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TET2 facilitates PPARγ agonist–mediated gene regulation and insulin sensitization in adipocytes

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Abstract

Emerging evidence indicates that epigenetic mechanisms like DNA methylation directly contribute to metabolic regulation. For example, we previously demonstrated that *de novo* DNA methyltransferase Dnmt3a plays a causal role in the development of adipocyte insulin resistance. Recent studies suggest that DNA demethylation plays an important role in the developmental process of adipocytes. However, little is known about whether DNA demethylase ten-eleven translocation (TET) proteins regulate the metabolic functions of adipocytes.

METHODS: The expression of Tet genes was assessed in the fractionated adipocytes of chow- and high fat diet–fed C57/Bl6 mice using qPCR and western blotting. The effect of *Tet2* gain- or loss-of-function in fully mature 3T3-L1 adipocytes in the presence/absence of Rosiglitazone (Rosi) and TNF-α on insulin sensitivity was using the insulin-stimulated glucose uptake and insulin signaling assays. Gene expression and DNA methylation analyses of PPARγ target genes was performed in the same setting. In addition, PPARγ reporter assays, co-immunoprecipitation assays, PPARγ ChIP-PCR analyses were performed.

RESULTS: We found that adipose expression of TET2, alone among its family members, was significantly reduced in diet-induced insulin resistance. TET2 gain-of-function was sufficient to promote insulin sensitivity while loss-of-function was necessary to facilitate insulin sensitization in response to the PPARγ agonist Rosiglitazone (Rosi) in cultured adipocytes. Consistent with this, TET2 was required for Rosi-dependent gene activation of certain PPARγ targets accompanied by changes in DNA demethylation at the promoter regions. Furthermore, TET2 was necessary to sustain PPARγ binding to target loci upon activation with Rosi via physical interaction with PPARγ.

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Author Contributions

BF, XM, SK conceived of the experimental plan. BF, XM, SD, and DY performed experiments and analyzed data. wrote manuscript. LRC, AP, and SF helped with experiments. BF and SK wrote manuscript.

CONCLUSIONS: Our data demonstrate that TET2 works as an epigenetic regulator of Rosi-mediated insulin sensitization and transcriptional regulation in adipocytes.

Keywords

insulin sensitivity; adipocytes; epigenetics; DNA demethylation; TET proteins

Introduction

Insulin resistance is a key underlying feature of type 2 diabetes and a frequent complication in multiple clinical conditions including obesity, aging, and cardiovascular disease. Extraordinary efforts have gone into defining the mechanisms that underlie insulin resistance, with most of the attention focused on insulin signal transduction and other mitochondrial and cytosolic pathways such as ER stress [1-5]. The data supporting the involvement of these non-mutually exclusive pathways are convincing, but there is still a great deal of uncertainty about the mechanisms by which cells and organisms become insulin resistant.

Environmental factors, such as diet and aging, are important risk factors for metabolic disorders. The intricate interaction between genes and the environment is mediated by epigenetic mechanisms, suggesting they play a major role in the etiopathogenesis of metabolic dysregulation. DNA methylation is an epigenetic mark involving the covalent transfer of a methyl group to the C-5 position (5mC) of cytosine by DNA methyltransferases (Dnmts) [1]. Mounting evidence points to a role for DNA methylation in the pathogenesis of metabolic disorders: 1) obesity in agouti mice is associated with reduced DNA methylation at the regulatory region of the *agouti* gene, leading to its ectopic expression during development [2]; 2) changes in DNA methylation at key metabolic genes, such as *Cox7a1* [3], *PPARGC1a* [4], *IGF-2* [5], and *Pomc* [6], associate with various metabolic insults including those from aging, obesity, anorexia, and prenatal exposure to famine; 3) recent genome-wide profiling studies have identified distinct global DNA methylation patterns that associate with obesity and diabetes [7-9]; 4) altered DNA methylation is linked to the transgenerational passage of metabolic disorders [9-11]; and 5) both pharmacological and genetic studies have shown that certain DNMTs directly contribute to adipose insulin resistance [10, 11].

DNA methylation was long thought to be a static epigenetic mark, but recent evidence demonstrates that it undergoes dynamic remodeling [12], which involves the ten-eleven translocation (TET) protein family acting as DNA demethylases. TET proteins (TET1, 2, and 3) oxidize 5mC to hydroxymethylcytosine (5hmC), which is then converted to unmethylated cytosine (5C) through base excision repair (BER) and thymidine DNA glycosylase (TDG). In contrast to DNA methylation, TET-mediated DNA demethylation associates with increased enhancer activity and transcription factor occupancy in different biological contexts. For example, 5mC enrichment at distal enhancers is inversely correlated with enhancer activity [13-15], whereas 5hmC enrichment is positively correlated with enhancer activity and Foxp3 binding in immune cells [16-18]. A recent large-scale functional association analysis from GWAS data identified a genetic variant within *TET2*

(rs9884482) that associates with fasting insulin level, suggesting an impact on insulin resistance [19]. Furthermore, recent global profiling studies have demonstrated that 5hmC colocalizes with PPARγ binding at enhancers in 3T3-L1 adipocytes [20], and PPARγ-positive nuclei sorted from visceral adipose tissue from healthy humans is strongly co-enriched with 5hmC [21]. Together, these studies suggest the presence of functional and physical interactions between TET proteins and PPARγ.

Here, we found that adipocyte expression of *Tet2* is downregulated in obesity. Our gain- and loss-of-function studies in 3T3-L1 adipocytes found that TET2 was required for Rosiglitazone (Rosi)-mediated insulin sensitization. Consistent with this, Tet2-knockdown adipocytes had decreased expression of some of the key PPARγ target genes important for insulin sensitivity in response to Rosi. TET2-dependent gene regulation of PPARγ targets involved site-selective DNA demethylation at the promoter regions. Mechanistically, we show that TET2 and PPARγ physically interact and that TET2 is required to maintain DNA binding of PPARγ upon ligand activation.

Methods

Cell culture:

3T3-L1 preadipocytes were obtained from ATCC and maintained and differentiated as described [22]. These cells had the ability to differentiate and were confirmed to be mycoplasma negative. To generate lentivirus particles, lentiviral constructs were cotransfected with pMD2.G- and psPAX2-expressing plasmids into 293T cells. After 48 hr, the virus-containing supernatant was collected, filtered through 0.45-mm filters, and added to mature 3T3-L1 adipocytes for 24 hr along with 8 mg/ml polybrene. Immortalized human preadipocyte cell line was cultured as described [23]. Transduction efficiency was determined by comparing to cells transduced in parallel with a GFP-expressing lentivirus.

^{3}H-2-DG assay:

Mature 3T3-L1 adipocytes were incubated in serum-free DMEM for 4–6 hr. Cells were then washed three times with KRH buffer (12 mM HEPES, pH 7.4, 121 mM NaCl, 5 mM KCl, 0.33 mM $CaCl_2$, and 1.2 mM $MgSO_4$) and incubated for 20 min in KRH buffer in the absence or presence of 50 nM insulin. Cells were treated with 2-deoxy-d-[2,6-^{3}H]-glucose (0.33 mCi/ml) for another 10 min. Glucose uptake was stopped quickly by three rapid washes with KRH buffer containing 200 mM glucose and 10 mM cytochalasin B on ice. Cells were solubilized in 0.1% SDS for 30 min, and radioactivity was measured by liquid scintillation counting.

Antibodies:

Antibodies were purchased from Cell Signaling Technology (PPARγ, Cat: 2443S, Lot: 4; Akt, Cat: 9272; Lot: 22; pAkt [S473], Cat: 9271, Lot: 12; IRS-1, Cat: 2382; Lot: 4), from Thermo Fisher Scientific (β-Actin: MA5-14739; pIRS1[pY612]: Cat: 44–816G, Lot: 1100477B), from Proteintech Group (TET2, Cat: 21207-1-AP, Lot: 00047680), Millipore (Ser82 PPARγ), from Santa Cruz Biotechnology (c-Myc, Cat: SC-40, Lot: F1917).

RNA extraction and quantitative PCR:

Total RNA was extracted from cells or tissues using TRIzol reagent according to the manufacturer's instructions. cDNA was reverse-transcribed from 1 μg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative PCR (qPCR) was performed with SYBR Green qPCR Master Mix (Bioneer) using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Primer sequences are listed in Supplementary Table 1. The relative amount of mRNA normalized to 36B4 was calculated using the delta-delta method [24].

Plasmids:

Tet2-CD (#79554) and Tet2-HxD (#79611) cDNA was purchased from Addgene, PCR amplified, and subcloned into pCDH vector with the XbaI and EcoRI sites. sgRNAs that targeted *Tet2* were cloned into lentiCRISPR v2 vector. Hairpin and sgRNA sequences are shown in Supplementary Table 1.

hMeDIP-qPCR:

Genomic DNA was sheared using a Covaris S220 to an average of 200–800 bp. Two micrograms of denatured DNA was incubated with 2 mg of anti-5-hydroxymethylcytidine antibody (EpiGentek) in IP buffer (10 mM Na-Phosphate pH 7.0, 0.14 M NaCl, 0.05% Triton X-100) for 2 hr at 4°C. Antibody-bound DNA was collected with 20 ml of Dynabeads anti-mouse IgG (Invitrogen Dynal AS) for 1 hr at 4°C on a rotating wheel and successively washed five times with washing buffer (0.1% SDS, 1 Triton X-100, 2 mM EDTA, 20 mM Tris-HCI pH 8.1, 150 mM NaCl) and twice with TE (10 mM TrisCl, 1 mM EDTA pH 8.0). DNA was recovered in 125 ml of digestion buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.5% SDS, 35 mg proteinase K) and incubated for 3 hr at 65°C. Recovered DNA was used for qPCR analysis. Primers for MeDIP-qPCR studies are listed in Supplementary Table 1. All data were normalized to input.

ChIP-qPCR:

Cells were crosslinked with 1% formaldehyde for 10 min at room temperature. Cross-linked chromatin was sonicated using an S220 Ultrasonicator (Covaris) to generate DNA fragments of ~150–600 bp. Inputs were taken from cleared lysates, and the rest were rotated O/N at 4°C with PPARγ (Cell Signaling Technology, 2443S) and IgG antibodies to precipitate protein:DNA complexes. An aliquot of 20 μl of pre-washed Dynabeads Protein G was added per IP and rotated 1 hr at 4°C. Beads were successively washed in low-salt RIPA buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Triton x-100, 0.1% SDS, 140 mM NaCl, 0.1% Na deoxycholate), high-salt RIPA buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Triton x-100, 0.1% SDS, 500 mM NaCl, 0.1% Na deoxycholate), LiCl buffer (250 mM LiCl, 0.5% NP40, 0.5% Na deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.0]) and TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA). Each reaction was then incubated in digestion buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl, 0.5% SDS, proteinase K) for a minimum of 4 hr at 65°C to reverse cross-links. DNA was recovered using a phenol-chloroform extraction. Real-time qPCR primers are listed in Supplementary Table 1. All data were corrected for multiple hypothesis testing. All data were normalized to input.

Co-immunoprecipitation:

Co-immunoprecipitation (Co-IP) was performed according to a modified protocol as previously described[22]. Briefly, 293T cells were transfected with various DNA constructs using Lipofectamine 3000 (Invitrogen). A day after transfection, cells were lysed with Triton X lysis buffer (Boston Bio Products) containing 50 mM Tris-HCL, pH 7.4, 150 mM NaCl 1% Triton X-100, 5 mM EDTA, plus protease inhibitors (complete, Roche). 500 mg of protein was incubated with the appropriate antibodies overnight. The next day, protein A/G Dynabeads (Thermo Fisher Scientific) were added and incubated for 1 hr, washed with lysis buffer five times and PBS once. Beads were eluted with non-reducing SDS/PAGE loading buffer and subjected to SDS/PAGE and Western blotting.

Western blot analysis:

Whole-cell protein lysates were prepared according to the manufacturer's protocol using RIPA lysis buffer and protease inhibitor cocktail. 20–30 μg of protein was resolved using 4–20% Tris-glycine gradient gels and transferred to PVDF membrane. After blocking with 5% non-fat dried milk in PBSTween (0.25%), membranes were incubated with the appropriate primary antibodies against FLAG (Sigma), MYC, PPARγ (Cell signaling technology), or TET2 (Proteintech Group). Anti-goat and anti-mouse or rabbit IgG–peroxidase conjugate (Sigma) were used to detect primary antibodies.

Luciferase reporter assays:

Reporter plasmids were co-transfected with expression vectors of Tet2-CD, Tet2-HxD, and PPARγ into 293T cells using a Profection kit (Promega Corporation). The Renilla reference plasmid was co-transfected to normalize transfection efficiency, and the mass of transfected plasmids was balanced with empty vector (pCDH-EGFP). 48 hr after transfection, cells were harvested and measured using the Dual-Luciferase Reporter Assay System (Promega Corporation).

Results

Tet2 expression is enriched in adipocytes, and adipocyte *Tet2* expression is diminished in diet-induced obesity.

To explore the potential role of TET proteins in adipocytes, we assessed their relative expression between the adipose and stromal-vascular fraction (SVF), which contains preadipocytes (Fig. 1, Supplemental Fig. 1). Overall, *Tet3* expression was highest, followed by *Tet2* and *Tet1*, in all three tested adipose depots and in both fractions (Supplemental Fig. 1). However, only *Tet2* was expressed more in adipocytes than in SVF (Fig. 1A-C). Moreover, both mRNA and protein expression of *Tet2* in adipocytes was significantly reduced in a model of diet-induced obesity (DIO) (Fig. 1E, G). This implies a regulatory role for TET2 in the metabolic function of mature adipocytes.

TET2 plays a necessary and sufficient role for Rosi-mediated insulin sensitization in cultured adipocytes.

To investigate the potential role of TET2 in the regulation of insulin sensitivity, we performed a 2-deoxyglucose uptake assay in fully mature 3T3-L1 adipocytes that were lentivirally transduced with single-guide RNAs specific for *Tet2* and an empty vector (Supplemental Fig. 2). In control adipocytes, TNF-α, a mediator of insulin resistance, potently inhibited insulin-stimulated glucose uptake, whereas the TZD compound Rosiglitazone (Rosi) enhanced it (Fig. 2A). *Tet2**knockdown adipocytes had a similar basal insulin sensitivity, and as expected, TNF-α caused insulin resistance similar to control cells (Fig. 2A). However, Rosi largely failed to improve insulin sensitivity in the presence and absence of TNF-α (Fig. 2A). This result suggests that TET2 plays a necessary role in Rosi-mediated improvement of insulin sensitivity in cultured adipocytes.

Next, we tested whether TET2 gain-of-function is sufficient to improve insulin sensitivity in adipocytes. Overexpressing the catalytic domain of the wild-type allele (Tet2-CD) was sufficient to increase insulin-stimulated glucose uptake (Fig. 2B). Previous studies report a catalytic and non-catalytic function for *Tet2*; therefore, we asked whether its DNA demethylase activity is critical for facilitating insulin sensitivity by overexpressing the catalytically inactive allele Tet2-HxD [25], which largely lacks DNA demethylase activity (Supplemental Fig. 3). To our surprise, Tet2-HxD still promoted insulin-stimulated glucose uptake, albeit to a lesser extent than wild type (Fig. 2B). To test whether the role of TET2 is conserved in human adipocytes, we performed gain-of-function study using immortalized human preadipocytes [23], Similar to 3T3-L1 adipocytes, overexpression of both TET2-CD and HxD significantly increased insulin-stimulated glucose uptake (Fig. 2C).

Together, these results indicate that TET2 facilitates insulin sensitivity both in a DNA demethylase-dependent and -independent manner. Despite a clear phenotypic difference in insulin-stimulated glucose uptake between these models of TET2 gain- and loss-of-function, there was a marginal difference in insulin signal transduction in both settings (Supplemental Fig. 4).

TET2 facilitates the transcriptional activity of PPARγ in both a catalytic-dependent and independent manner.

TET-dependent DNA demethylation associates with transcriptional regulation in several biological contexts including adipogenesis [26-29]. To determine the role of TET2 in Rosi-mediated transcriptional regulation, we measured a set of PPARγ target genes including those important to the regulation of insulin sensitivity [46]. Consistent with the insulin-stimulated glucose uptake assay, *Tet2*-knockdown adipocytes had reduced ability to acutely stimulate the transcription of PPARγ targets in response to Rosi, with and without TNF-α (Fig. 3). For example, Rosi-induced expression of *Fabp4, Adipoq,* and *Fgf21* was not seen in *Tet2*-knockdown adipocytes (Fig. 3A-D). Of note, this was not due to the loss of PPARγ expression (Supplemental Fig. 5). Conversely, the expression of these PPARγ target genes was significantly increased by the overexpression of Tet2-CD and less potently by Tet2-HxD (Fig. 3E-H). Consistent with these expression patterns, we observed reduced DNA

demethylation activity at several CpG-rich regions of the *Adipoq* and *Fabp4* promoters upon TET2 loss-of-function (Fig 4).

Tet2 promotes PPARγ-driven transcriptional activity through a physical interaction.

To directly assess whether TET2 regulates PPARγ-driven transcriptional activity, we used a reporter assay with PPARγ X3-TK-luc, which contains 3 copies of the PPARγ-binding DR1 site, and a plasmid with the Glut4 2.4-kb promoter (Fig. 5A, B). Overexpression of Tet2-CD or Tet2-HxD alone significantly increased the activity of both reporter plasmids, and the effect was additive when co-expressed with PPARγ (Fig. 5A, B).

PPARγ colocalizes with 5hmC in adipocytes [20]; therefore, we reasoned that TET2 and PPARγ may physically interact, which could lead to TET2 regulating the transcriptional activity of PPARγ. Indeed, we found that the tagged version of TET2 and PPARγ co-immunoprecipitated in 293T cells in the presence and absence of Rosi (Fig. 5C). Furthermore, the physical interaction of the native proteins was confirmed in 3T3-L1 adipocytes (Fig. 5D). These results suggest that TET2 works in the same protein complex as PPARγ, modulating its transcriptional activity. Further, we investigated whether TET2 may affect post-translational modifications (PTM) of PPARγ [30-32], which are important for the regulation of transcriptional activity of PPARγ. In result, we did not see a major difference in the levels of Ser283 or acetylation of PPARγ in both TET2 gain- and loss-of-function settings (Supplemental Fig. 5F, G).

TET2 is required for PPARγ to maintain DNA binding at selective target *loci* in response to Rosi.

TET proteins and DNA demethylation at gene regulatory regions affects the DNA binding affinity of transcription factors. Thus, we examined PPARγ binding affinity at previously characterized PPARγ binding sites [23] after Rosi treatment using ChIP-PCR. PPARγ binding was mostly intact with TET2 gain-of-function (Supplemental Fig. 6), suggesting TET2 alone is not sufficient to increase PPARγ binding to target loci. Also, overall, the basal level of PPARγ binding was intact in *Tet2*-knockdown cells (Fig. 6). However, PPARγ binding affinity was greatly weakened by Rosi treatment in *Tet2*-knockdown cells compared to the control (Fig. 6), which suggests that TET2 is required to sustain PPARγ binding upon ligand activation. Together, these results suggest that TET2 plays an important role in facilitating the Rosi-stimulated transcriptional program by sustaining PPARγ binding.

Discussion

We previously demonstrated a causal role for DNMT3a in the development of adipocyte insulin resistance both *in vitro* and *in vivo* [11]. Here, we revealed an opposing functional role for TET2 as a modulator of insulin sensitivity. In particular, we demonstrated that TET2 plays an important role in Rosi-mediated insulin sensitization and transcriptional regulation in adipocytes. Although the functional roles of Dnmt3a and TET2 are opposite in the regulation of insulin sensitivity, their underlying mechanisms, such as gene targets, do not seem to converge. We did not find obvious PPARγ target genes using RNA-Seq on DNMT3a gain- or loss-of-function models.

Emerging evidence indicates that epigenetic control directly contributes to metabolic regulation. For example, administering pan-inhibitors for histone deacetylase has beneficial metabolic effects in both mice and humans, such as increased energy expenditure and insulin sensitivity and secretion [33-36]. In line with pharmacological studies, mutant mouse models carrying loss-of-function histone modifiers (e.g., *Mll2, Ehmt1, Jmhd2a, Lsd1*) have profoundly different whole-body metabolism[37-41].

PPARγ is the master transcription factor of adipogenesis, as evidenced by its necessary and sufficient role [42,43]. The expression of PPARγ is highly induced during adipogenesis, and it is expressed primarily in adipose tissue [44,45], In addition to development, PPARγ plays a pivotal role in the regulation of numerous genes important for adipocyte functions such as lipid metabolism, adipokine secretion, and insulin sensitivity [46]. TZDs, which are synthetic ligands of PPARγ, exert a powerful insulin-sensitizing effect *in vitro* and *in vivo* [47], primarily working on the adipose tissue [48,49]. It has been proposed that the insulin-sensitizing effect of Rosi involves the regulation of key adipocyte target genes including adiponectin [50] and FGF-21 [51] while suppressing the expression of genes promoting insulin resistance including TNF-α [52], *Resistin* [53], and *Rbp4* [54]. Thus, it is critical to fully understand how TZDs regulate the transcriptional program of PPARγ to achieve healthy adipose development and metabolism.

It is relatively well understood how TZDs activate PPARγ at the molecular level. For example, ligand activation of PPARγ recruits coactivators such as SRC-1, CBP, p300, and MED1 [55-58]. However, it is poorly understood whether ligand activation of PPARγ involves epigenetic mechanisms. Recent studies suggest that TET protein–mediated DNA demethylation is necessary for adipogenesis *in vitro.* In addition, 5hmC enrichment overlaps with enhancer peaks induced during adipogenesis [20], and it's enriched in PPARγ-positive nuclei in association with other chromatin modifiers [21]. Consistent with this, PPARγ induces DNA demethylation at the Plin promoter region during adipogenesis [59]. These studies strongly suggest that TET-mediated DNA demethylation is important in adipocyte development. By contrast, our study focuses on the functional role of TET2 in the metabolic function of mature adipocytes.

During the revision of our manuscript, Wu et al published their work by revealing a novel axis between TET2 and AMPK in the regulation of glucose homeostasis [60]. In that study, authors demonstrated that hyperglycemia destabilizes the TET2 through the inhibition of AMPK-mediated TET2 phosphorylation at Ser99, which lead to downregulation of global 5hmC levels in diabetic patients. Further, hyperglycemia promoted tumor growth was suppressed by TET2 and that anti-tumor effect of Metformin requires the AMPK–TET2–5hmC axis. Together, their findings suggest that TET2 is an epigenetic sensor of glucose levels in tumor. It will be highly interesting to find out whether such regulatory loop operates in non-oncogenic state.

In summary, our study identified a novel role for TET2 as an epigenetic regulator of PPARγ ligand-mediated insulin sensitization and transcriptional regulation in adipocytes. Future studies will be required to fully elucidate the full range of TET2-dependent transcriptional

effects on the genomic targets of PPARγ as well as the *in vivo* role of TET2 in adipose and whole-body metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

Rosi	Rosiglitazone
Tet	Ten-eleven translocation
Dnmts	DNA methyltransferases
HFD	High fat diet

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Highlights

1. *Tet2* expression is enriched in the adipose fraction, and adipocyte *Tet2* expression is diminished in diet-induced obesity.
2. TET2 plays a necessary and sufficient role in a PPARγ agonist's mediation of insulin sensitization in cultured adipocytes.
3. TET2 facilitates the transcriptional activity of PPARγ in both a catalytic-dependent and independent manner.
4. TET2-mediated gene regulation of PPARγ involves concordant changes in DNA demethylation at the promoter regions.
5. TET2 is required for PPARγ to maintain DNA binding at selective target loci in response to a PPARγ agonist.

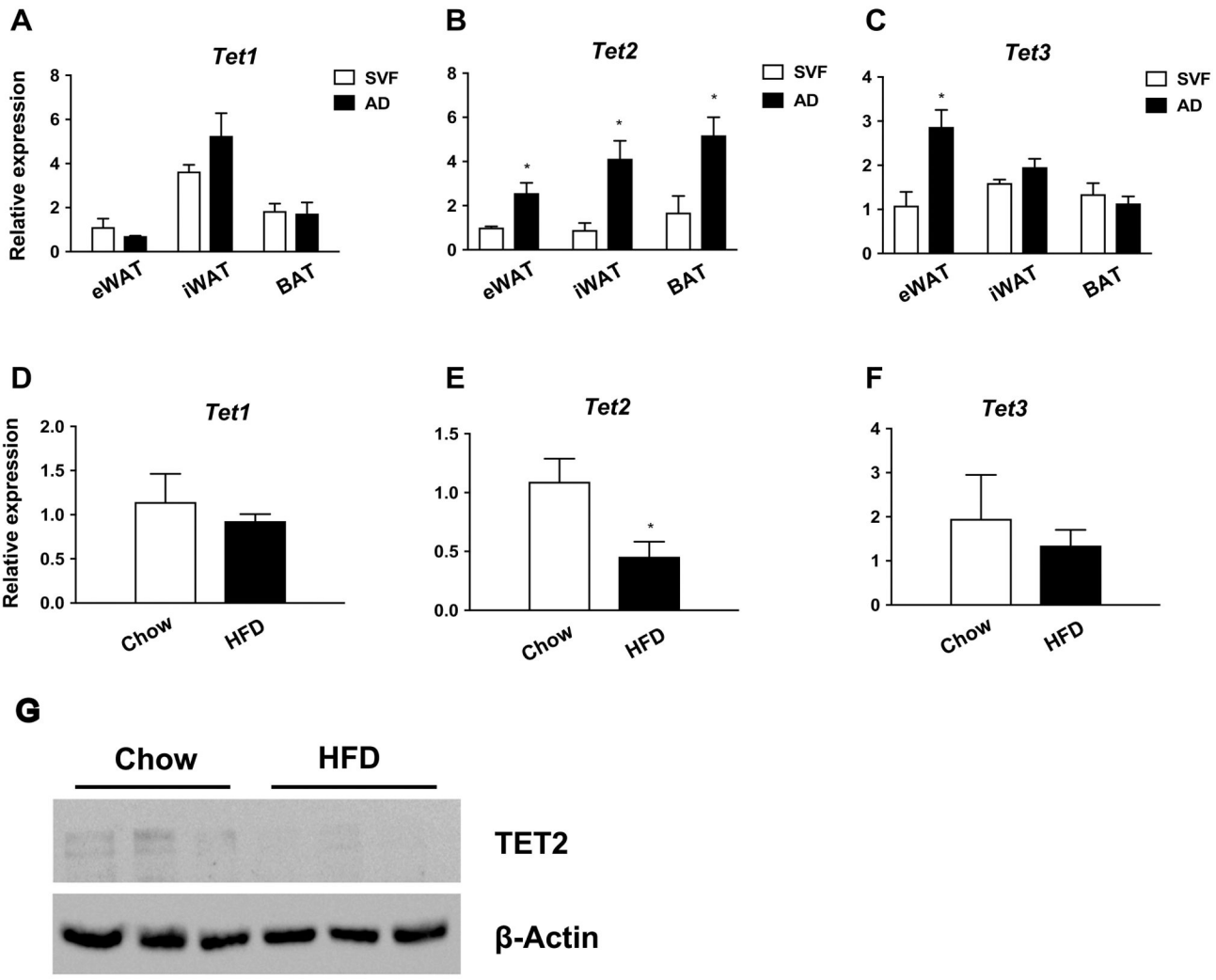

Figure 1. *Tet2* expression is enriched in adipocytes, and adipocyte *Tet2* levels are reduced in obesity.

(*A-C*) *Tet* expression was measured by q-PCR in the stromal vascular fraction (SVF) and adipose fraction (AD) of the eWAT, iWAT, and BAT of chow-fed C57BI/6 mice (n= 3, p < 0.05, Student's t-test, mean ± s.e.m.); (**D-F**) *Tet* expression in fractionated adipocytes from chow- and high fat diet–fed (HFD) C57Bl/6 mice (n = 3, p < 0.05, Student's t-test, mean ± s.e.m.); (**G**) Western blot showing TET2 protein levels in fractionated adipocytes from chow vs. HFD mice. eWAT: epididymal WAT; iWAT: inguinal WAT; BAT: brown adipose tissue.

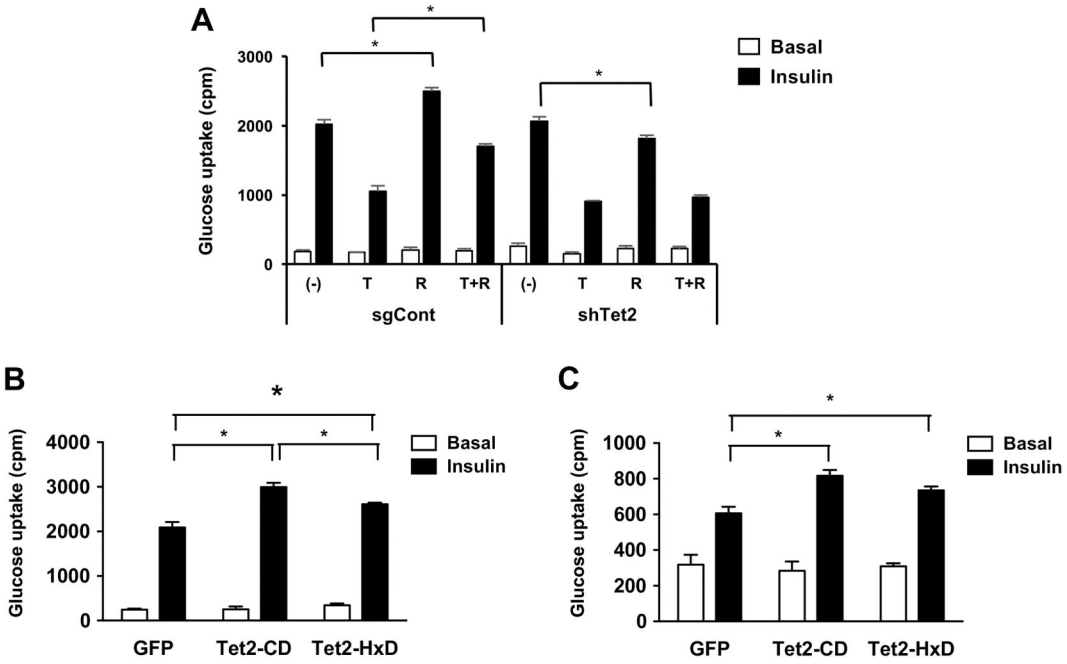

Figure 2. TET2 is necessary and sufficient to facilitate Rosi-mediated insulin sensitization *in vitro*.

(**A**) Mature 3T3-L1 adipocytes at day 8 were lentivirally transduced with single-guide RNA specific for *Tet2* (sgTet2) or an empty plasmid vector (sgCont). Two days after transduction, cells were treated with TNF (4 ng/ml), Rosi (100 nM), or both for 2 additional days and assessed for insulin-stimulated glucose uptake (^{3}H-2-DG assay, n = 6, $P < 0.05$). (**B, C**) Basal and insulin-stimulated glucose uptake in mature 3T3-L1 adipocytes (**B**) and immortalized human adipocytes (**C**) transduced with lentivirus expressing the catalytic domain of wild-type (Tet2-CD) or mutated (Tet2-HxD) *Tet2* or GFP ($n = 6$, $p < 0.05$, Student's *t*-test, mean ± s.e.m.).

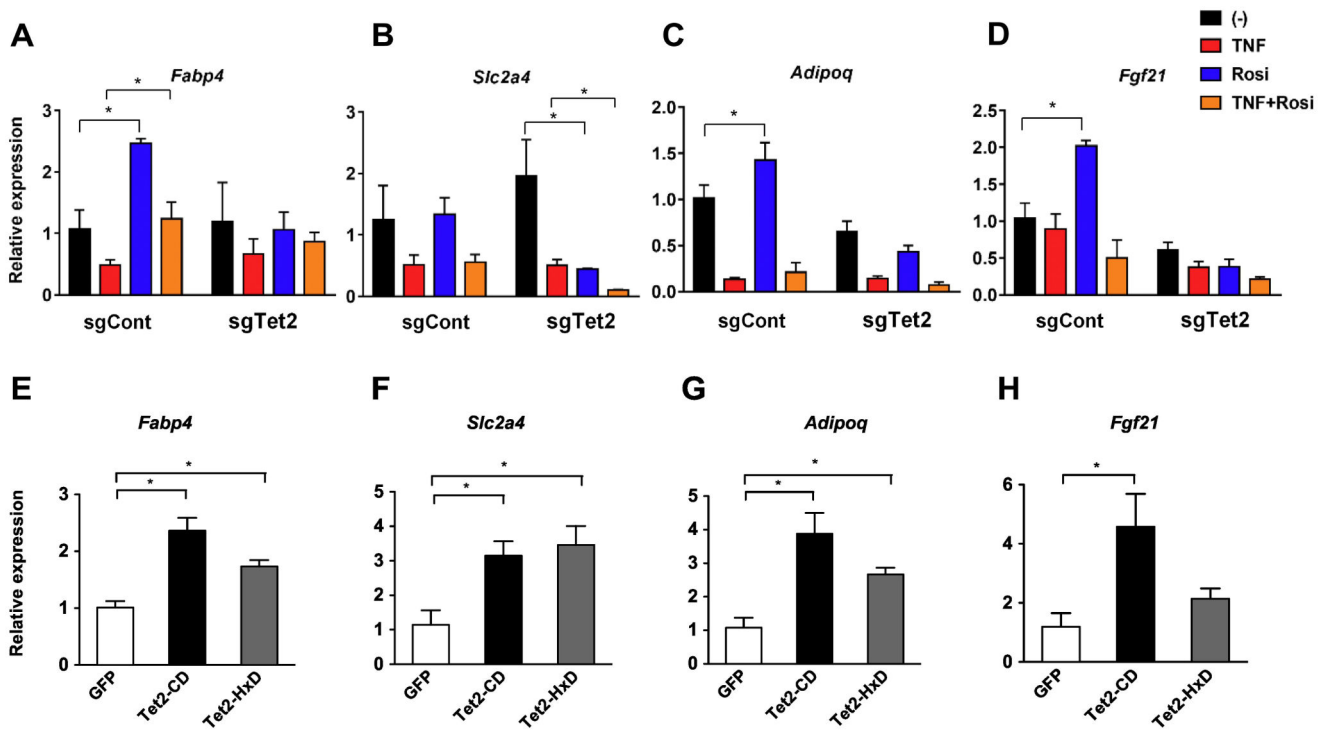

Figure 3. TET2 is required for Rosi-dependent gene regulation of PPARγ targets.

Mature 3T3-L1 adipocytes were transduced with *Tet2*-knockdown (**A-D**) or -overexpressor (**E-H**) lentiviruses. Two days after transduction, cells were treated with TNF (4 ng/ml), Rosi (1 μM), or both for 4 hours. Gene expression of a set of PPARγ target genes was determined by q-PCR ($n = 3$, $p < 0.05$, Student's *t*-test, mean ± s.e.m.).

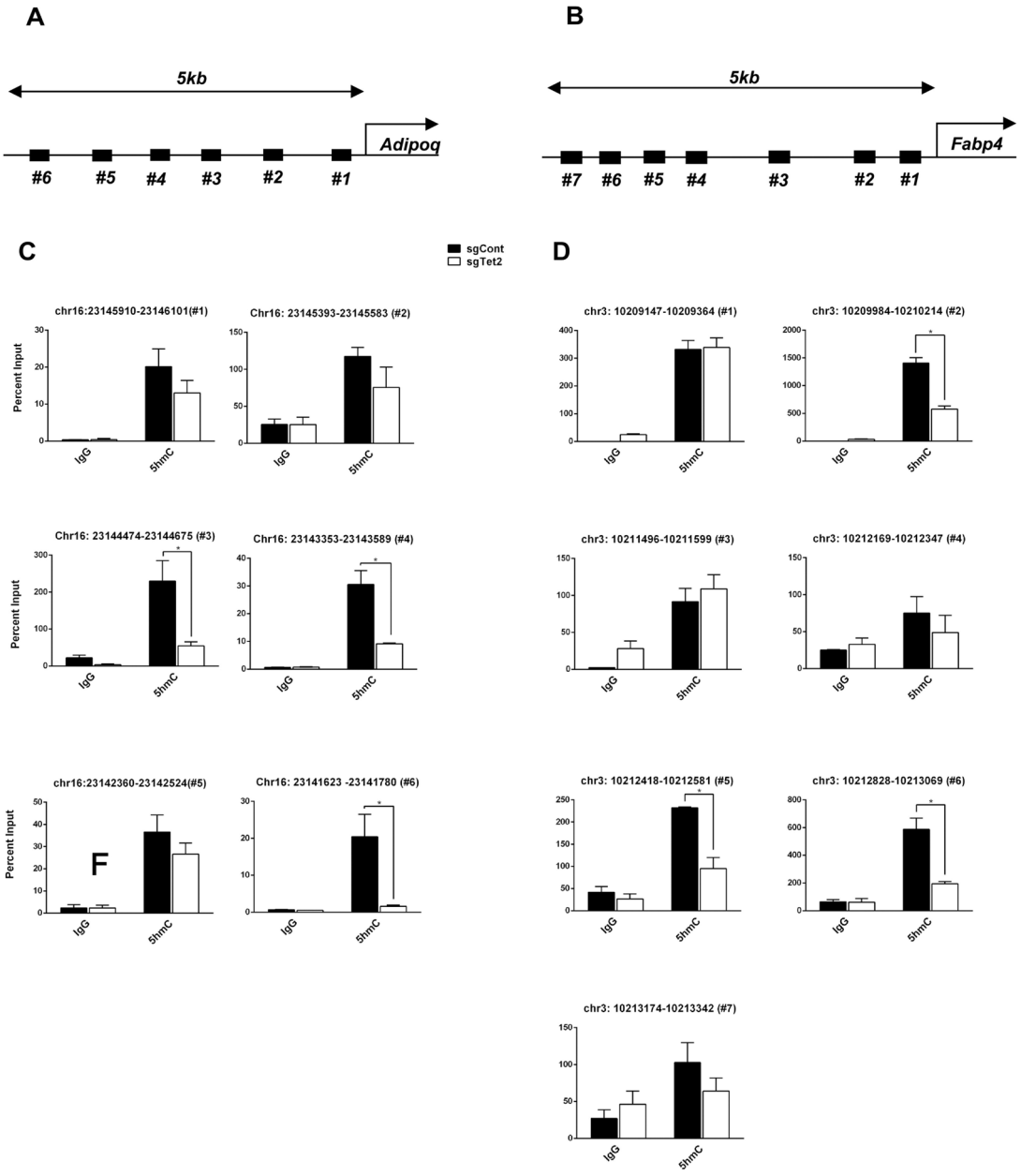

Figure 4. TET2 loss-of-function leads to reduced DNA demethylation at the promoter regions of *Adipoq* and *Fabp4*.

Schematic showing the tested regions for hMeDIP-qPCR at *Adipoq* (**A**) and *Fabp4* (**C**). Target regions were chosen using MethFinder (http://www.urogene.org/methprimer) on regions proximal (5 kb) to the *Adipoq* or *Fabp4* transcriptional start site. (**B, D**) Genomic DNA extracted from mature 3T3-L1 adipocytes transduced with *Tet2*-overexpressor or -knockdown lentiviruses was subjected to hMeDIP-qPCR using anti-5hmC and IgG antibodies, ($n = 3$, $p < 0.05$, Student's *t*-test, mean ± s.e.m.).

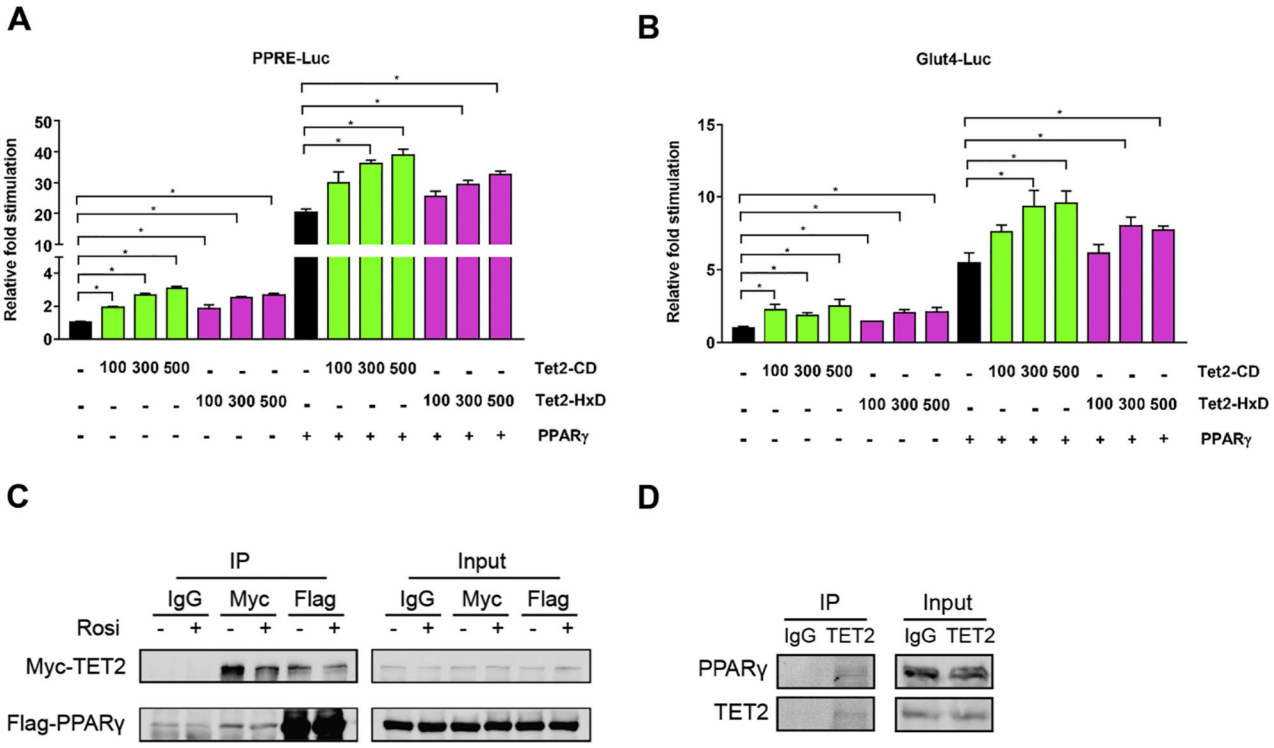

Figure 5. TET2 facilitates the transcriptional activity of PPARγ via physical interaction.

(**A, B**) 293T cells were co-transfected with vectors expressing Tet2-CD, Tet2-HxD, PPARγ, and reporter plasmids containing 3 copies of PPREs (**A**) and 2.4 kb of the Glut4 promoter (**B**). We measured Luciferase and Renilla activity 24 hr after transfection. Shown is the relative fold stimulation of luciferase activity after assessing transfection efficiency with Renilla ($n = 3$, $p < 0.05$, Student's t-test, mean ± s.e.m.). (**C**) 293T cells transfected with plasmids expressing Flag-PPARγ and Myc-TET2. At 24 hr after transfection, cells were treated with 1 μM Rosi or DMSO for 1 hour. Cell lysates were harvested and immunoprecipitated using anti-Flag, anti-Myc, or IgG and blotted with anti-Flag- and Myc-antibodies ($n = 3$, $p < 0.05$, Student's t-test, mean ± s.e.m.). (**D**) Mature 3T3-L1 adipocytes were harvested, and endogenous TET2 protein was immunoprecipitated using anti-TET2 antibody and blotted with anti-PPARγ and TET2 antibodies ($n = 3$, $p < 0.05$, Student's t-test, mean ± s.e.m.).

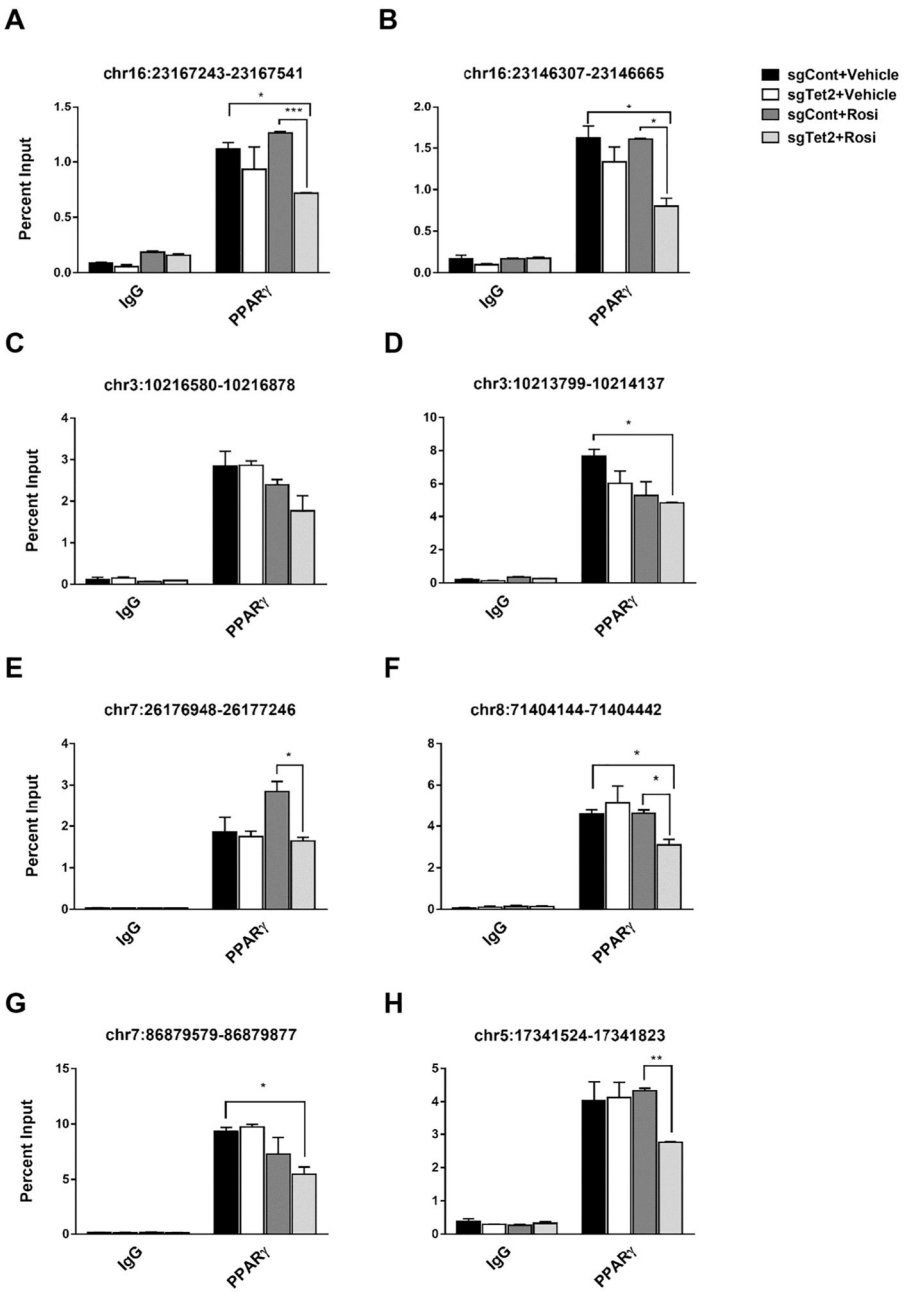

Figure 6. TET2 is necessary to sustain PPARγ binding to target loci upon ligand activation with Rosi.

Mature 3T3-L1 adipocytes were lentivirally transduced with single-guide RNA specific for *Tet2* (sgTet2) or with empty plasmid vector (sgCont). Two days after transduction, cells were treated with Rosi (1 μM) or DMSO for 1 hour. ChIP-qPCR analysis was performed using PPARγ antibody. Primers amplifying the individual PPREs are indicated ($n = 3$, $p < 0.05$, Student's *t*-test, mean ± s.e.m.).