PPARγ-K107 SUMOylation regulates insulin sensitivity but not adiposity in mice

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The nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) is a master regulator of adipocyte differentiation and is the target for the insulin-sensitizing thiazolidinedione (TZD) drugs used to treat type 2 diabetes. In cell-based and in vitro studies, the transcriptional activity of PPARγ is inhibited by covalent attachment of small ubiquitin-related modifier (SUMOylation) at K107 in its N terminus. However, whether this posttranslational modification is relevant in vivo remains unclear. Here, using mice homozygous for a mutation (K107R) that prevents SUMOylation at this position, we demonstrate that PPARγ is SUMOylated at K107 in white adipose tissue. We further show that in the context of diet-induced obesity PPARγ-K107R–mutant mice have enhanced insulin sensitivity without the corresponding increase in adiposity that typically accompanies PPARγ activation by TZDs. Accordingly, the PPARγ-K107R mutation was weaker than TZD treatment in stimulating adipocyte differentiation in vitro. Moreover, we found that both the basal and TZD-dependent transcriptomes of inguinal and epididymal white adipose tissue depots were markedly altered in the K107R-mutant mice. We conclude that PPARγ SUMOylation at K107 is physiologically relevant and may serve as a pharmacologic target for uncoupling PPARγ’s beneficial insulin-sensitizing effect from its adverse effect of weight gain.

PPARγ | SUMOylation | adipose tissue | insulin | rosiglitazone

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor family of ligand-activated transcription factors that is activated by various fatty acids and their metabolites (1). Two forms of PPARγ, PPARγ1 and PPARγ2, which differ by a 30-aa N-terminal extension in PPARγ2, are derived from alternative promoter usage and mRNA splicing of the PPARγ gene. While PPARγ1 is expressed at low levels in many tissues, PPARγ2 is enriched in adipocytes, where it serves as a master regulator of fat cell differentiation (1). In mature adipocytes, PPARγ2 regulates a program of genes involved in glucose and lipid homeostasis. Mutations in PPARγ cause lipodystrophy and severe insulin resistance in humans, and PPARγ is the molecular target for the thiazolidinedione (TZD) diabetes drugs, including rosiglitazone and pioglitazone (1, 2). Thus, PPARγ regulates systemic insulin sensitivity.

The transcriptional activity of PPARγ is regulated by a variety of posttranslational modifications including covalent attachment of small ubiquitin-related modifier (SUMO) to either K107 in the N terminus or K395 in the ligand-binding domain (numbering according to PPARγ2 sequence) (3). PPARγ SUMOylation at K107 strongly represses PPARγ transcriptional activity in vitro even though only a small percentage of PPARγ protein is modified (4–6). The mechanism may involve either recruitment of corepressor proteins or dissociation of coactivator proteins. Ligand-dependent PPARγ SUMOylation at K395 occurs in macrophages, where it is believed to target PPARγ to genes involved in inflammation to inhibit clearance of corepressor complexes, thereby maintaining the genes in a repressed state (7).

The metabolic regulatory hormone FGF21 is induced by TZDs and fasting/refeeding in white adipocytes, where it stimulates glucose uptake and insulin sensitivity (8, 9). We previously reported a positive regulatory loop wherein FGF21 activates PPARγ in isolated adipocytes by inhibiting its SUMOylation at K107 (10). However, whether K107 SUMOylation regulates PPARγ transcriptional activity in vivo remains unclear. In this report we evaluate the physiologic consequences of mutating K107 to prevent PPARγ SUMOylation at this residue in mice.

Results

K107R Mice Have Increased Insulin Sensitivity. Mice with a lysine-to-arginine substitution at codon 107 of PPARγ were generated by homologous recombination. Mice heterozygous and homozygous for the K107R mutation were born at the expected Mendelian ratio and appeared normal except for decreased body weight and liver size. Heterozygous and homozygous K107R mice had improved insulin sensitivity in both euglycemic–hyperinsulinemic clamp and oral glucose tolerance tests (Fig. 1). This was attributable to increased glucose uptake in white adipose tissue, but not liver or muscle, and was associated with increased expression of genes that regulate glucose metabolism (Fig. S1). Consistent with these observations, K107R mice had reduced body weight and liver size (Fig. 1). These results suggest that covalent modification of PPARγ at K107 is a physiologically relevant mechanism regulating adipocyte function.

Significance

Covalent attachment of small ubiquitin-related modifier (SUMOylation) is a posttranslational modification that regulates the activity of many transcription factors. The nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) is a master regulator of adipogenesis and the target of the thiazolidinedione (TZD) diabetes drugs. Although the TZDs have potent insulin-sensitizing properties, their use is limited by adverse effects such as weight gain. Here we provide unequivocal evidence that PPARγ is SUMOylated in mice at lysine-107 and that preventing this modification selectively enhances the insulin-sensitizing activity of PPARγ by regulating the expression of a discriminate network of genes in white adipose tissue. Our work suggests that inhibiting PPARγ SUMOylation may be a strategy for designing safer PPARγ drugs.

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Data deposition: RNA-sequencing data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (accession no. GSE120332).

See QnAs on page 12081.

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ratio and appeared normal and healthy. Total PPARγ levels were unchanged in inguinal and epididymal white adipose tissue (iWAT and eWAT, respectively) and in interscapular brown adipose tissue (BAT) in heterozygous and homozygous K107R mice (Fig. 1). However, SUMOylated PPARγ levels were reduced by half in iWAT and eWAT in heterozygous K107R mice and were virtually undetectable in homozygous K107R mice (Fig. 1). We did not detect SUMOylated PPARγ in BAT (Fig. 1). Phosphorylation at S112, which inhibits PPARγ transcriptional activity at least in part by enhancing K107 SUMOylation (11), was unaffected by the K107R mutation (Fig. 1). These data demonstrate that PPARγ is SUMOylated in vivo and that there is little or no SUMOylation of PPARγ on residues other than K107 in adipose tissue.

Homozygous K107R mice (hereafter referred to as “K107R mice”) fed a normal chow diet had body weights that were indistinguishable from WT littermates (Table 1). The K107R mice had a modest decrease in fasted plasma glucose concentrations but no changes in plasma insulin, triglyceride, nonesterified fatty acid (NEFA), cholesterol, adiponectin, or leptin levels (Table 1). When challenged with a high-fat diet, K107R mice gained slightly less weight than their WT littermates (Fig. 1B and Table 1) without accompanying changes in either adiposity or lean mass (Fig. 1C). This result was unexpected, given that PPARγ activation with TZDs causes weight gain due to increased adiposity and fluid retention (1, 2). After 19 wk on the high-fat diet, there were no differences between WT and K107R mice in plasma glucose, triglyceride, cholesterol, adiponectin, or leptin concentrations (Table 1), nor were there changes in iWAT or eWAT histology (Fig. 1D). Notably, however, the diet-induced obese (DIO) K107R mice had lower plasma insulin and NEFA levels than their WT littermates (Table 1).

To determine whether the K107R mutation affects insulin sensitivity, we performed hyperinsulinemic–euglycemic clamp

Fig. 1. PPARγ is SUMOylated at K107 in WAT. (A) Immunoblotting for SUMO1-conjugated PPARγ (SUMO1), S112-phosphorylated PPARγ-(p-PPARγ), and total PPARγ following PPARγ immunoprecipitation from eWAT and iWAT and interscapular BAT from individual WT (K/K), K107R heterozygous (K/R), and homozygous (R/R) mutant mice. Band intensities were quantified by ImageJ and normalized to WT except for SUMO1-PPARγ levels in BAT, which were normalized to SUMO1 in WT iWAT. Molecular mass is indicated on the right. (B) Body weights of WT and homozygous K107R mice fed a high-fat diet (HFD) for the indicated times. (C) Fat and lean content of WT and homozygous K107R mice after 19 wk on a high-fat diet. Values were normalized to body weight. (D) H&E staining of iWAT and eWAT from WT and homozygous K107R mice after 19 wk on a high-fat diet. For B and C, n = 6 mice per group. *P < 0.05.
experiments using weight-matched DIO WT (55.2 ± 1.9 g) and K107R (53.8 ± 2.5 g) mice. K107R mice had a doubling in both the glucose infusion rate and whole-body glucose uptake rate (Fig. 2A). There were significant increases in glucose uptake into gastrocnemius and diaphragm muscle and iWAT and similar trends in soleus muscle and eWAT, iWAT, and BAT (Fig. 2B). There was no difference between WT and K107R mice in basal glucose production (Fig. 2A). Thus, preventing PPARγ SUMOylation increases whole-body insulin sensitivity by stimulating glucose disposal principally in muscle.

**Comparison of K107R and Rosiglitazone Effects in Vivo.** To examine the relationship between PPARγ regulation by SUMOylation and TZDs, DIO WT and K107R mice were administered rosiglitazone or vehicle for 1 wk by oral gavage, and plasma insulin, glucose, triglycerides, NEFAs, and adiponectin concentrations

### Table 1. Metabolic parameters in PPARγ-K107R mice

| Parameter            | WT mice on chow diet | K107R mice on chow diet | P value  | DIO WT mice | DIO K107R mice | P value |
|----------------------|----------------------|-------------------------|----------|--------------|----------------|---------|
| Body weight, g       | 30.3 ± 2.6           | 25.3 ± 1.1              | 0.12     | 55.3 ± 2.4   | 49.3 ± 1.0     | 0.030*  |
| Glucose, mg/dL       | 135 ± 4.7            | 120 ± 4.1               | 0.032*   | 167 ± 7.6    | 175 ± 7.5      | 0.51    |
| Insulin, ng/mL       | 1.00 ± 0.25          | 0.94 ± 0.13             | 0.84     | 2.09 ± 0.27  | 1.37 ± 0.15    | 0.036*  |
| Cholesterol, mg/dL   | 150 ± 13.8           | 126 ± 13.4              | 0.25     | 202 ± 15.9   | 181 ± 22.0     | 0.44    |
| Triglyceride, mg/mL  | 25.7 ± 2.0           | 29.7 ± 1.5              | 0.25     | 96.9 ± 22.5  | 77.9 ± 5.4     | 0.39    |
| NEFA, mEq/mL         | 0.715 ± 0.046        | 0.562 ± 0.088           | 0.15     | 0.817 ± 0.055| 0.624 ± 0.028  | 0.0069**|
| Adiponectin, mg/mL   | 10.6 ± 1.8           | 9.4 ± 1.3               | 0.33     | 11.9 ± 1.5   | 10.6 ± 0.60    | 0.40    |
| Leptin, ng/mL        | 6.41 ± 2.57          | 2.57 ± 0.84             | 0.18     | 64.4 ± 6.3   | 60.2 ± 11.7    | 0.71    |

n = 5 to 8 mice per group.

*P < 0.05.

**P < 0.01.

Fig. 2. K107R mice have increased insulin sensitivity. (A) Glucose infusion rate, whole-body glucose uptake, basal glucose production, and basal endogenous glucose production were measured under hyperinsulinemic-euglycemic clamp conditions in DIO WT and K107R mice. (B) Glucose uptake under clamp conditions into muscle depots (Upper Row) and in eWAT, iWAT, and BAT (Lower Row). n = 8 or 9 mice per group. *P < 0.05; **P < 0.01; ***P < 0.001.
were measured (Fig. 3 A–E). As expected, K107R mice had lower plasma insulin and NEFA levels, which were further reduced by rosiglitazone treatment (Fig. 3 A and D). The K107R mice had no changes in plasma glucose, triglycerides, or adiponectin (Fig. 3 B, C, and E). Rosiglitazone treatment lowered plasma triglyceride levels and increased adiponectin levels to a comparable degree in both WT and K107R mice (Fig. 3 C and E).

Thus, blocking SUMOylation and administering rosiglitazone have additive effects on a subset of PPARγ-regulated metabolic parameters, including plasma insulin and NEFA levels. Rosiglitazone has additional effects on plasma triglyceride and adiponectin concentrations that were not seen in K107R mice.

In an i.p. glucose tolerance test, K107R mice had decreased plasma insulin concentrations compared with WT mice (Fig. 3F). While plasma insulin concentrations decreased further in K107R mice treated with rosiglitazone, they were the same as those in WT mice administered rosiglitazone (Fig. 3F). Plasma glucose concentrations were also decreased to a similar extent in WT and K107R mice administered rosiglitazone (Fig. 3G). In an insulin tolerance test, plasma glucose concentrations trended lower in K107R mice than in WT mice (Fig. 3H), consistent with the increased insulin sensitivity observed in the hyperinsulinemic-euglycemic clamp studies. Rosiglitazone treatment decreased plasma glucose levels even further in both WT and K107R mice (Fig. 3H). We conclude that while K107R improves insulin sensitivity and glucose tolerance, it is less efficacious than rosiglitazone treatment.

To further compare PPARγ regulation by SUMOylation and TZDs, we performed comprehensive whole-transcriptome RNA sequencing (RNA-seq) analysis with RNA prepared from iWAT and eWAT of DIO WT and K107R mice administered either rosiglitazone or vehicle for 1 wk. Using stringent criteria [≥ twofold or greater change in expression, false-discovery rate (FDR) ≤ 0.05], we identified genes whose expression was either increased or decreased by K107R (compared with WT/vehicle), rosiglitazone (compared with WT/vehicle), or K107R + rosiglitazone (compared with K107R/vehicle) (Datasets S1 and S2). In iWAT, 80, 108, and 233 genes were increased by K107R, rosiglitazone, and K107R + rosiglitazone, respectively (Fig. 4 A and B). While there was surprisingly little overlap in the genes induced by K107R or rosiglitazone alone, the two together induced an additional 153 genes (Fig. 4A and B). In eWAT, many more genes were induced by K107R (406) than by rosiglitazone (67), with only 19 genes in common (Fig. 4C and D). Combining K107R and rosiglitazone induced an additional 202 genes (Fig. 4C and D). Among the genes induced by K107R alone in iWAT was Fgf21 (Dataset S1), which likely contributes to K107R’s insulin-sensitizing effect. Other metabolism-related genes induced by K107R alone in both iWAT and eWAT are elongation of very long-chain fatty acids-like 3 (Elov3), HMG-CoA synthase 2 (Hmgcs2), perilipin 5, and serine palmitoyltransferase long-chain base subunit 3. Additional K107R-induced genes involved in carbohydrate and lipid metabolism are highlighted in Datasets S1 and S2.

In iWAT, the expression of 62, 105, and 375 genes was decreased by K107R, rosiglitazone, or K107R + rosiglitazone, respectively (Fig. 4 A and B). Combining K107R with rosiglitazone repressed an additional 288 genes not seen with either K107R or rosiglitazone alone. In eWAT, 174, 106, and 440 genes were repressed by K107R, rosiglitazone, or K107R + rosiglitazone, respectively; 439 genes were repressed only by the combination of K107R + rosiglitazone (Fig. 4C and D). Thus, K107R and rosiglitazone act together to repress large numbers of genes.

As expected, gene set enrichment analysis (GSEA; FDR ≤ 0.05) showed that rosiglitazone induced genes involved in adipogenesis and lipid metabolism and repressed genes involved in inflammation and lymphocyte activation in both iWAT and eWAT (Fig. 4B and D and Datasets S3 and S4). K107R enhanced rosiglitazone’s...
inductive effects in both WAT depots (Fig. 4 B and D), consistent with its serving as a TZD sensitizer. The effects of K107R and rosiglitazone on inflammation genes were more complicated. In iWAT, the combination of K107R and rosiglitazone repressed many more inflammation-related genes than either did alone (Fig. 4B). However, this profile was not observed in eWAT (Fig. 4D), suggesting that K107R and rosiglitazone have depot-specific effects on inflammation.

Interestingly, K107R induced genes involved in cell lineage determination, including those related to muscle and kidney, in eWAT and to a lesser extent in iWAT (Fig. 4 B and D and Datasets S3 and S4). In contrast, these gene sets were unaffected.

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**Fig. 4.** RNA-seq analysis of iWAT and eWAT. RNA-seq analysis was performed using RNA prepared from iWAT or eWAT of WT and K107R mice treated with vehicle or rosiglitazone for 7 d by oral gavage. Criteria for gene selection were a twofold or greater change in expression and FDR ≤0.05. (A and C) Venn diagrams showing the number and overlap of genes whose expression was increased or decreased by K107R (relative to WT), rosiglitazone (relative to vehicle), or K107R + rosiglitazone (relative to K107R/vehicle). (B and D) Unsupervised hierarchical clustering analysis of the RNA-seq data summarized in A and C. Shown to the right of each panel are the major gene categories represented in the analysis as determined by GSEA (see Datasets S1 and S2 for a list of the differentially expressed genes in the K107R, rosiglitazone, and K107R + rosiglitazone comparisons in iWAT and eWAT, respectively, and Datasets S3 and S4 for a list of the names and statistics for the GSEA gene sets with the most positive or most negative enrichment scores for iWAT and eWAT, respectively).
by rosiglitazone alone and were repressed by the combination of rosiglitazone and K107R (Fig. 4 B and D). These data reveal an unexpected combinatorial relationship between PPARγ SUMOylation and TZD binding in preventing the ectopic expression of transcripts normally present in other mesenchymal tissues. The biological significance of these intriguing findings remains to be determined.

Inductive Effects of K107R Are Adipocyte Autonomous. We examined the effect of K107R on adipocyte differentiation using stromal vascular cells derived from WT and K107R iWAT. Cells were differentiated using insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) in the absence or presence of rosiglitazone. In time-course studies, K107R had no significant effect on Cepha, Cebpβ, and Pparg mRNA expression during the differentiation process (Fig. 5A). In contrast, rosiglitazone increased Cepba expression in WT cells on day 2 and Cebpβ expression in both WT and K107R cells on days 6 and 8 (Fig. 5A). Accordingly, rosiglitazone accelerated neutral lipid accumulation compared with K107R, which had a more modest effect on its own (Fig. 5B). As expected, PPARγ was not SUMOylated in K107R adipocytes (Fig. 5C). Interestingly, PPARγ SUMOylation increased in response to rosiglitazone in WT adipocytes (Fig. 5C), supporting the existence of a feedback regulatory loop. We conclude that the K107R mutation has little effect on adipocyte differentiation in vitro.

We next sought to determine whether genes that are regulated by K107R in iWAT in vivo are similarly regulated in isolated adipocytes in vitro. In an effort to recapitulate our in vivo conditions, stromal vascular cells were differentiated for 8 d using the insulin/dexamethasone/IBMX mixture and subsequently were incubated for 48 h in medium containing either vehicle or rosiglitazone. Based on our RNA-seq analysis, we selected genes from various groups shown in Fig. 4 A that were induced by K107R and/or rosiglitazone in iWAT and compared their regulation in vivo and in vitro by qPCR. These genes included Hmgcs2 (induced by K107R), integrin subunit alpha D (Itgaid; induced by K107R), oxidized low-density lipoprotein receptor 1 (Olr1; induced by K107R + rosiglitazone), Fgf21 (induced by K107R, K107R + rosiglitazone), enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrase (Ehhadh; induced by rosiglitazone, K107R + K107R), Elovl3 (induced by K107R, rosiglitazone, K107R + rosiglitazone), and pyruvate dehydrogenase kinase 4 (Pdk4; induced by K107R, rosiglitazone, and K107R + rosiglitazone). Similar induction profiles in response to K107R and/or rosiglitazone were seen in iWAT, eWAT, and isolated iWAT adipocytes (Fig. 6 A). Thus, K107R regulates the expression of these genes in an adipocyte-autonomous manner. Consistent with these findings, knockdown of the E2 ubiquitin conjugating enzyme, UBC9, which is required for PPARγ-TZD binding in preventing the ectopic expression of transcripts normally present in other mesenchymal tissues. The biological significance of these intriguing findings remains to be determined.

We performed a similar qPCR analysis on genes repressed by K107R and/or rosiglitazone in iWAT. These genes included Gstα3 (repressed by K107R), WNT1 inducible signaling pathway protein 2 (Wisp2; repressed by rosiglitazone), nerve growth factor (Ngf; repressed by K107R and rosiglitazone), BPI fold-containing family B member 6 (Bpifb6; repressed by K107R, rosiglitazone, and K107R + rosiglitazone), and nitric oxide synthase 2 (Nos2; repressed by K107R, rosiglitazone, and K107R + rosiglitazone). These genes showed the expected regulation in response to K107R and/or rosiglitazone in iWAT and, with the exception of Gsta3, in eWAT (Fig. 7). The repression profile for Gsta3 was reproduced in isolated adipocytes, while Wisp2 showed a similar pattern of regulation in vitro and in vivo. However, the repression of Ngf and Nos2 was not...
Fig. 6. K107R induces gene expression in an adipocyte-autonomous manner. (A) Comparison of gene regulation by K107R and rosiglitazone in vivo and in vitro. For the in vivo analysis, RNA was prepared from iWAT and eWAT from WT and K107R mice administered rosiglitazone or vehicle by oral gavage for 7 d. For the in vitro analysis, RNA was prepared from WT and K107R stromal vascular cells derived from iWAT and differentiated for 8 d using the insulin/dexamethasone/IBMX mixture and subsequently incubated for 48 h with either rosiglitazone or vehicle. Gene expression was measured by qPCR. Cycle threshold (Ct) values are shown for WT/vehicle conditions. (B) Immunoblotting for SUMO1-conjugated PPARγ (SUMO1) and total PPARγ following PPARγ immunoprecipitation (IP) (Top Two Rows) and total PPARγ, UBC9, and β-actin in total cell extract (Bottom Three Rows) from WT and K107R adipocytes treated with a control or UBC9 siRNA. (C) Quantification of Fgf21 and Elovl3 gene expression by qPCR using RNA prepared from WT and K107R adipocytes exposed to either the UBC9 or control siRNA. Ct values are shown for WT/control siRNA conditions. For B and C, WT and K107R stromal vascular cells were treated with control or UBC9 siRNA for 2 d in growth medium. The cells were subsequently incubated for 2 d in differentiation medium containing insulin, dexamethasone, IBMX, and rosiglitazone, followed by 2 d in medium containing only insulin and rosiglitazone. *P < 0.05; **P < 0.01; ***P < 0.001.
recapitulated in vitro, and Bpifb6 mRNA was below the limits of detection in isolated adipocytes (Fig. 7). We conclude that K107R and rosiglitazone can repress gene expression through either adipocyte-autonomous or adipocyte-nonautonomous mechanisms of action, depending on the gene.

**Discussion**

K107 SUMOylation markedly inhibits PPARγ transcriptional activity in vitro (4–6). However, whether PPARγ K107 SUMOylation is relevant in vivo has remained unclear. While we previously showed that FGF21 inhibits PPARγ SUMOylation in murine WAT (10), another group failed to detect SUMOylated PPARγ under similar conditions (12). Here, using homozygous K107R mice, we demonstrate unequivocally that PPARγ is SUMOylated in WAT and that this postranslational modification has significant physiologic consequences. Among the genes induced by K107R in WAT was Fgf21. Thus, the FGF21–PPARγ pathway is a self-reinforcing regulatory loop.

K107R recapitulates a subset of rosiglitazone’s and FGF21’s metabolic actions in DIO mice. These include reducing plasma NEFA concentrations and increasing insulin sensitivity. However, unlike rosiglitazone and FGF21, K107R did not induce circulating adiponectin concentrations, nor did it decrease plasma triglyceride levels. In glucose tolerance and insulin tolerance assays, K107R was less efficacious than rosiglitazone. Interestingly, unlike TZDs, which cause weight gain by increasing adiposity and fluid retention (1, 2), K107R modestly decreased body weight in mice fed a high-fat diet. We did not observe changes in histology or Ucp1 mRNA levels in WAT from K107R mice, suggesting that browning of WAT is unlikely to be the mechanism. These findings suggest that it may be possible to pharmacologically uncouple PPARγ’s insulin-sensitizing actions from weight gain by targeting PPARγ SUMOylation status.

RNA-seq studies with WAT revealed two interesting effects of blocking K107 SUMOylation. First, the K107R mutation sensitized WAT to rosiglitazone’s induction of metabolism-related genes. Since FGF21 inhibits PPARγ SUMOylation, these data are consistent with previous studies showing that FGF21-KO mice are refractory to the insulin-sensitizing actions of TZDs (10) and that FGF21 and TZDs cooperate in inducing glucose uptake into adipocytes in vitro (13). Second, K107R caused ectopic expression of genes involved in the differentiation and function of other cell lineages (e.g., muscle and kidney). These findings suggest an unexpected role for PPARγ SUMOylation in repressing genes normally expressed in other tissues.

In addition to SUMOylation, PPARγ’s transcriptional activity is also regulated by other postranslational modifications, including phosphorylation. Like SUMOylation at K107, PPARγ phosphorylation at the nearby residue, S112, inhibits its transcriptional activity (1–3). In cell-based experiments, mutating S112 to prevent phosphorylation causes a corresponding decrease in PPARγ SUMOylation, indicating that PPARγ is regulated by a
coordinate phosphorylation–SUMOylation switch (11). Accordingly, there are strong similarities between K107R- and S112A-mutant mice (14). When challenged with a high-fat diet, both have reduced plasma NEFA levels and are refractory to diet-induced insulin resistance without weight gain. However, S112A mice also had increased plasma adiponectin concentrations and decreased plasma triglyceride concentrations (14), which we did not observe in K107R mice. In addition, we found no evidence of S12 phosphorylation in K107R mice, indicating that SUMOylation does not regulate phosphorylation at this site. Thus, while PPARy may be regulated similarly by phosphorylation and SUMOylation, S112 phosphorylation and K107 SUMOylation are also likely to have distinct physiologic effects.

PPARy is also phosphorylated at S273 and acetylated at K268 in somatic cell lines (15, 16). Interestingly, PPARy translocation and phosphorylation with fewer side effects. Currently, K107R recapitulates the insulin-sensitizing actions of resoligotane, albeit with reduced efficacy, and that it enhances the effect of rosiglitazone on genes involved in metabolism. Importantly, K107R improves insulin sensitivity without increasing body weight or adiposity. Thus, inhibiting PPARy K107 SUMOylation may provide a mechanism for generating novel PPARy-targeted insulin-sensitizing drugs with fewer side effects.

Materials and Methods

Generation of PPARy-K107R Mice. The PPARy-K107R targeting vector was constructed using a recombineering-based method (19). A 10,268-bp genomic DNA fragment containing exons 2 and 3 of the PPARy2 gene was transferred from BAC clone RP23-207Q20 to a vector containing the diptheria toxin negative-selection gene. The point mutation corresponding to K107R was synthesized and cloned into the S′ end of an frt-Neo-frt cassette. The lengths of the S′ and S homologous arms were 5,473 bp and 4,434 bp, respectively. The neo cassette was inserted in intron 3, 192 bp downstream of the start codon. The vector was electroproporated into E1 129Sv/Ethi hybrid ES cells derived by the Janelia Transgenic Facility. The G418-resistant ES cells were cloned by nested PCR using primers outside the construct paired with primers inside the inserted cassette. The primer sequences were 3′ arm forward primers, PPARy2 Scr F1: GACACTGACTACTCTCGAG and PPARy2 Scr F2: TCGATTCACTTGGCACGCA. Reverse primers were PKG R1: TTGGATGGGTATGTCGGCA and PKG R2 TAAAGGGATCCCTCGAG. The arm forward primers were Neo F3: CTCTCCTGGTATCGGTA and Neo F4: ACGAGGTCTCTGAGGAT. Reverse primers were PPARy2 Scr R3: CTGGTCCTGAAATGCTCGAC and PPARy2 Scr R4: AATCCTGCTGACCTGGAA. The PCR′ ES cells were aggregated with eight-cell CD-1 strain embryos. The neo cassette was removed by breeding germline chimeras with ROSA26FLP1 homozygous mice. The point mutation corresponding to K107R was recombined into the S′ end of an frt-Neo-frt cassette. The lengths of the S′ and S homologous arms were 5,473 bp and 4,434 bp, respectively. The neo cassette was inserted in intron 3, 192 bp downstream of the start codon. The vector was electroproporated into E1 129Sv/Ethi hybrid ES cells derived by the Janelia Transgenic Facility. The G418-resistant ES cells were cloned by nested PCR using primers outside the construct paired with primers inside the inserted cassette. The primer sequences were 3′ arm forward primers, PPARy2 Scr F1: GACACTGACTACTCTCGAG and PPARy2 Scr F2: TCGATTCACTTGGCACGCA. Reverse primers were PKG R1: TTGGATGGGTATGTCGGCA and PKG R2 TAAAGGGATCCCTCGAG. The arm forward primers were Neo F3: CTCTCCTGGTATCGGTA and Neo F4: ACGAGGTCTCTGAGGAT. Reverse primers were PPARy2 Scr R3: CTGGTCCTGAAATGCTCGAC and PPARy2 Scr R4: AATCCTGCTGACCTGGAA. The PCR′ ES cells were aggregated with eight-cell CD-1 strain embryos. The neo cassette was removed by breeding germline chimeras with ROSA26FLP1 homozygous females (Jackson Laboratories stock no. 003946). Correct targeting was confirmed by the lack of a deficiency phenotype.

Plasma Measurements. WT and K107R mice fed the high-fat diet for 19 wk were fasted for 4 h, and blood was collected from the tail vein for measuring glucose levels. Glucose was determined at 95 °C for 5 min and filtered using Spin-X centrifuge tubes (Costar). The immunoprecipitated samples and total extracts were resolved by SDS/PAGE and blotted onto a nitrocellulose membrane, and the proteins of interest were detected by antibodies against PPARγ

ELISA kits were used to measure insulin (Crystal Chem, Inc.) and adiponectin and leptin (Millipore).

Glucose Tolerance and Insulin Tolerance Tests. Glucose tolerance tests were performed on mice that were fasted for 16 h and i.p. injected with 2 g/kg glucose. Insulin tolerance tests were performed in mice that were fasted for 4 h and i.p. injected with 0.75 U/kg insulin (Sigma).

Hyperinsulinemic–Euglycemic Clamp Studies. Studies were performed as described (20) using WT and K107R mice fed the high-fat diet for 19 wk. Briefly, mice were fasted for 4 h before the experiments. At t = -90 min, continuous infusion of [3H]2-deoxyglucose (0.05 μCi/min) was started to measure gluco

ulence, and basal blood samples were collected from the tail vein at t = -15 and t = -5 min. Continuous infusion of insulin (4 μU kg-1 min-1) was started at t = 0 with the tracer infusion increased to 0.1 μCi/min. Blood glucose levels were monitored every 10 min, and the blood glucose concentration was maintained at ~150 mg/dl by infusion of 50% dextrose. At steady state, between 80 and 120 min, tail vein blood was collected every 20 min to determine glucose turnover.

In Vitro Adipocyte Differentiation. Preadipocytes were derived from the stromal vascular fraction of neonatal inguinal adipose tissue as described (10). Briefly, the isolated fat pads were digested with 0.2% collagenase II (Worthington) in HBSS containing 2% BSA at 37 °C for 1 h. The tissue was cultured by pipetting and centrifuged at 300 × g for 2 min. The PPARy and SUMOylation, phosphorylation, and acetylation all provide a means for separating PPARy’s beneficial effects from its adverse adipogenic effects.

In closing, we show that PPARy is SUMOylated at K107 in WAT and that this regulates PPARy transcriptional activity. We further demonstrate that the K107R mutation recapitulates the insulin-sensitizing actions of resoligotane, albeit with reduced efficacy, and that it enhances the effect of resoligotane on genes involved in metabolism. Importantly, K107R improves insulin sensitivity without increasing body weight or adiposity. Thus, inhibiting PPARy K107 SUMOylation may provide a mechanism for generating novel PPARy-targeted insulin-sensitizing drugs with fewer side effects.
RNA-Seq. Groups of K107R and WT littermates fed the high-fat diet for 19 wk were administered either vehicle (1% methylcellulose) or rosiglitazone (10 mg kg⁻¹ d⁻¹) by oral gavage for 1 wk (n = 6 mice per group). Total RNA from iWAT or eWAT from each group was prepared, pooled, and run on an Agilent TapeStation 4200 system to confirm quality (RNA integrity number > 8). RNA-Seq libraries were generated using DNase-treated total RNA (4 μg) and the TrueSeq Stranded mRNA kit (Illumina). Poly-A RNA was purified and fragmented, and cDNA was synthesized. cDNA was A-tailed, and indexed adapters were ligated. After ligation, samples were PCR amplified and purified with Ampure XP beads (Beckman Coulter), and the quality was confirmed using the Agilent TapeStation 4200 system. Before normalization and pooling, samples were quantified by Qubit and then were run on the Illumina NextSeq 500 sequencer using V2 reagents. Raw data were then demultiplexed and converted to FASTQ files using Bcl2fastq (v2.17, Illumina). The FASTQ files were checked for quality using FastQC (v0.11.2) (21) and FASTQ Screen (v0.4.4) (22). FASTQ files were mapped to the mm10 assembly of the mouse genome using TopHat (23); fragments per kilobase per million mapped reads (FPKM) calculation and differential expression analysis were performed using Cufflinks (v 2.2.1) (24). RNA-seq data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus databank (accession no. GSE120332). We used the union set of differentially expressed genes with fold change ≥2 and adjusted P value ≤ 0.05 across the three different comparisons (WTvehicle vs. K107R/vehicle, WT/vehicle vs. WT/rosiglitazone, and K107R/vehicle vs. K107R/rosiglitazone) in iWAT or eWAT for visualization. Heatmaps were generated using log2-transformed fold changes, and hierarchical clustering was performed using the Euclidean distance metric and the ward.D2 method using the heatmap.2 function available in the gplots R package. To analyze the enrichment of gene sets in rank-ordered gene lists obtained for the expression changes in the three different comparisons in iWAT or eWAT samples, we performed GSEA (25). We used the signal-to-noise ratio metric to rank the genes.

gPCR. RNA from adipose tissue or isolated adipocytes was purified using the RNeasy Lipid Tissue Mini Kit (Qiagen) or RNA Stat-60 (Ambion), respectively. cDNA was synthesized from 2 μg total RNA using High-capacity cDNA Transcription Kits (Thermo Fisher Scientific) with random primers. gPCR was performed with the primer sets listed in SI Appendix, Table S1.

RNAi. The control SMARTpool of nontargeting siRNA (D-001810-10-05) and siRNA specific for the RNA encoding UBC9 (D-040661-02-0005) pooled with sequences GGGAAGGGGCUUGUCUCA, GGGUCAGCAGAAGGCGAAGG, CUACACAAUUCACGUCCAA, and AGAUCAUAUGGCUCGGUA were purchased from Dharmacon. Preadipocytes isolated from WT and K107R neonatal mice were seeded onto 10-cm or 24-well dishes and were cultured for 3 d. The siRNAs were transfected using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) with OptiMEM according to the manufacturer’s protocol, and the siRNA-transfected cells were further cultured another 2 d. Adipogenesis was induced by culturing those cells for 2 d with DMEM supplemented with 10% non-heart-inactivated FBS, 5 mg/mL insulin, 1 μM dexamethasone, 0.5 mM IBMX, and 1 μM rosiglitazone followed by culturing for 2 d with DMEM + 10% FBS supplemented with insulin and rosiglitazone only. Immunoprecipitation and detection of proteins of interest were performed as described above by using the cells on 10-cm dishes. The cells on 24-well dishes were used for isolation of RNA for gPCR.

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