprising a consensus cdk5 phosphorylated site.

46. The method of claim 41, wherein the level of expression of the marker in the sample is assessed by detecting the presence in the sample of a transcribed polynucleotide or portion thereof.

47. The method of claim 46, wherein the transcribed polynucleotide is an mRNA or a cDNA.

48. The method of claim 46, wherein the step of detecting further comprises amplifying the transcribed polynucleotide.

49. The method of claim 41, wherein the level of expression of the marker in the sample is assessed by detecting the presence in the sample of a transcribed polynucleotide which anneals with the marker or anneals with a portion of a polynucleotide under stringent hybridization conditions.

50. The method of claim 29, wherein the marker is phosphorylated Ser-273 on murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog.

51. The method of claim 29, wherein the metabolic disorder is selected from the group consisting of: glucose intolerance, insulin resistance, hypertension, dyslipidemia, obesity, type II diabetes, hyperglycemia, hyperinsulinemia, elevated systolic and diastolic blood pressure, hypertrigliceridemia, hypercholesterolemia, and body mass index greater than 30.

52. The method of claim 29, wherein PPAR gamma comprises the amino acid sequence set forth in SEQ ID NOS:1-7.

53. A method of treating a subject afflicted with a metabolic disease comprising administering to the subject a compound that binds PPAR gamma and which selectively promotes anti-diabetic activity, thereby treating the subject afflicted with the metabolic disease.

54. The method of claim 53, wherein said compound inhibits Ser-273 phosphorylation of murine PPAR gamma 2 or a corresponding serine residue in a murine PPAR gamma 2 homolog.

55. The method of claim 53, wherein the compound is administered in a pharmaceutically acceptable formulation.

56. The method of claim 55, wherein the pharmaceutically acceptable formulation is an oral formulation.

57. The method of claim 53, wherein the compound is a small molecule.

58. A compound for treating a metabolic disorder by binding PPAR gamma and selectively promoting anti-metabolic disorder activity in a cell type, wherein the compound inhibits phosphorylation of Ser-273 on PPAR gamma.

59. The compound of claim 58, wherein the compound has less than 30% of the PPAR gamma agonist function of rosiglitazone.

60. The compound of claim 58, wherein the compound has an EC50 binding affinity for PPAR gamma less than 1-200 nM.

61. The compound of claim 58, wherein the compound upregulates expression of a marker listed in Table 1 at least 30% relative to rosiglitazone under identical conditions and/or downregulates expression of a marker listed in Table 2 at least 30% relative to rosiglitazone under identical conditions.

62. The compound of claim 58, wherein the metabolic disorder is selected from the group consisting of: glucose intolerance, insulin resistance, hypertension, dyslipidemia, obesity, type II diabetes, hyperglycemia, hyperinsulinemia, elevated systolic and diastolic

blood pressure, hypertrigliceridemia, hypercholesterolemia, and body mass index greater than 30.

63. The compound of claim 58, wherein the compound is selected from the group consisting of: m-benzyl indoles, MRL-20, MRL-24, nTZDpa, SR145, SR147, Mbx-102, MK-0533, and BVT.13.

64. An isolated nucleic acid molecule encoding a murine peroxisome proliferator activated receptor gamma 2 polypeptide having a non-phosphorylatable amino acid at position Ser-273 or a homolog thereof having a non-phosphorylatable amino acid at the corresponding serine residue position in the murine PPAR gamma 2 polypeptide, or a complement thereof.

65. The isolated isolated nucleic acid molecule of claim 64, which encodes a polypeptide having the amino acid sequence set forth in SEQ ID NO:2 or 3, and further encoding a non-phosphorylatable amino acid at position Ser-273, or a complement thereof.

66. The isolated nucleic acid molecule of claim 64 or 65 further encoding a heterologous polypeptide.

67. A vector comprising the nucleic acid molecule of any one of claims 64-66.

68. The vector of claim 67, which is an expression vector.

69. A host cell comprising the vector of claim 68.

70. A method for producing a protein comprising culturing the host cell of claim 69 in a suitable medium until the protein is produced.

71. The method of claim 70, further comprising isolating the protein from the medium or the host cell.

72. An isolated protein comprising a murine peroxisome proliferator activated receptor gamma 2 polypeptide having a non-phosphorylatable amino acid at position Ser-273 or a homolog thereof having a non-phosphorylatable amino acid at the corresponding serine residue position in the murine PPAR gamma 2 polypeptide.

73. The isolated protein of claim 72 comprising a polypeptide having the amino acid sequence set forth in SEQ ID NO:2 or 3, and further encoding a non-phosphorylatable amino acid at position Ser-273.

74. The isolated protein of claim 72 or 73 operatively linked to a heterologous polypeptide.

75. A method of making an isolated hybridoma which produces an antibody that specifically binds to murine PPAR gamma 2 phosphorylated at Ser-273 or a corresponding serine residue in a murine PPAR gamma 2 homolog or a fragment thereof, the method comprising:

a) immunizing a mammal using a composition comprising said murine PPAR gamma 2 phosphorylated at Ser-273 or a corresponding serine residue in a murine PPAR gamma 2 homolog or a fragment thereof;

b) isolating splenocytes from the immunized mammal;

c) fusing the isolated splenocytes with an immortalized cell line to form hybridomas; and

d) screening individual hybridomas for production of an antibody which specifically binds with said polypeptide thereof to isolate the hybridoma.

76. An antibody produced by a hybridoma according to the method of claim 75.

77. An isolated antibody or antigen binding portion thereof that specifically binds to a polypeptide comprising the amino acid sequence of a murine PPAR gamma 2 phosphorylated at Ser-273 or a corresponding serine residue in a murine PPAR gamma 2 homolog or a fragment thereof.

78. The isolated antibody or antigen binding portion thereof of claim 77, wherein the antibody or antigen binding portion thereof specifically binds the epitope having the amino acid sequence: KTTDK(pS)PFVIYDC.

79. The antibody or antigen binding portion thereof of claim 77 which is a monoclonal antibody.

80. The antibody or antigen binding portion thereof of claim 77 which is a polyclonal antibody.

81. The antibody or antigen binding portion thereof of claim 77 which is a chimeric or a humanized antibody.

82. The antibody or antigen binding portion thereof of claim 77 which is detectably labeled.

83. The antibody or antigen binding portion thereof of claim 77, wherein the antibody or antigen binding portion thereof comprises an effector domain.

84. The antibody or antigen binding portion thereof of claim 77, wherein the antibody or antigen binding portion thereof comprises an Fc domain.

85. The antibody or antigen binding portion thereof of claim 77, wherein the antibody or antigen binding portion thereof is a single-chain antibody.

86. The antibody or antigen binding portion thereof of claim 77, wherein the antibody or antigen binding portion thereof is a Fab fragment.

87. A kit for assessing the ability of a compound to inhibit phosphorylation of PPAR gamma comprising one or more reagents for specifically detecting murine PPAR gamma 2 phosphorylated at Ser-273 or a corresponding serine residue in a murine PPAR gamma 2 homolog.

88. A non-human animal model comprising a mutated PPAR gamma 2 gene encoding a protein incapable of being phosphorylated at serine 273 or a corresponding serine residue in a murine PPAR gamma 2 homolog.

89. The non-human animal model of claim 88, wherein the mutation comprises a mutation of said serine to a non-phosphorylatable amino acid.

90. The non-human animal model of claim 88, wherein said animal is heterozygous or homozygous for the mutated PPAR gamma 2 gene encoding a protein incapable of being phosphorylated at serine 273 or a corresponding serine residue in a murine PPAR gamma 2 homolog.

91. The non-human animal model of claim 88, wherein the animal is a knock-in or a transgenic animal.

92. The non-human animal model of claim 88, wherein the animal is a rodent and/or a mouse.

93. A method for identifying genes regulated in a subject, comprising:

(a) expressing a mutated PPAR gamma gene encoding a protein incapable of being phosphorylated on Serine 273 or a corresponding serine residue in a murine PPAR gamma 2 homolog;

(b) determining the level of expression and/or activity of one or more candidate target genes of said mutated PPAR gamma gene; and

(c) identifying genes which exhibit significantly altered expression and/or activity relative to a control.

94. A method for classifying a sample according to a predicted treatment outcome comprising comparing:

a) the level of expression of a marker or a plurality of markers in a biological sample; and

b) the level of expression of the marker or plurality of markers in a control sample,

wherein the marker or plurality of markers are selected from the group consisting of the markers listed in Tables 1 and 2 and the difference between the level of expression of the marker or plurality of markers in the biological sample and the control sample classifies the biological sample according to the predicted treatment outcome.

95. A method for identifying a subject likely to have a predicted treatment outcome comprising comparing:

a) the level of expression of a marker or a plurality of markers in a biological sample from the subject; and

b) the level of expression of the marker or plurality of markers in a control sample,

wherein the marker or plurality of markers are selected from the group consisting of the markers listed in Tables 1 and 2 and the difference between the level of expression of the marker or plurality of markers in the biological sample from the subject and the control sample predicts the likelihood of a predicted treatment outcome in the subject.

96. The method of claim 94 or 95, wherein the predicted treatment outcome is selected from the treatment outcomes listed in Tables 1 and 2.

97. The method of claim 94 or 95, wherein the predicted treatment outcome is the likelihood of increased glucose sensitivity.

98. The method of claim 94, wherein the tissue sample is obtained after a PPAR gamma ligand has been administered to the subject.

99. The method of claim 95, wherein the biological sample is obtained after a PPAR gamma ligand has been administered to the subject.

Figure 1

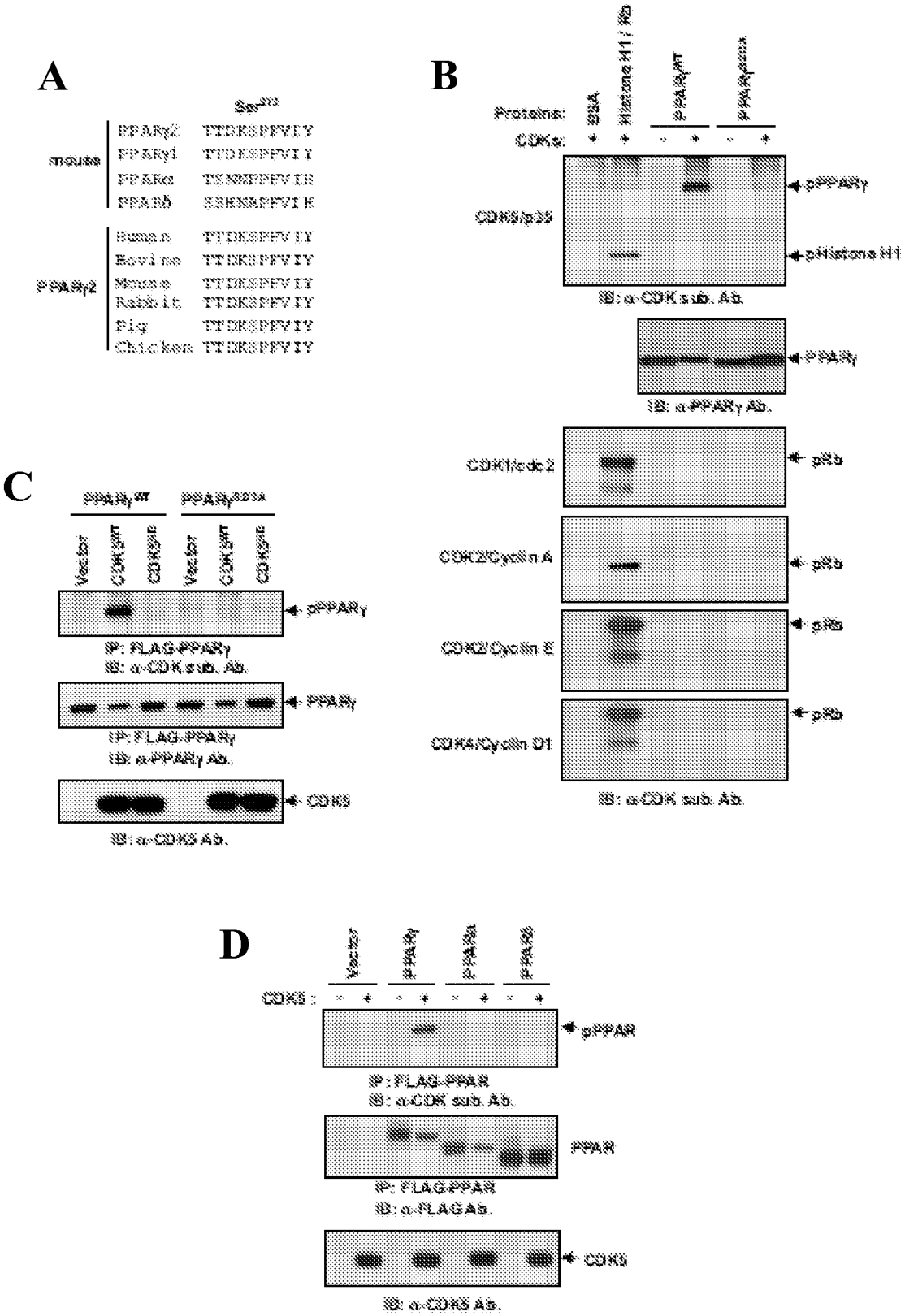

A
mouse
PPARγ2 TTDKSPFVIY
PPARγ1 TTDKSPFVIY
PPARα TSNKPPFVIR
PPARδ SSHNAPFVIE
PPARγ2
Human TTDKSPFVIY
Bovine TTDKSPFVIY
Mouse TTDKSPFVIY
Rabbit TTDKSPFVIY
Pig TTDKSPFVIY
Chicken TTDKSPFVIY
B
Proteins:
BSA
Histone H1
CDKs:
CDK5/p35
pPPARγ
pHistone H1
IB: α-CDK sub. Ab.
IB: α-PPARγ Ab.
CDK1/cdc2
CDK2/Cyclin A
CDK2/Cyclin E
CDK4/Cyclin D1
pRb
C
Vector
CDK5WT
CDK5KE
IP: FLAG-PPARγ
IB: α-CDK sub. Ab.
IB: α-PPARγ Ab.
IB: α-CDK5 Ab.
D
Vector
CDK5 :
pPPAR
PPAR
IP: FLAG-PPAR
IB: α-CDK sub.Ab.
IB: α-FLAG Ab.
IB: α-CDK5 Ab.

Figure 2

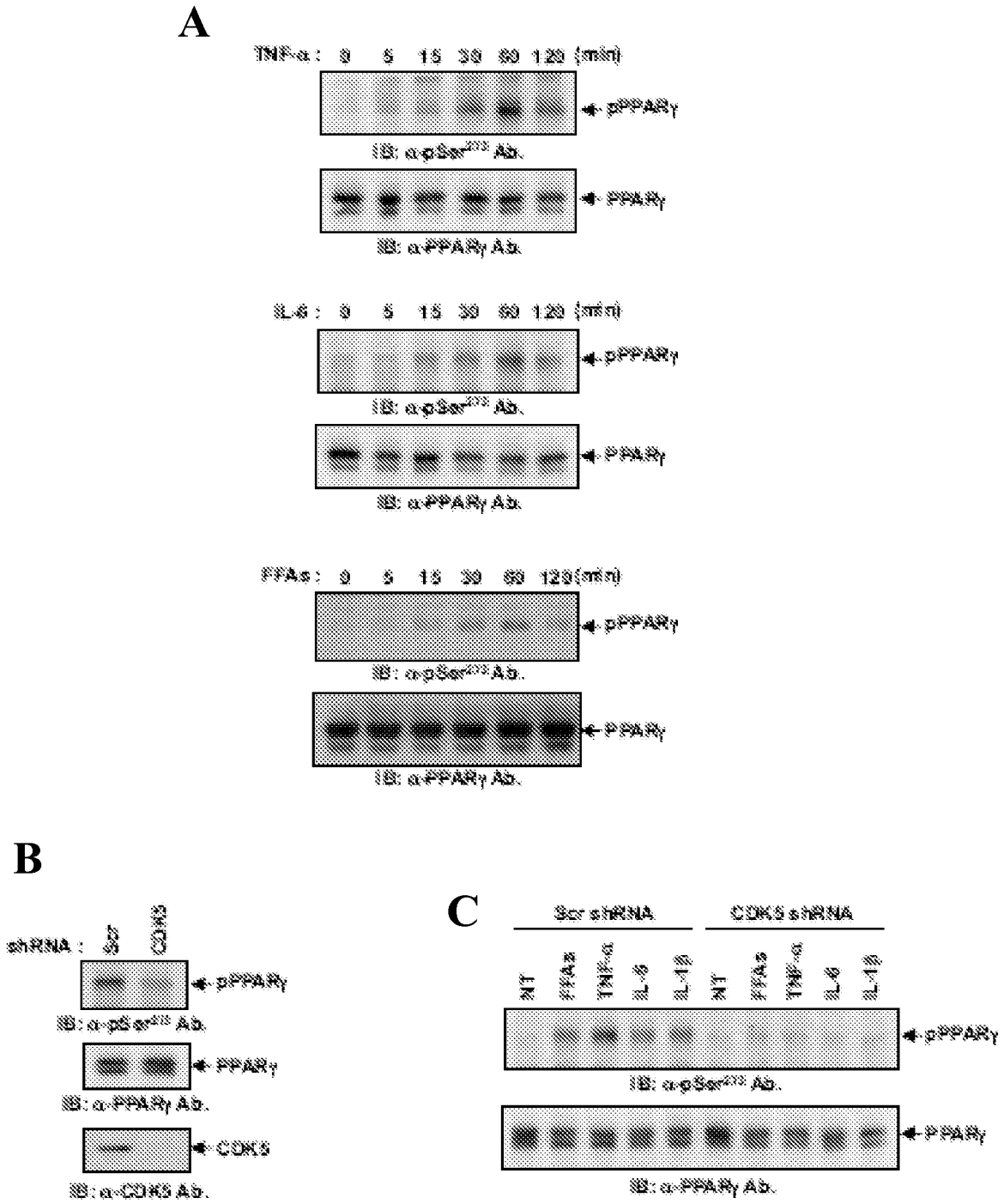

A
TNF-α : 0 5 15 30 60 120 (min)
pPPARγ
IB: α-pSer273 Ab.
PPARγ
IB: α-PPARγ Ab.
IL-6 : 0 5 15 30 60 120 (min)
pPPARγ
IB: α-pSer273 Ab.
PPARγ
IB: α-PPARγ Ab.
FFAs : 0 5 15 30 60 120(min)
pPPARγ
IB: α-pSer273 Ab.
PPARγ
IB: α-PPARγ Ab.
B
shRNA : Scr CDK5
pPPARγ
IB: α-pSer273 Ab.
PPARγ
IB: α-PPARγ Ab.
CDK5
IB: α-CDK5 Ab.
C
Scr shRNA
CDK5 shRNA
NT
FFAs
TNF-α
IL-6
IL-1β
NT
FFAs
TNF-α
IL-6
IL-1β
pPPARγ
IB: α-pSer273 Ab.
PPARγ
IB: α-PPARγ Ab.

Figure 3

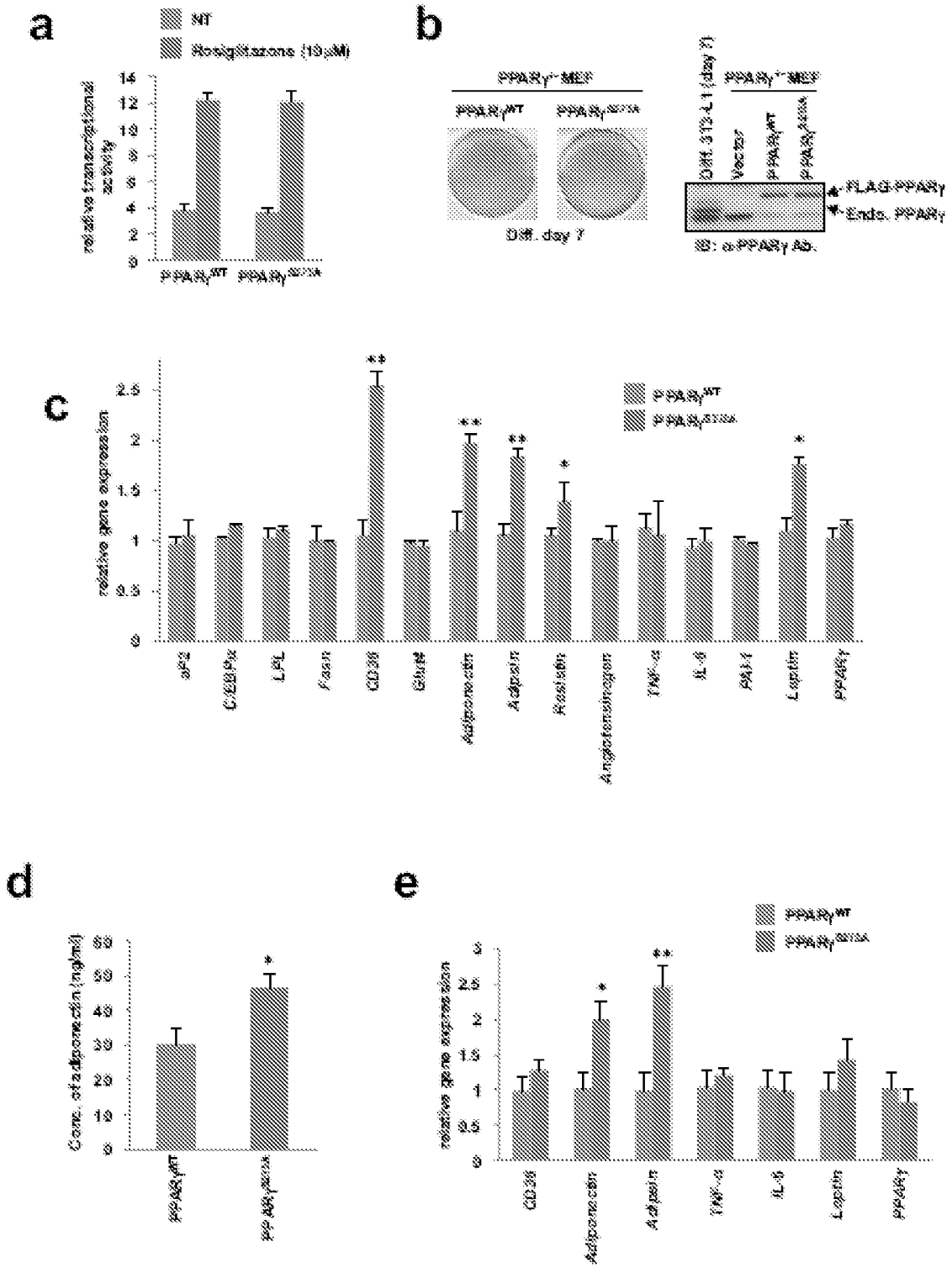
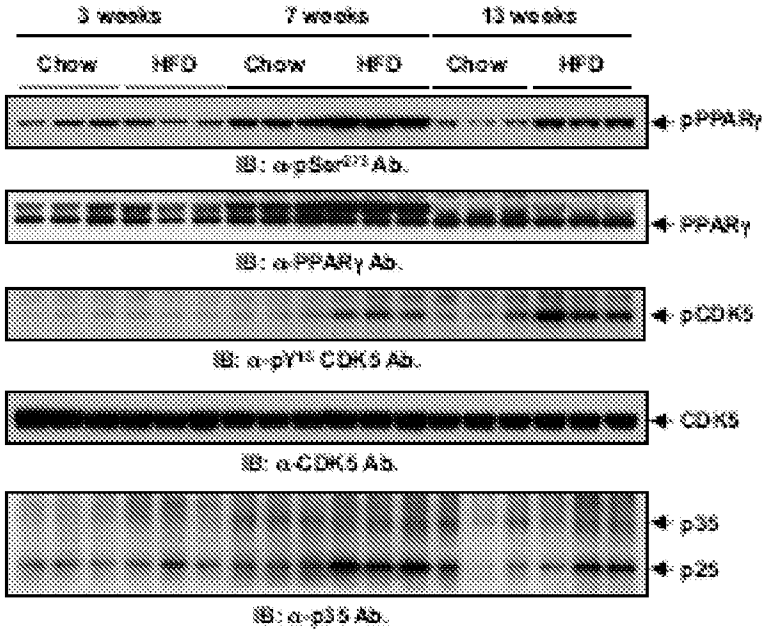

a
NT
Rosiglitazone (10μM)
relative transcriptional activity
b
Diff. day 7
Diff. 3T3-L1 (day 7)
Vector
FLAG-PPARγ
Endo. PPARγ
IB: α-PPARγ Ab.
c
relative gene expression
aP2
C/EBPα
LPL
Fasn
CD36
Glut4
Adiponectin
Adipsin
Resistin
Angiotensinogen
TNF-α
IL-6
PAI-1
Leptin
PPARγ
d
Conc. of adiponectin (ng/ml)
e
relative gene expression
CD36
Adiponectin
Adipsin
TNF-α
IL-6
Leptin
PPARγ

Figure 4

A



B

	7 weeks Chow	7 weeks HFD	13 weeks Chow	13 weeks HFD
Weight (grams)	26.2 ± 0.3	33.8 ± 1***	29.4 ± 0.6	44.5 ± 1.1***
Glucose (mg/dl)	88.2 ± 6.4	92.6 ± 5.4	115.4 ± 8.9	124.4 ± 6.5
Insulin (ng/ml)	0.1 ± 0.03	0.18 ± 0.06	0.24 ± 0.03	1.07 ± 0.15**

C

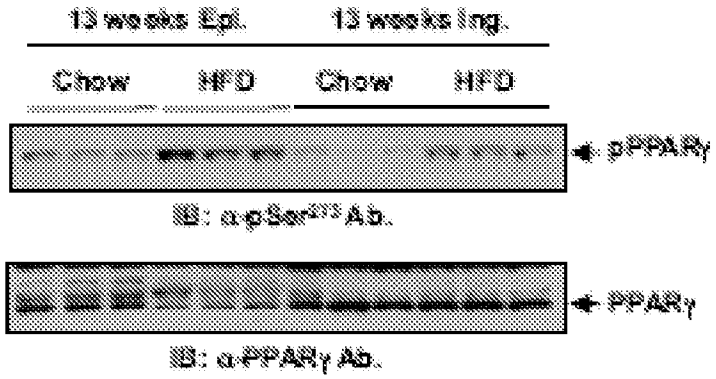


Figure 5

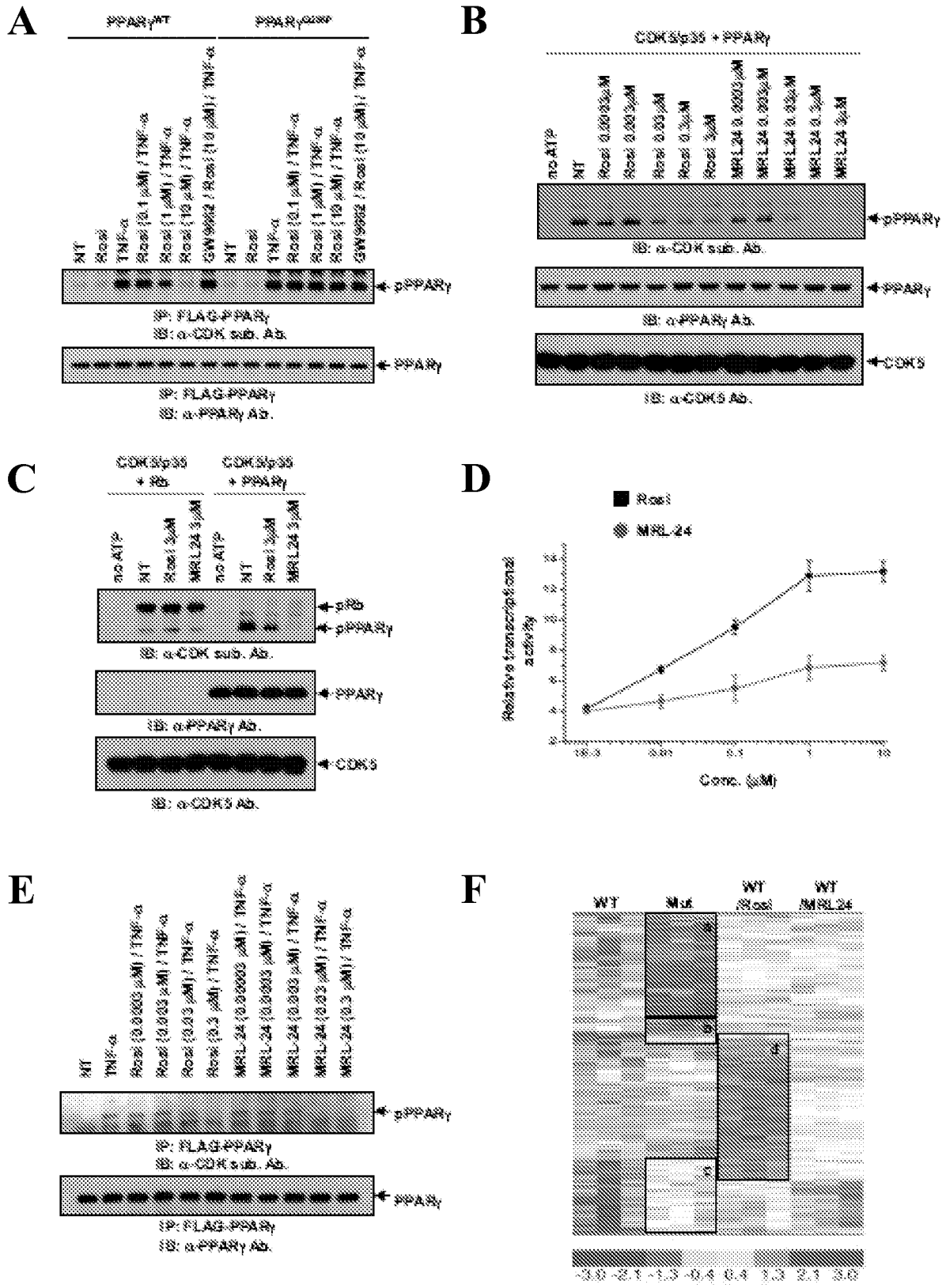
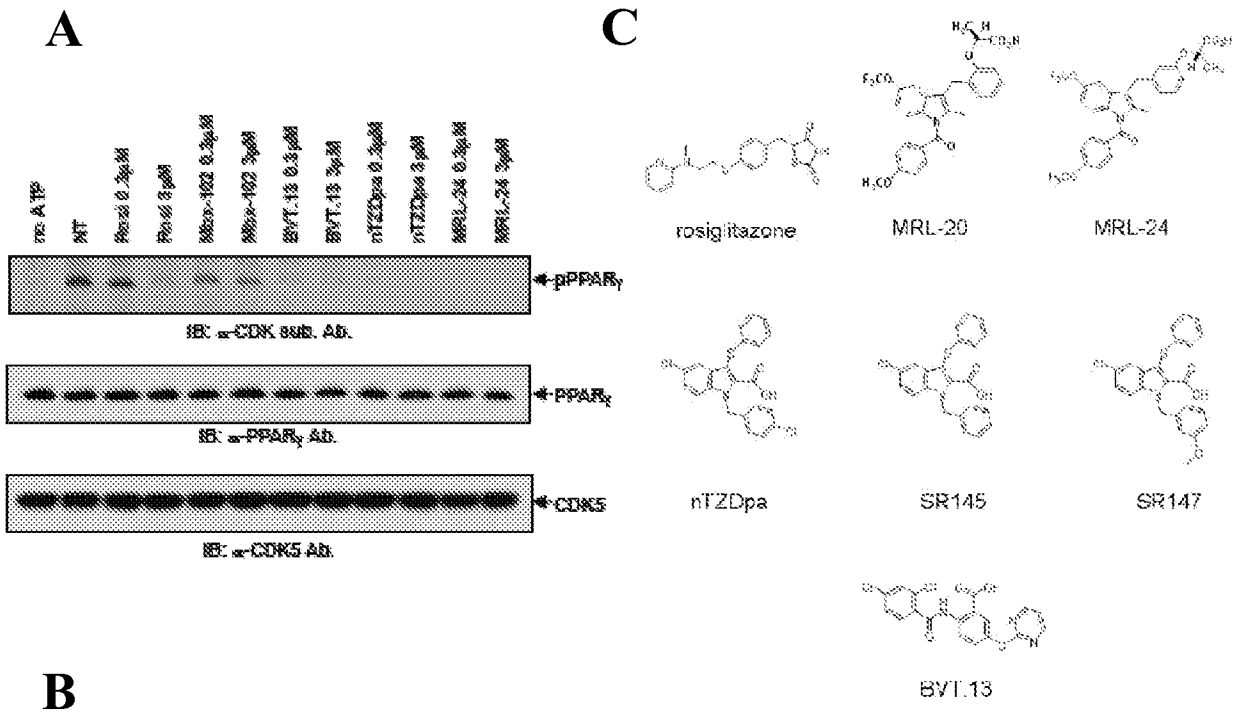

A
B
C
D
E
F
pPPARγ
PPARγ
CDK9
pRb
Rosi
MRL-24
Conc. (μM)
WT
Mut
WT (Rosi)
WT MRL24

Figure 6


A
no ATP
WT
Rosi 0.3μM
Rosi 3 μM
Mbx-102 0.3μM
Mbx-102 3μM
BVT.13 0.3 μM
BVT.13 3μM
nTZDpa 0.3μM
nTZDpa 3 μM
MRL-24 0.3μM
MRL-24 3μM
pPPARγ
IB: α-CDK sub. Ab.
PPARγ
IB: α-PPARγ Ab.
CDK5
IB: α-CDK5 Ab.
C
rosiglitazone
MRL-20
MRL-24
nTZDpa
SR145
SR147
BVT.13

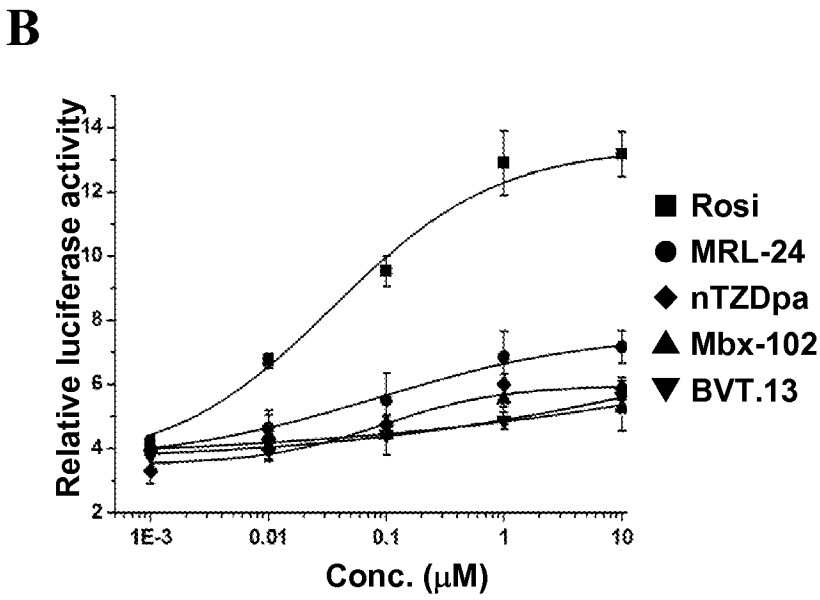

B
Relative luciferase activity
14
12
10
8
6
4
2
1E-3
0.01
0.1
1
10
Conc. (μM)
Rosi
MRL-24
nTZDpa
Mbx-102
BVT.13

Figure 7

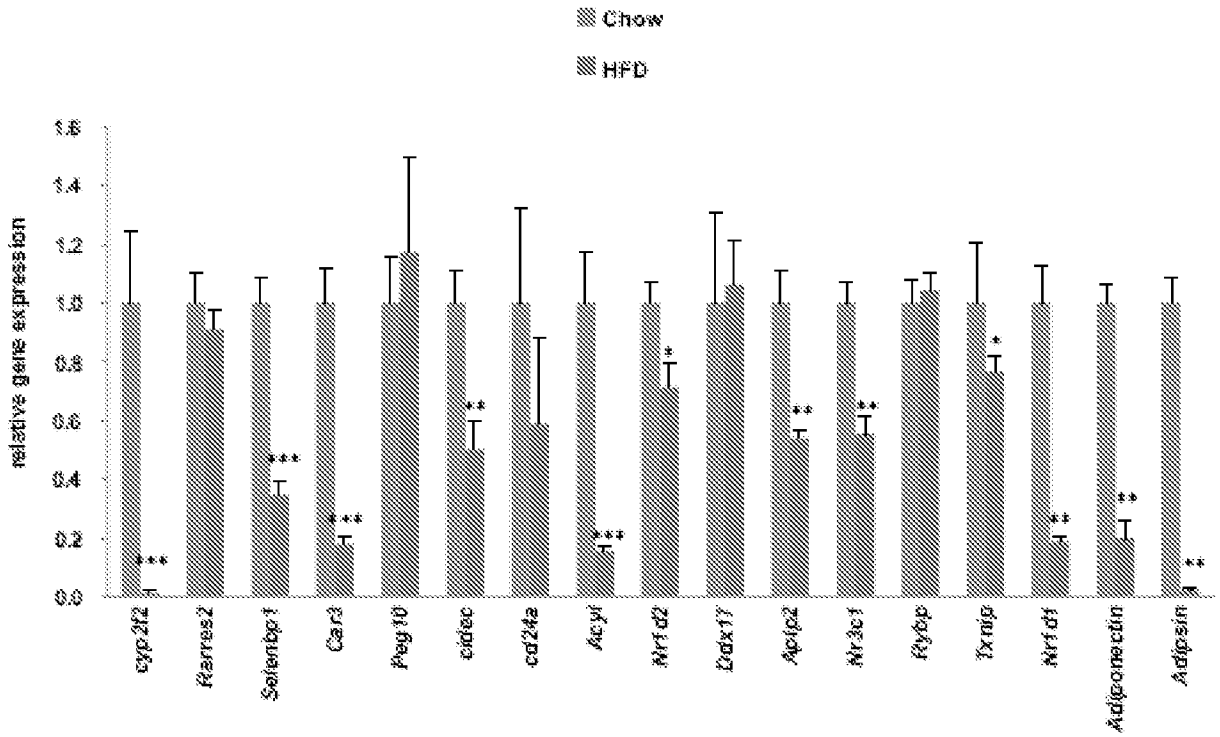

Chow
HFD
relative gene expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
cyp2f2
Rarres2
Selenbp1
Car3
Peg10
cidec
cd24a
Acyl
Nr1d2
Ddx17
Aqlp2
Nr3c1
Klybp
Txnip
Nr1d1
Adiponectin
Adipsin

Figure 8

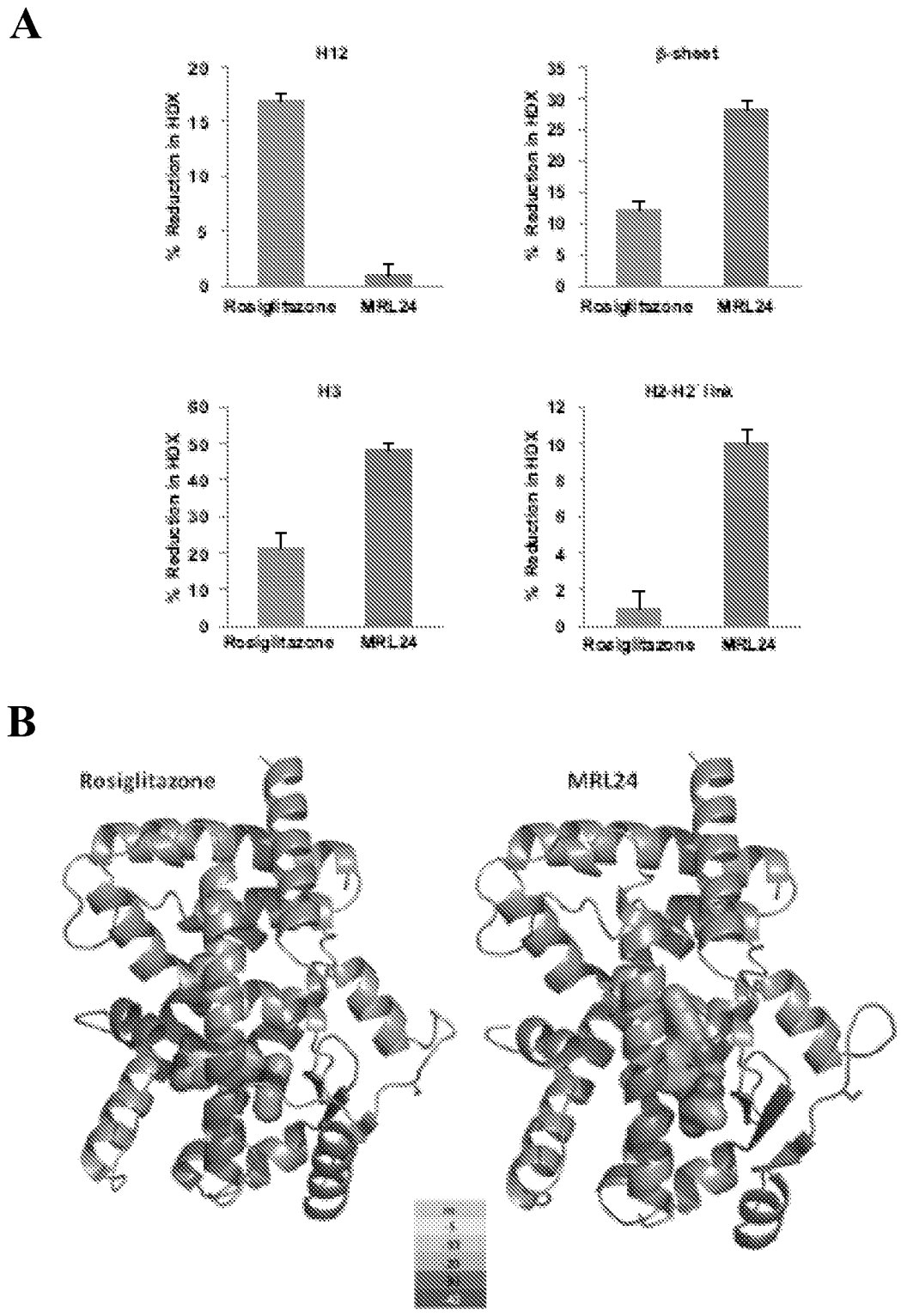

A
H12
β-sheet
H3
H2-H2' link
% Reduction in HDX
Rosiglitazone
MRL24
B
Rosiglitazone
MRL24

Figure 9

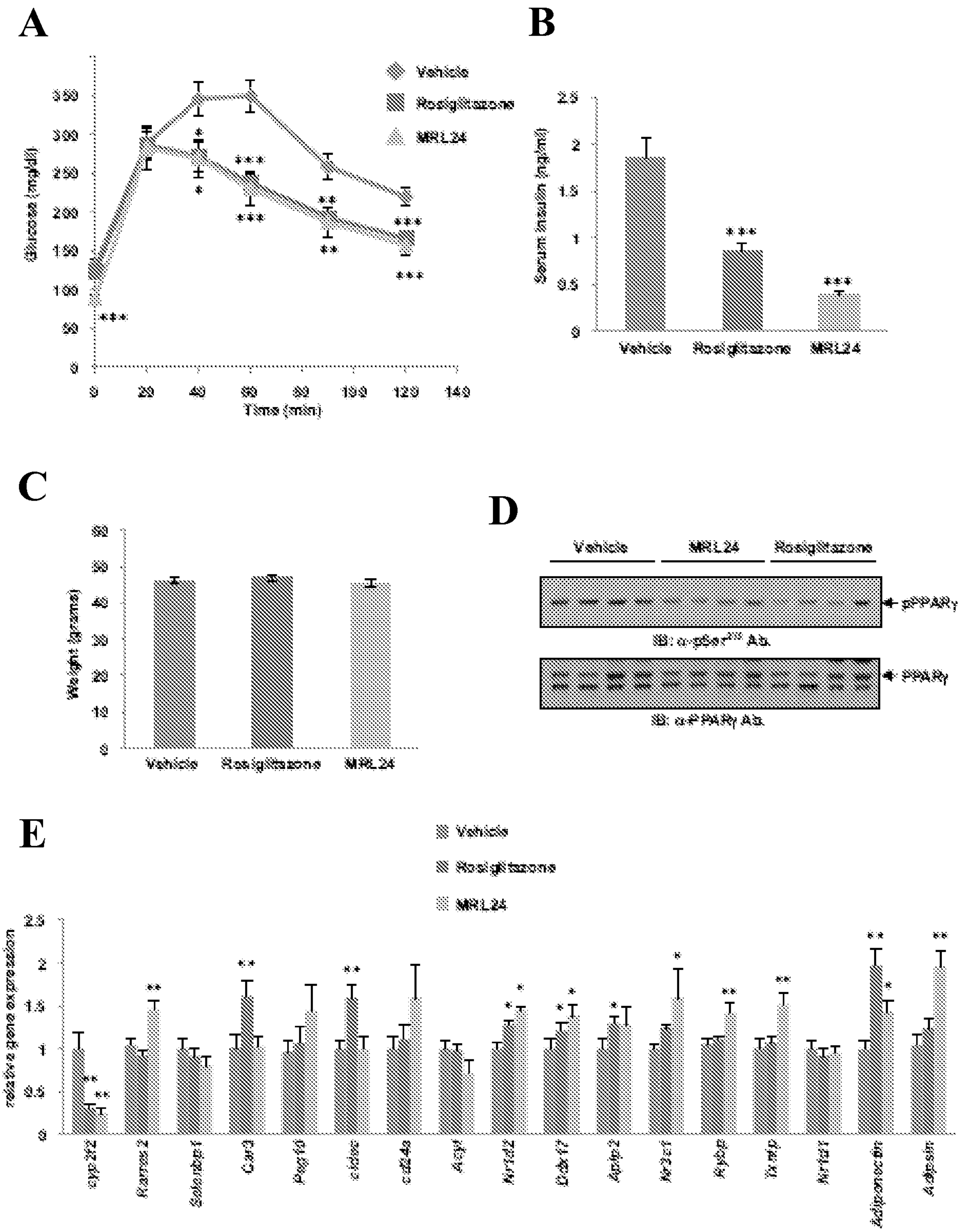

A
Vehicle
Rosiglitazone
MRL24
Glucose (mg/dl)
Time (min)
B
Serum insulin (ng/ml)
Vehicle
Rosiglitazone
MRL24
C
Weight (grams)
Vehicle
Rosiglitazone
MRL24
D
Vehicle
MRL24
Rosiglitazone
pPPARγ
PPARγ
IB: α-PPARγ Ab.
E
Vehicle
Rosiglitazone
MRL24
relative gene expression
cyp2f2
Rarres2
Selenbp1
Car3
Peg10
cidec
cd24a
Acyl
Nr1d2
Ddx17
Aplp2
Nr3c1
Rybp
Txnip
Nr1d1
Adiponectin
Adipsin

Figure 10

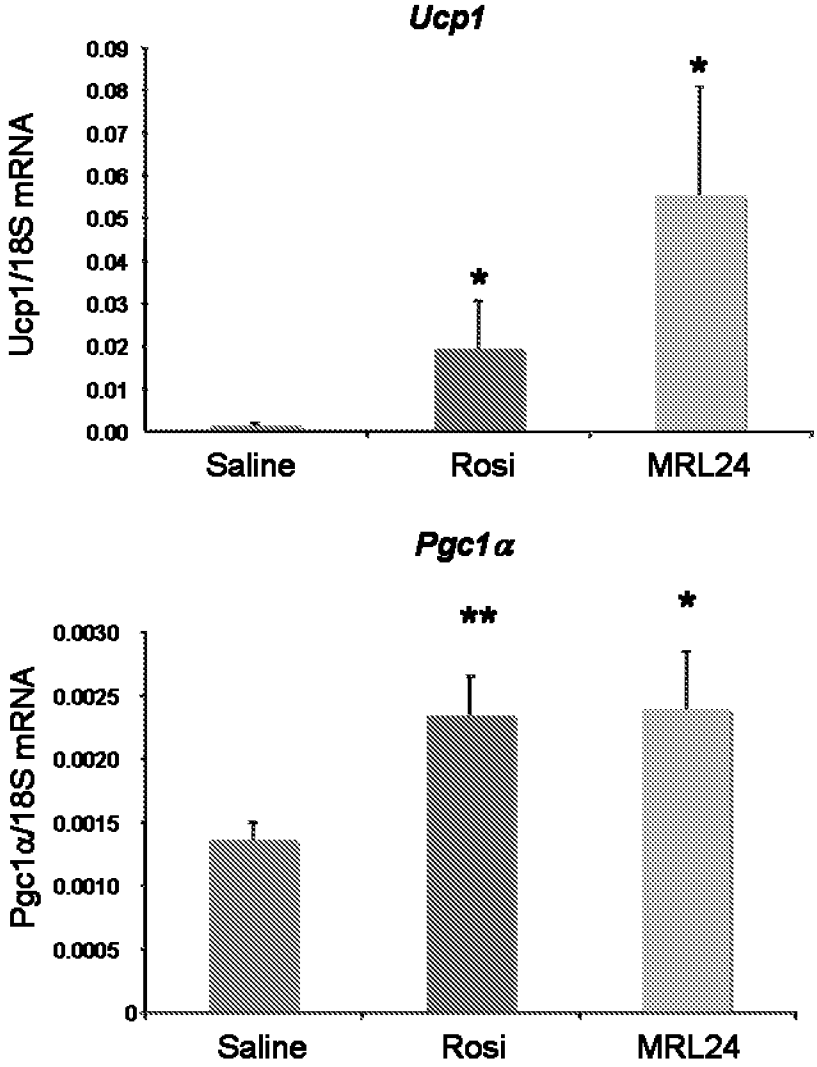

Ucp1
Ucp1/18S mRNA
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
*
*
Saline
Rosi
MRL24
Pgc1α
Pgc1α/18S mRNA
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0
**
*
Saline
Rosi
MRL24

Figure 11

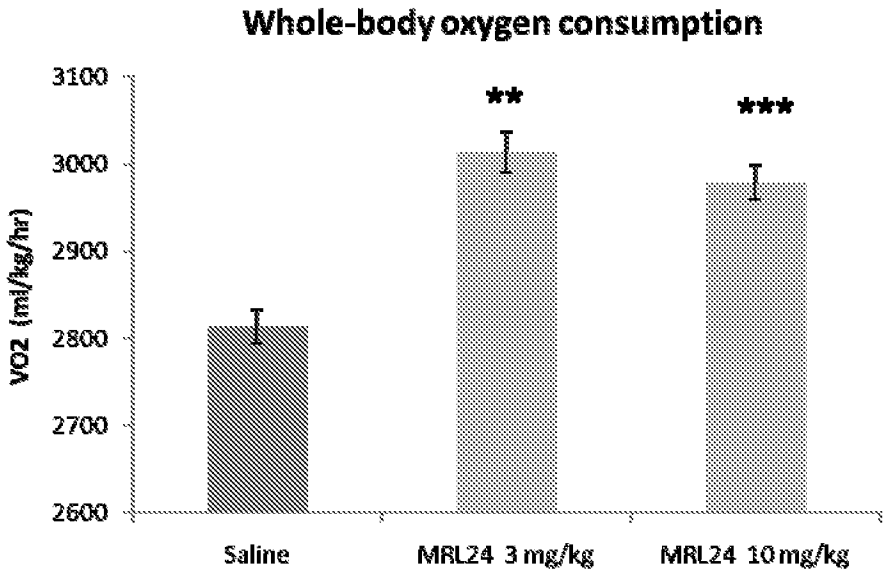
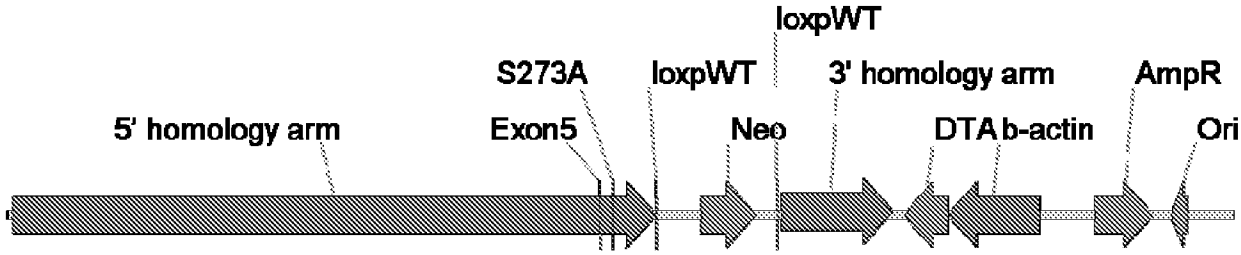

Whole-body oxygen consumption
VO2 (ml/kg/hr)
3100
3000
2900
2800
2700
2600
**
***
Saline
MRL24 3 mg/kg
MRL24 10 mg/kg

Figure 12

A


loxpWT
S273A
loxpWT
3' homology arm
AmpR
5' homology arm
Exon5
Neo
DTA
b-actin
Ori

B

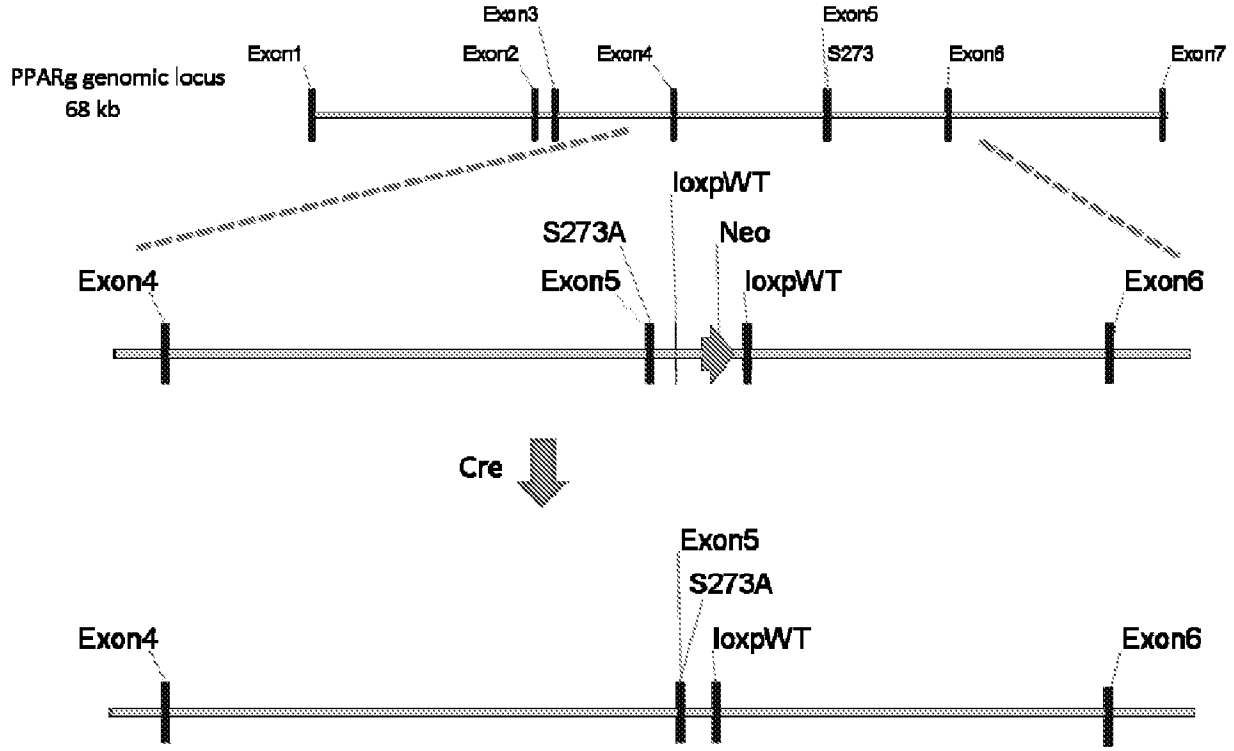

Exon3
Exon5
PPARg genomic locus
68 kb
Exon1
Exon2
Exon4
S273
Exon6
Exon7
loxpWT
S273A
Neo
Exon4
Exon5
loxpWT
Exon6
Cre
Exon5
S273A
Exon4
loxpWT
Exon6

Figure 13

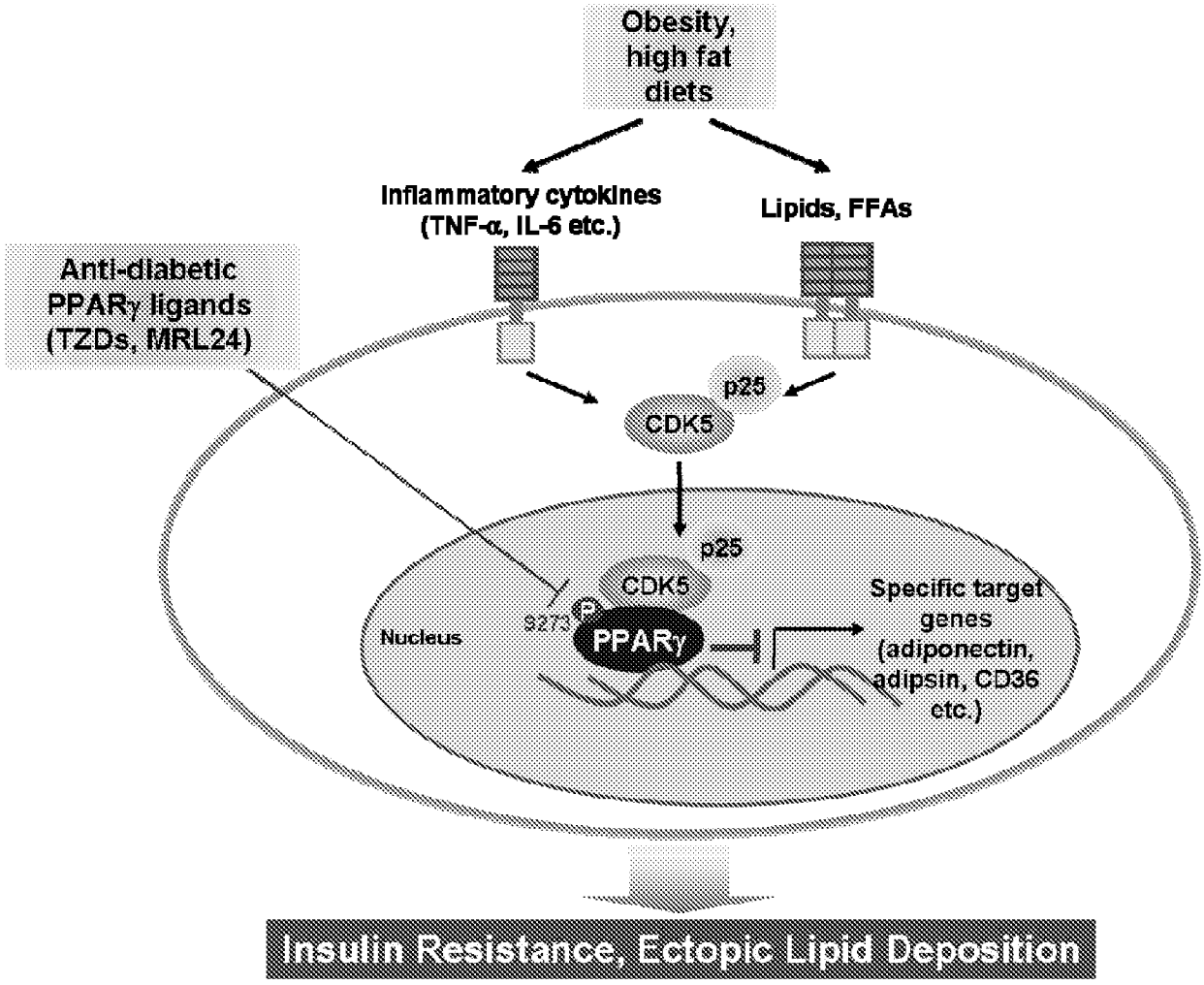

Obesity, high fat diets
Inflammatory cytokines (TNF-α, IL-6 etc.)
Lipids, FFAs
Anti-diabetic PPARγ ligands (TZDs, MRL24)
p25
CDK5
p25
CDK5
Nucleus
S273
P
PPARγ
Specific target genes (adiponectin, adipsin, CD36 etc.)
Insulin Resistance, Ectopic Lipid Deposition

Figure 14

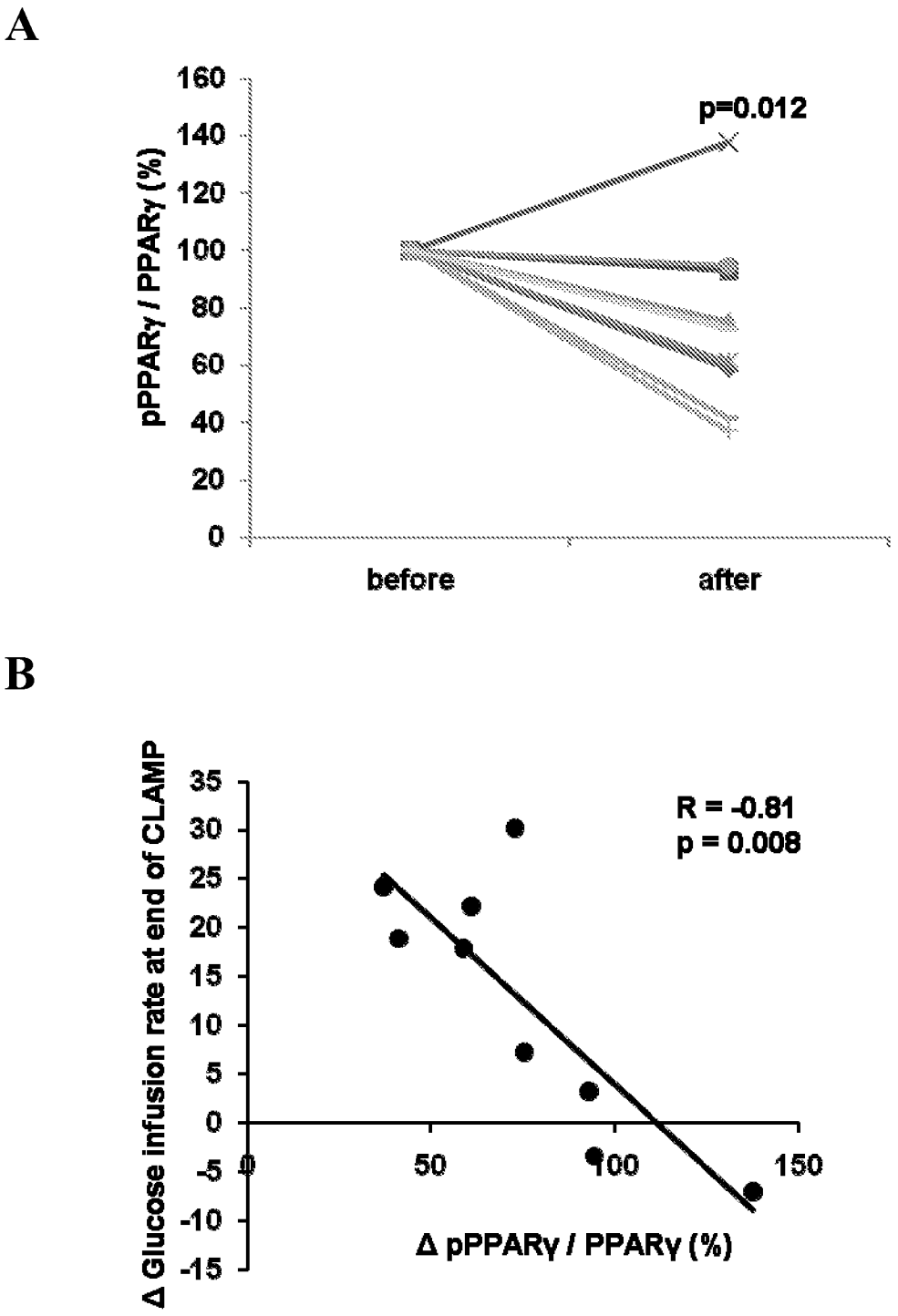

A
160
140
120
100
80
60
40
20
0
pPPARγ / PPARγ (%)
p=0.012
before
after
B
35
30
25
20
15
10
5
0
-5
-10
-15
Δ Glucose infusion rate at end of CLAMP
R = -0.81
p = 0.008
0
50
100
150
Δ pPPARγ / PPARγ (%)

Figure 14 (cont.)

C

		Delta phosphoPPARg	Delta GIR	Delta TG	Delta Weight	Delta V02max	Delta FPG	Delta OGGT	Delta FPI
Delta phosphoPPARg	Pearson Correlation	1	-,812	-,656	,238	-,638	,729	-,063	,804
	Sig. (2-tailed)		,008	,055	,537	,065	,026	,871	,009
	N	9	9	9	9	9	9	9	9
Delta GIR	Pearson Correlation	-,812	1	,437	,196	,666	-,840	-,366	-,788
	Sig. (2-tailed)	,008		,240	,613	,050	,005	,333	,012
	N	9	9	9	9	9	9	9	9
Delta TG	Pearson Correlation	-,656	,437	1	-,284	,194	-,294	,284	-,664
	Sig. (2-tailed)	,055	,240		,459	,617	,442	,459	,051
	N	9	9	9	9	9	9	9	9
Delta Weight	Pearson Correlation	,238	,196	-,284	1	-,282	-,032	-,365	,346
	Sig. (2-tailed)	,537	,613	,459		,462	,934	,335	,362
	N	9	9	9	9	9	9	9	9
Delta V02max	Pearson Correlation	-,638	,666	,194	-,282	1	-,528	-,102	-,601
	Sig. (2-tailed)	,065	,050	,617	,462		,144	,794	,087
	N	9	9	9	9	9	9	9	9
Delta FPG	Pearson Correlation	,729	-,840	-,294	-,032	-,528	1	,298	,762
	Sig. (2-tailed)	,026	,005	,442	,934	,144		,436	,017
	N	9	9	9	9	9	9	9	9
Delta OGGT	Pearson Correlation	-,063	-,366	,284	-,365	-,102	,298	1	,130
	Sig. (2-tailed)	,871	,333	,459	,335	,794	,436		,738
	N	9	9	9	9	9	9	9	9
Delta FPI	Pearson Correlation	,804	-,788	-,664	,346	-,601	,762	,130	1
	Sig. (2-tailed)	,009	,012	,051	,362	,087	,017	,738	
	N	9	9	9	9	9	9	9	9