A noncanonical PPARγ/RXRα-binding sequence regulates leptin expression in response to changes in adipose tissue mass

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Leptin expression decreases after fat loss and is increased when obesity develops, and its proper quantitative regulation is essential for the homeostatic control of fat mass. We previously reported that a distant leptin enhancer 1 (LE1), 16 kb upstream from the transcription start site (TSS), confers fat-specific expression in a bacterial artificial chromosome transgenic (BACTG) reporter mouse. However, this and the other elements that we identified do not account for the quantitative changes in leptin expression that accompany alterations of adipose mass. In this report, we used an assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) to identify a 17-bp noncanonical peroxisome proliferator-activated receptor gamma (PPARγ)/retinoid X receptor alpha (RXRα)-binding site, leptin regulatory element 1 (LepRE1), within LE1, and show that it is necessary for the fat-regulated quantitative control of reporter (luciferase) expression. While BACTG reporter mice with mutations in this sequence still show fat-specific expression, luciferase is no longer decreased after food restriction and weight loss. Similarly, the increased expression of leptin reporter mouse associated with obesity in ob/ob mice is impaired. A functionally analoguous LepRE1 site is also found in a second, redundant DNA regulatory element 13 kb downstream of the TSS. These data uncouple the mechanisms conferring qualitative and quantitative expression of the leptin gene and further suggest that factor(s) that bind to LepRE1 quantitatively control leptin expression and might be components of a lipid-sensing system in adipocytes.

leptin | adipose tissue | PPARγ/RXRα | weak binding | lipid sensing

Leptin is an adipocyte hormone that maintains homeostatic control of adipose tissue mass and functions as an afferent signal in a negative feedback loop. Leptin deficiency leads to extreme obesity in both mice and humans, while leptin treatment of wild-type mice reduces fat mass (1–7). Weight gain leads to an increased leptin level that, in turn, inhibits food intake and returns adipose tissue mass to the starting point. Similarly, weight loss decreases the leptin level, leading to increased food intake and increased fat mass. Thus, the quantitative changes in leptin expression associated with changes in nutritional state are critical for the proper functioning of this system. Consistent with this, the levels of leptin RNA and plasma leptin are highly correlated with adipocyte cell size and cellular lipid content (8–10). This has suggested that, analogous to a cholesterol sensing system in liver and other cell types (11, 12), there might be a lipid-sensing mechanism (or another mechanism, such as sensing of cell size) in adipocytes that adjusts the level of leptin gene expression to changes in the level of lipid stores. However, neither the molecular mechanisms controlling the change in leptin expression levels nor the elements of this putative signal transduction pathway are known (13).

To address this question, we initiated efforts to define cis elements and trans factors that control the quantitative expression of the leptin gene. Defining DNA sequence-binding sites has been crucial for identifying numerous transcription factors, including SP1 (14) and NF-κB (15). This approach is also analogous to that used to identify the aforementioned cholesterol-sensing system. In that case, studies of LDL receptor expression revealed that the levels of cholesterol in the endoplasmic reticulum membrane regulate cleavage and nuclear transport of the SREBP transcription factor, in turn, controlling the expression of genes that regulate cholesterol metabolism. However, analogous studies...
of leptin require that expression be monitored in vivo because cultured adipocytes, which have a very low lipid content, express an approximately 1,000-fold lower level of leptin RNA than do fat cells in vivo (16). Previous efforts to map cis elements controlling leptin expression have thus used bacterial artificial chromosome transgenic (BACTG) reporter animals with luciferase inserted at the transcription start site (TSS) of the leptin gene (17). Through a comprehensive deletion analysis, we previously found that a BACTG encompassing 31 kb (−22 to +8.8 kb) of the leptin locus showed fat-specific luciferase expression (18). Similar to the endogenous gene, luciferase expression was reduced after 48 h of food deprivation, while its expression was increased after crossing of the reporter mice to ob/ob mice that express very high levels of leptin RNA. We also found that a 3′ BACTG extending from −762 bp to +18 kb was able to drive reporter expression in a manner similar to the 5′ 31-kb (−22 to +8.8 kb) BACTG, while a BACTG extending from −762 bp to +8.8 kb lost fat-specific reporter expression. Subsequent studies have identified two redundant elements that can independently confer cell-specific expression of leptin in adipocytes: Leptin enhancer 1 (LE1) is localized between −16.5 and −16.1 kb upstream of the leptin TSS, while LE2 is located between +13.6 and +13.9 kb downstream of the leptin TSS. However, because deletion of these elements ablates fat-specific expression altogether, it is not possible to assess the role of these specific sequences to quantitatively regulate leptin expression. Similarly, while other factors, including C/EBPα and SPI (19, 20), FOSL2 (21), and NF-Y (18), have been reported to play a role in leptin expression, none have been shown to quantitatively regulate this gene.

In this report, we employed the assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) (22) as part of an unbiased screen to identify sites of transcription factor binding in inguinal white adipose tissue (iWAT) from fed, food-restricted (48 h), and ob/ob mice. Deep sequencing revealed a highly conserved noncanonical footprint for a PPARγ/RXRα-binding site within LE1 [referred to hereafter as leptin regulatory element 1 (LepRE1)] in adipose tissue nuclei derived from ob/ob mice, but not from adipose tissue of wild-type mice. Point mutations in this regulatory sequence in the 5′ reporter BACTG (−22 to +8.8 kb) abrogated quantitative regulation of luciferase in adipose tissue from fasted and obese mice. A functionally equivalent PPARγ/RXRα-binding site was also found within LE2, potentially explaining the functional redundancy of these elements. Purified PPARγ/RXRα binds weakly to these sequences, and both gain-of-function and loss-of-function mutations within the core sequence affect the regulated expression of the reporter. In addition, mutations in the adjacent PPARγ extension site disrupt the proper quantitative control of reporter expression. These data suggest a model in which the quantitative regulation of leptin expression depends on the stabilization of PPARγ/RXRα binding to an otherwise weak binding site by an accessory factor binding to the adjacent extension site.

**Results**

**A Fat-Regulated Footprint, LepRE1, Is a Noncanonical PPARγ/RXRα-Binding Site.** To find potential LepREs that respond to changes in fat mass, we isolated nuclei from inguinal iWAT of fasted, fed, and ob/ob mice and generated genome-wide footprints using ATAC-seq. In this method, isolated nuclei are incubated with a hyperactive transposase loaded with adaptor sequences so that DNase-hypersensitive regions can be PCR-amplified using the adaptor sequences as primers. Deep sequencing of the PCR products yields a genome-wide inventory of sequences from accessible regions of chromatin (22). Furthermore, analysis of the heights of the peaks of the DNA sequence reads reveals DNA footprints indicative of protein binding. Using this method, we identified six peaks within −22 to +8.8 kb of the leptin gene that showed a threefold or higher signal in adipose tissue nuclei from ob/ob mice compared with nuclei from fed and fasted mice (Fig. 1A). These six peaks included the aforementioned LE1 between −16.5 and −16.1 kb of the TSS, the proximal promoter around the TSS, and four regions within the first intron. The differences in access of the transposase to the proximal promoter and the transcribed regions in the first exon are consistent with the higher level of expression of this gene in ob vs. wild-type adipose tissue.

We thus focused on the LE1 sequence at −16 kb because it is not transcribed and also because a 400-bp deletion of LE1 in a BACTG (−22 to +8.8 kb) abolishes fat-specific expression of a luciferase reporter. Within LE1, there is a 101-bp segment (mm9, chr6: 28993757–28993857) that is almost identical among 20 placental mammal species, which is consistent with the finding that fat-specific expression of leptin is only evident in mammals (23). Within this 101-bp region of LE1, there is a small 17-bp footprint in ob/ob but not wild-type adipose tissue nuclei. The
footprint showed two apparent peaks, suggesting that two (or more) proteins bound there. We refer to this footprinted sequence as LepRE1 (Fig. 1A).

We noticed that six consecutive base pairs (out of 13) of the footprinted sequence of LepRE1 were identical to the RXRα-binding sequence [referred to as the conserved direct repeat 1 (DR1) half-site] of the Fabp4/aP2 gene enhancer. This binding site is referred to as the peroxisome proliferator response element (PPRE)/adipocyte regulatory factor response element 7 (also known as ARE7), and this binding site is composed of a direct repeat of two DR1 half-sites with a 1-nt spacer (24, 25) (Fig. 1B). PPARγ, a member of the nuclear hormone receptor superfamily, is the master regulator of adipogenesis (26, 27). This transcription factor binds as a heterodimer with RXRα, another nuclear receptor, to a DR1 site (28). However, in LepRE1, the next 6-bp sequence, 3′ to the conserved DR1 (i.e., the second half-site), had only very limited homology to DR1 as would typically be found in a canonical PPARγ-binding sequence or PPRE.

Thus, the footprint we found in the −16-kb upstream region of the leptin gene is not a canonical PPRE (DR1) in that a single DR1 half-site was followed by a nonhomologous sequence (Fig. 1B and SI Appendix, Fig. S1A). Indeed, because of this, none of the current algorithms that identify DNA-binding sites identified LepRE1 as a PPARγ/RXRα-binding sequence at all. However, as shown below, this sequence can bind to a PPARγ/RXRα heterodimer, albeit more weakly than does a canonical binding site.

We confirmed that the PPARγ/RXRα protein complex could bind to this sequence using an EMSA assay in which purified PPARγ and RXRα were incubated with LepRE1 oligonucleotides labeled with infrared dye (IRDye) 700. FLAG-tagged mouse RXRα2 and human RXRα proteins were purified from baculovirus-infected Sf9 cells. We found that the purified protein bound to this sequence with an affinity one-tenth that of a canonical DR1 PPRE. In these studies, we used the ARE6-binding site rather than the aforementioned ARE7-binding site because more extensive mutagenesis has been performed for this sequence (24) (Fig. 1C). ARE6 and ARE7 share six of 13 core DR1 nucleotides, and both provide high-affinity sites for PPARγ binding to the Fabp4/aP2 enhancer (25). This gel shift was abolished after coincubation with an excess of unlabeled wild-type DNA fragments, while unlabeled DNA fragments with point mutations in the RXRα/RXRα-binding sequence, particularly the AACT/AGTT part of the conserved DR1 half-site of LepRE1, no longer competed with the labeled probe in the gel shift (Fig. 1D).

In contrast, unlabeled DNA fragments with point mutations in the sequences 3′ to the conserved DR1 half-site and the PPARγ-binding sequence (TCCGCA/TGGCGA) as well as mutations in the extension sequence for PPARγ (Fig. 1D) competed for binding in a similar manner to wild-type oligonucleotides. The extension sequence is adjacent to the nonconserved half-site with the sequence GAAT/ATT. It previously has been suggested that this site binds other transcription factors, the identities of which have not been determined (29). These results show that the RXRα-binding portion of LepRE1 is required for the (weaker) binding of PPARγ/RXRα to this sequence (Fig. 1D). These in vitro EMSA findings are also consistent with data from ChIP-sequencing (ChIP-seq) analyses identifying sites of interaction of PPARγ with RXRα (Fig. 1E). PPARγ/RXRα binding to the leptin gene in adipocytes in vivo (Fig. 2A and SI Appendix, Fig. S1B).

### Fat-Specific Expression in Reporter Mice with LepRE1 Mutations

Previous studies have shown that PPARγ, at least by itself, is not sufficient to induce a high level of leptin expression. For example, leptin expression is extremely low in cultured adipocytes and in brown fat despite the fact that they express high levels of this transcription factor (16). To confirm that LepRE1 and LepRE2 are functional PPREs, we cotransfected PPARγ and RXRα into HEK293T cells expressing a luciferase reporter construct. We found that PPARγ and RXRα can indeed activate the LE1 and LE2 enhancers upstream of a luciferase reporter with an ∼10-fold induction relative to control experiments without cotransfection of PPARγ and RXRα (Fig. 3D). Interestingly, treatment with the RXR ligand 9-cis-Retinoic acid decreases the LE1-driven and LE2-driven luciferase activity. The PPARγ ligand rosiglitazone also decreases reporter expression, although to a lesser extent than 9-cis-Retinoic acid (Fig. 3D). These results are...
Fig. 2. A fat-regulated footprint, LepRE2, is homologous to LepRE1. (A) ATAC-seq results of the inguinal fat from fed and fasted B6 mice, and PPARγ ChIP-seq in the inguinal fat from fed B6 mice (32, 33) between −0.762 and +18 kb sequences of the leptin locus are shown. Regions with a threefold change in nuclei from fed vs. fasted mice are highlighted. The +13-kb region of the leptin gene is enlarged to show the calculated footprint score (blue) and conservation score within mammals (green). The LepRE2 sequence is denoted by a red box. (B) Sequence comparison of LepRE1 and LepRE2. (Left) LepRE2 sequence denoted by the red box in A is shown. The conserved nucleotides are colored red. PPARγ- and RXRα-binding sequences are boxed based on the solved crystal structure (40). (C) EMSA of purified PPARγ/RXRα with IRDye 700-labeled PPRE (ARE6) and LepRE2 oligos. The shifted bands were quantified with ImageStudioLite and normalized to PPARγ/RXRα. * indicates point mutation in LepRE2 that affects the binding to purified PPARγ/RXRα proteins.

consistent with the finding that thiazolidinediones inhibit leptin expression (34–36).

We thus investigated whether this noncanonical PPARγ/RXRα-binding site is required for proper qualitative and quantitative expression of leptin by making point mutations in LepRE1 in the reporter BACTG that extends from −22 to +8.8 kb. In this BACTG, luciferase is inserted by homologous recombination into the TSS. Three LepRE1 mutants were characterized: (i) a gain-of-function PPARγ/RXRα-binding mutant LepRE1 (GOF) DR1, in which a canonical DR1 half-site has replaced the second noncanonical 3′-binding site; (ii) a loss-of-function PPARγ/RXRα-binding mutant LepRE1 (LOF) in which a point mutation was introduced into the highly conserved DR1 half-site sequence (i.e., the RXRα-binding region that is required for binding) (Fig. 1D); and (iii) an extension mutant LepRE1 (EXT) in which a point mutation was introduced in the 5′-flanking sequence upstream of the core DR1. As mentioned, previous studies suggest the 5′-extension site of PPARγ may be a target of other binding proteins (29). EMSA assays confirmed that the DR1 GOF mutant bound to purified PPARγ/RXRα more strongly than did the wild-type sequence, that the LOF mutant failed to bind, and that the EXT mutant bound with similar affinity to the wild-type oligonucleotide (Fig. 3B).

We next made multiple BACTG reporter lines for each of the three different LepRE1 mutants. All of the lines for the GOF DR1, LOF, and EXT mutants expressed leptin luciferase reporter exclusively in adipose tissue, albeit with lower baseline levels of expression vs. the wild-type reporter mice. In temporal analyses (SI Appendix, Fig. S2), we also noticed a progressive diminution in luciferase expression between 3 and 8 wk of age, with the relative level of luciferase expression in the LepRE1 DR1 reporter mice decreasing from 120% of the wild-type BACTG to 20–30% that of the control construct by 8 wk of age, after which time the expression level was stable. At baseline, the level of luciferase expression in the LepRE1 LOF reporter mice was fat-specific but expressed at a considerably lower level of ~4–8% that of the wild-type construct. Finally, luciferase expression in the LepRE1 EXT

Fig. 3. Fat-specific expression in reporter mice with LepRE1 mutations. (A) PPARγ/RXRα complex activates both LE1 and LE2 enhancers in transfected HEK293T cells in a dual-luciferase reporter assay. (B) Series of point mutations were introduced into LepRE1 for functional studies in vitro and in vivo. Sequence alignment of PPRE (ARE7), a LepRE1 DR1 GOF mutation, wild-type (WT) LepRE2 and oligos with point mutations using IRDye 700-labeled WT LepRE2 in the presence of purified PPARγ/RXRα. * indicates point mutation in LepRE2 that affects the binding to purified PPARγ/RXRα proteins.
Dysregulation of Leptin Reporter Expression After Weight Loss and Weight Gain. Weight loss after a period of food restriction is associated with a decrease in leptin gene expression and leptin plasma level. After 2 d of food restriction, similar to expression of the endogenous gene, expression of the leptin luciferase reporter in the 5’ wild-type BACTGs (~22 to +8.8 kb) decreased 3.3-fold (3.1 \times 10^7 \text{ p}^{-1} \text{ s}^{-1} \text{ units of total flux}) relative to the average expression level before fasting (Fig. 4A). In contrast, the level of luciferase expressed from the GOF mutant (with the stronger PPARγ/RXRα-binding site, LepRE1 DR1) did not decrease after 2 d of food restriction. Similarly, the reporter animals with a point mutation in the 5’ extension site (LepRE1 EXT) also showed a stable level of luciferase expression after a fast. Importantly, as shown previously (Fig. 3B), this mutation in the extension sequence did not alter the binding of PPARγ/RXRα to LepRE1. Finally, the reporter animals with a loss of function of PPARγ/RXRα binding (LepRE1 LOF) showed a 1.6-fold decrease of luciferase expression after fasting (1.1 \times 10^7 \text{ p}^{-1} \text{ s}^{-1} \text{ total flux}), and the magnitude of this decrease was 27.7-fold lower than that seen in wild-type mice after 2 d of food restriction (Fig. 4A and B). The body weight and fat mass of each of the groups (11–13 wk old) was the same, showing that the altered reporter expression was not a result of differences in adipose tissue mass (Fig. 4C).

We next analyzed the effect of these mutations on reporter expression in obese animals by mating the aforementioned reporter lines to ob/ob mice. The ob/ob mice carry a mutation in the leptin coding sequence and show a dramatic compensatory increase in the level of expression of leptin RNA. As above, no significant differences in body weight were observed in the mutant reporter animals vs. mice carrying the wild-type reporter (Fig. 5A). Nine-week-old ob/ob mice had an average body weight of 42 g, which is significantly higher than the 21-g average body weight in 9-wk-old ob/+ mice. As previously reported, the level of the luciferase reporter was 9.6-fold higher (7.6 \times 10^7 \text{ p}^{-1} \text{ s}^{-1} \text{ total flux}) in ob/ob transgenic animals expressing the wild-type reporter relative to the level of reporter expression in nonobese ob/+ mice.

In contrast, and relative to control mice, the increase in the levels of luciferase expression in 9-wk-old ob/ob mice carrying the LOF reporter was 20.6-fold lower (3.7 \times 10^7 \text{ p}^{-1} \text{ s}^{-1} \text{ total flux}) than the increase in 9-wk-old ob/ob mice carrying the wild-type reporter. Similarly, in ob animals carrying the EXT reporter construct, the increase of luciferase expression in ob/ob mice was 15.8-fold lower (4.8 \times 10^7 \text{ p}^{-1} \text{ s}^{-1} \text{ total flux}) than the increase in luciferase expression in ob/ob mice with the wild-type reporter (Fig. 5 B and C).

We found that the increased luciferase expression with obesity was impaired (albeit to a lesser extent) in the DR1 BACTG reporter mice, as the increased expression (3.9 \times 10^7 \text{ p}^{-1} \text{ s}^{-1} \text{ total flux}) of this reporter construct in ob/ob mice was 1.9-fold lower than the expression in ob/ob mice from the wild-type construct (Fig. 5 B and C). To reconfirm that the induction of luciferase expression from the LepRE1 DR1 reporter line was impaired, a second cohort of 11-wk-old animals was analyzed. Eleven-week-old ob/ob and ob/+ mice had average body weights of 49 g and 25 g, respectively. While the body weights of the LepRE1 wild-type and DR1 ob/ob animals were similar, the increase in luciferase expression from the DR1 reporter [5.3 \times 10^7 \text{ p}^{-1} \text{ s}^{-1} \text{ total flux}] was 1.7-fold lower than the increase in expression from the wild-type reporter [9.1 \times 10^7 \text{ p}^{-1} \text{ s}^{-1} \text{ total flux}] (SI Appendix, Fig. S4).

Finally, we tested whether additional factors in a nuclear extract from ob/ob adipose tissue can interact with the PPARγ/RXRα:LepRE1 complex. We found that addition of a nuclear extract from ob/ob adipocyte supershifted the PPARγ/RXRα:LepRE1 complex in an EMSA assay, while nuclear extracts from wild-type adipose tissue did not (SI Appendix, Fig. S4).

In summary, while leptin reporter mice with mutations in LepRE1 show fat-specific expression of luciferase (albeit with lower baseline levels), all three mutants show an impaired response to nutritional changes. The DR1 and EXT mutations fail to reduce luciferase expression after fasting and also show an impairment in the increased expression normally seen on an ob/ob background. The LOF BACTG reporter mice show a lesser reduction of reporter expression after weight loss and also show a profound defect in the increase of luciferase expression with obesity. Overall, these data suggest that trans factors binding to this noncanonical PPARγ-binding site and the adjacent EXT sequence play a critical role in controlling the quantitative expression of the leptin gene.

**Discussion**

Quantitative control of leptin expression is critical for the homeostatic control of adipose tissue mass. However, neither the transcription mechanism nor the signal transduction pathway that regulates the level of leptin expression is known. Their elucidation has been confounded by the finding that the key regulatory elements regulating leptin expression appear to be responsible for its tissue-specific expression in fat. We thus sought to identify transcription factor-binding sites whose footprints (reflecting occupancy) differed in obese vs. wild-type animals (Fig. L4).

Here, we report the identification and functional characterization of a specific PPARγ-binding site (LepRE1) that is responsible.

**Fig. 4.** Dysregulation of leptin reporter expression after weight loss. (A) Individual whole-body luciferase levels before fasting (day 0) and after 2 d of fasting (day 2) for the wild-type BACTG mice (~22 to +8.8 kb) and the DR1 (GOF), LOF, and EXT mutants are shown. The mice were between 11 and 13 wk old. (B) Difference between day 2 and day 0 whole-body luciferase level (Day 2–Day 0) for individual mice is shown. WT, wild type. (C) Body weight for corresponding individual mice on day 0. In A–C, WT (n = 21), DR1 (n = 21), LOF (n = 13), EXT (n = 12). ns, P > 0.05. ****P < 0.0001.
for the quantitative control of the leptin gene without affecting its fat-specific expression.

Previous studies from our laboratory have shown that redundant sequences in the extreme 5' and 3' regions of the gene, greater than 10 kb from the TSS, can confer fat-specific expression of the leptin gene (18). However, because mutations in these regions interfered with the fat-specific expression of this gene, it was impossible to define the sequences that quantitatively regulated leptin expression. This problem also applied to studies of other transcription factors, including C/EBPα and SP1 (19, 20), FOSL2 (21), and NFY (18), each of which has been reported to play a role in tissue-specific leptin expression in adipose tissue. Thus, in contrast to the binding sites for these other factors, LepRE1 and the factors that bind to it uncouple the mechanisms conferring quantitative expression of the leptin gene from its fat-specific expression.

LepRE1 and a functionally redundant element in the 3' region of the leptin gene (LepRE2, Fig. 2) show weak binding to PPARγ, raising the possibility that an additional stabilizing factor is necessary for its binding. PPARγ binding to the LepRE1 and LepRE2 sites is not seen in macrophages (33) (SI Appendix, Fig. S1), adding further evidence that there is an accessory factor that enables binding in adipocytes. PPARγ, forming an obligate heterodimer with RXRα, is a master regulator of adipogenesis (26, 27). PPARγ expression is highly correlated with leptin expression (37), and an adipose-specific PPARγ deletion reduces leptin expression, although this reduction is thought to be secondary to a defect in lipolysis (38). However, PPARγ by itself is not sufficient for a high level of leptin expression. For example, leptin expression is extremely low in cultured adipocytes despite the fact that they express high levels of this transcription factor (16). In addition, thiazolidinediones were found to inhibit leptin expression despite activating PPARγ in cultured adipocytes and rodents (34-36).

The finding of a noncanonical PPARγ/RXRα-binding sequence (LepRE1) and the effect of cognate mutations in impairing the nutritional regulation of the leptin gene provide evidence that an additional factor(s) is necessary for PPARγ-regulated expression. The canonical PPARγ/RXRα-binding PPRE motif known as the DR1 site is a strong target for binding of this transcription factor (28). The LepRE1 contains an RXRα-binding sequence identical to that in the PPRE (ARE7), but the other half is diverged (Fig. 1B). This alteration explains why this sequence was not identified as a binding site by current algorithms. These sequence changes also render the PPARγ/RXRα binding much weaker and explain why PPARγ alone is not sufficient to induce a high level of leptin expression in cultured adipocytes. Furthermore, a PPARγ ligand, rosiglitazone (and an RXRα ligand, 9-cis-Retinoic acid), decreases the expression of both LE1-driven and LE2-driven luciferase activity (Fig. 3A). Such results could explain why thiazolidinediones inhibit leptin expression despite being thought of as PPARγ agonists. These data are also consistent with previous results with the estrogen receptor, where ligands can reduce expression of a target gene in the absence of a functional coactivator (39). While there were other potential noncanonical PPARγ/RXRα-binding sites in the LE1 region, LepRE1 is the only one that was identified in the footprinting studies using ATAC-seq. Nonetheless, even though the LepRE1 site was functionally validated and found to be necessary, as mentioned above, it is still possible that other sites could also contribute.

A functional requirement of the PPARγ/RXRα complex for quantitative transcriptional regulation of leptin by binding to LepRE1 is suggested by the following evidence: (i) Purified PPARγ/RXRα proteins bound IRDye 700-labeled LepRE1 with sequence specificity in an (in vitro) EMSA assay (Fig. 1 C and D); (ii) ChIP-seq analysis identified PPARγ binding to LepRE1 in vivo (30-33) (Fig. 1J and SI Appendix, Fig. S1B); (iii) at baseline, the level of luciferase expression in the LepRE1 LOF reporter mice was considerably lower, with a fat-specific luciferase expression level 4-8% that of the wild-type construct (Fig. 3 and SI Appendix, Fig. S2); and (iv) the LOF BACTG reporter mice showed a lesser reduction of reporter expression after fasting and an impairment in the increase of luciferase expression in ob/ob animals (Fig. 5).

As mentioned above, these data suggest that an additional factor is required to stabilize PPARγ binding to this site to regulate the quantitative level of leptin expression. Our data further suggest that this putative accessory factor binds to the adjacent extension sequence (and potentially part of the nearby DR1 half-site for PPARγ) because mutations in this sequence (LepRE1 EXT) and the nearby PPARγ-binding sequence (LepRE1 DR1) do not alter the specificity of reporter expression in fat but do impair the effect of fasting or obesity. The mutation in the extension sequence also dramatically decreases the baseline level of reporter expression, further suggesting that it provides a binding site for a factor that is necessary for high-level expression of leptin in vivo. We also noticed that fat nuclear extracts from ob/ob mice can supershift a purified PPARγ/RXRα:LepRE1 complex in an EMSA assay (SI Appendix, Fig. S4), suggesting that an additional factor(s) in ob/ob adipocytes is part of the complex. Efforts to identify this other factor(s) are
underway. However, we have not ruled out the possibility that a conformational change in PPARγ itself could potentially affect its binding to the extension site as the PPARγ DNA-binding domain includes a C-terminal helix that inserts into the minor groove of this extension sequence (as shown in the PPARγ/RXRα crystal structure) (40). There are numerous other instances in which gene expression is controlled by the stabilization of weak binding. For example, Escherichia coli RNA polymerase alone binds fairly weakly to the classic lac promoter and requires cooperative binding with another low-affinity partner (CAMP-CAP) for high-level expression of β-galactosidase in the absence of glucose (41). The requirement for accessory factors to facilitate PPARγ-mediated gene expression also has a precedent in brown fat (42). Brown fat expresses high levels of PPARγ but does not express UCP1 unless the PGC-1 coactivator is also expressed, although activation of the UCP1 promoter in brown fat also involves other factors such as PRDM16, MED1, and HDAC3 (33, 43, 44). Thus, the stabilization of PPARγ at a noncanonical site may provide a general mechanism for the control of a wide array of other PPARγ target genes. Indeed, previous studies have suggested that the 5′ extension site of PPARγ in the classic DR1 motif may indeed be involved in binding to other factors (29). Structural studies of the glucocorticoid receptor have shown how coregulatory proteins can alter transcription factor conformation in sequence selection (45). The nature of the accessory factor regulating leptin expression is unknown and under intense investigation. It is noteworthy that while the RXRα-binding sequences are identical between LepRE1 and PPARE (ARE7) of the Fabp4/aP2 gene, LepRE1 has a unique 5′ extension sequence that differs from the extension sequence of the PPARγ site that regulates the UCP1 gene.

We found an impairment in the decrease in reporter expression from the LepRE1 mutants after fasting, as well as a markedly diminished absolute increase in reporter expression from all three LepRE1 mutants after breeding to ob/ob mice. However, there was still a small relative increase in the expression from the LOF and EXT reporter constructs with obesity (Fig. 5C). This suggests that there may exist additional pathways that can partially up-regulate leptin transcription in ob/ob mice. This may be similar to the compensatory increase of leptin transcription from wild-type mice to ob/+ mice. Because the body weight difference between wild-type and ob/+ mice is almost undistinguishable, this additional leptin-independent pathway may not be associated with lipid content in fat.

The most parsimonious model to explain our findings is that the quantitative or qualitative state of an accessory factor(s) that binds to the extension site is altered in concert with changes in fat mass (or something that correlates with these changes) and, in turn, regulates the binding of PPARγ/RXRα binding to LepRE1, thus controlling transcription of the leptin gene (Fig. 6). The identification of this factor could thus potentially illuminate the nature of the adipocyte signal transduction pathway that is responsible for the regulated expression of leptin in parallel with changes in cellular lipid content. While several lines of evidence have suggested that cellular lipid content is sensed in adipocytes, the cellular mechanisms are not known. Lipid sensing has also been invoked as potentially regulating the activity of hypothalamic neurons and hepatic metabolism, although the underlying mechanism in these cell types is similarly unknown (46). Possible mechanisms could include regulation of a lipid metabolite, sensing of cell size (which would increase as lipid accumulates), effects of oxygen (the partial pressure of which could vary based on the distance of the nucleus from the capillaries), or other mechanisms. This mystery is analogous to the cholesterol-sensing problem for cells, which was resolved by defining the regulatory mechanisms and signal transduction pathway that regulates the transcription of LDL receptor (11, 12, 47, 48). Identification of the putative accessory factors that bind to the noncanonical PPARγ/RXRα site that we have implicated in the regulation of leptin expression could help resolve this conundrum and lead to the identification of the signal transduction mechanism that links changes in cellular lipid content, or its surrogate, to changes in gene expression and possibly other cellular functions. A deeper understanding of this putative lipid-sensing mechanism could be of general importance for understanding the mechanisms responsible for nutritionally mediated changes in cell function and gene expression.

Materials and Methods

Experimental Animals. All experiments were approved by The Rockefeller University Institutional Animal Care and Use Committee and were performed in accordance with the NIH guidelines. Both male and female mice (<3 wk old) were used for all studies. Mice were housed in a 12-h light/dark cycle with ad libitum access to food and water, except for fasting assays. All mouse lines are either C57BL/6J or FVB/NJ background. Male mice were used for ATAC-seq studies. Male and female mice were used for luciferase studies.

ATAC-Seq. Ten-week-old C57BL/6J and B6 ob/ob male mice were purchased from The Jackson Laboratory. Wild-type mice were fed or fasted for 2 d, and s.c. I.WAT was isolated from fasted, fed, and ob/ob mice. The fat tissue was minced with blades, dounced in homogenization buffer [20 mM Tricine (pH 7.8), 25 mM D-Sucrose, 15 mM NaCl, 60 mM KCl, 2 mM MgCl2, 0.5 mM spermidine], filtered with an EMD Millipore Nylon-Net Steriflip Vacuum Filter Unit (100 μm), and centrifuged at 18,000 × g for 5 min at 4 °C. The resulting nuclei pellet was washed once with tagmentation DNA buffer [10 mM Tris (pH 7.6) acetic acid, 5 mM MgCl2, 10% dimethylformamide] (49), followed by centrifugation at 500 × g for 5 min at 4 °C. After resuspension in the same tagmentation DNA buffer and counting using Trypan blue, an aliquot contacting 50,000 nuclei was performed by means of a transposition reaction using a Nextera DNA Library Preparation Kit (Illumina) and processed as described (22). A library from each mouse was sequenced in one lane using 50-bp × 2 paired-end reads on an Illumina HiSeq 2500 system.

Reads were aligned to the mm9 build and Ensemble gene model (NCBIM37) using Bowtie with parameters: X2000 and m1. Then, we processed the alignments using Samtools and adjusted the read start sites to represent the center of the transposon-binding event as previously described (22). All reads aligning to the + strand were offset by +4 bp, and all reads aligning to the − strand were offset by −5 bp. Reads in two libraries from two individual mice in each condition were mixed. After that, we found peaks in ob/ob samples with at least a threefold higher signal than fasted
conditions using Homer (50). Within the above peak region, footprint scores were calculated based on the Wellington method (51). The conservation score within mammal is downloaded from a University of California, Santa Cruz server and calculated from 20 placental mammal species (52). All of the results were viewed using the Integrative Genomics Viewer (53).

**Purification of PPARα and RXRs.** Baculoviruses expressing recombinant FLAG-tagged mouse PPARα (F-mPPARα) and human RXRα (F-hRXRα) were prepared as described (54). Sf9 cells were infected with each baculovirus to express the recombinant protein separately, and the soluble extract was purified by sonication in BC buffer [20 mM Hepes-KOH (pH 7.9), 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF] containing 100 mM KCl. After the ultracentrifugation, clear lysate was subjected to HiTrap Q (GE Healthcare), and bound proteins were eluted using a linear gradient of KCl.

EMSA. EMSA was performed using the LICOR Odyssey EMSA Buffer Kit. Basically, purified PPARα (10 ng) and RXRα (10 ng) were incubated in a 20-μL reaction volume with 5 mM Tris (pH 7.5), 25 mM KCl, 3 mM DTT, 0.25% Tween 20, 5 mM MgCl2, 1 μg of poly(dI-dC) (or 0.5 μg of salmon sperm DNA) and 200-nM-labeled DNA probe, with or without 300 nM unlabeled oligos for 20 min at room temperature. Samples were then mixed with 2 μL of 10× LICOR orange loading dye and loaded onto a 5% Mini-PROTEAN TBE Gel (BioRad). After an ~75-min run at 70 V, the gel was scanned with a LICOR Odyssey CLX Imaging System. The results were analyzed and quantified with Image Studio Lite (LICOR). The sequences of the 154-bp unlabeled and unlabeled oligos are shown in the Supporting Information. All oligos in this paper were ordered from Integrated DNA Technologies, Inc.

**Plasmids.** pTK-Renilla luciferase encoded the Renilla luciferase gene under control of the thymidine kinase promoter (Promega). pCMV-PPARα encoded the mouse PPARα under control of the CMV promoter (54). pCMV-RXRα encoded FLAG-tagged human RXRα under control of the CMV promoter (54). pCMV-PPARα and pCMV-RXRα were cloned into baculovirus transfer vectors pFastBac Dual (55). Sf9 cells were infected with each baculovirus to prepare recombinant baculoviruses pLE1- and pLE2-firefly luciferase was generated by cloning 279 bp of LE2 (TGG-CTAATGGCA) into pGL4.27 (Promega) between the XhoI and HindIII sites. pLE2-firefly luciferase was generated by cloning 115 bp of LE1 (GAGAACACTTAACAGCAAAGGTTAATCTTTGAAGTCCCTAAAGATTTGA) into pGL4.27 (Promega) between the BgIII and HindIII sites. pLE1-firefly luciferase was generated by cloning 54 bp of LE1 (TGG-CAGAAGCAAG) into pGL4.27 (Promega) between the XhoI and HindIII sites.

**Dual-Luciferase Reporter Assay.** The reporter assay was performed as described elsewhere (55, 56). On day 0, HEK293T cells were set up for experiments in 96-well plates (1500 cells per well). The reporter assay was performed as described elsewhere (55, 56). On day 0, HEK293T cells were set up for experiments in 96-well plates (1500 cells per well).

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