

## Mechanisms Regulating Adipocyte Expression of Resistin\*

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**Resistin, also known as Adipocyte Secreted Factor (ADSF) and Found in Inflammatory Zone 3 (FIZZ3), is a mouse protein with potential roles in insulin resistance and adipocyte differentiation. The resistin gene is expressed almost exclusively in adipocytes. Here we show that a proximal 264-base pair fragment of the mouse resistin promoter is sufficient for expression in adipocytes. Ectopic expression of the adipogenic transcription factor CCAAT/enhancer-binding protein (C/EBP $\alpha$ ) was sufficient for expression in non-adipogenic cells. C/EBP $\alpha$  binds specifically to a site that is essential for expression of the resistin promoter. Chromatin immunoprecipitation studies of the endogenous gene demonstrated adipocyte-specific association of C/EBP $\alpha$  with the proximal resistin promoter in adipocytes but not preadipocytes. C/EBP $\alpha$  binding was associated with the recruitment of coactivators p300 and CREB-binding protein and a dramatic increase in histone acetylation in the vicinity of the resistin promoter. The antidiabetic thiazolidinedione (TZD) drug rosiglitazone reduced resistin expression with an ED<sub>50</sub> similar to its K<sub>d</sub> for binding to peroxisome proliferator activated receptor  $\gamma$  (PPAR $\gamma$ ). Other TZD- and non-TZD PPAR $\gamma$  ligands also down-regulated resistin expression. However, no functional PPAR $\gamma$  binding site was found within 6.2 kb of the transcriptional start site, suggesting that if PPAR $\gamma$  is involved, it is either acting at a long distance from the start site, in an intron, or indirectly. Nevertheless, rosiglitazone treatment selectively decreased histone acetylation at the resistin promoter without a change in occupation by C/EBP $\alpha$ , CREB-binding protein, or p300. Thus, adipocyte specificity of resistin gene expression is because of C/EBP $\alpha$  binding, leading to the recruitment of transcriptional coactivators and histone acetylation that is characteristic of an active chromatin environment. TZD reduces resistin gene expression at least in part by reducing histone acetylation associated with the binding of C/EBP $\alpha$  in mature adipocytes.**

societies and is also associated with insulin resistance and diabetes (3). These functions of adipose tissue are mediated in part by secreted products including fatty acids as well as numerous protein products (4).

Adipocytes are highly differentiated cells, and numerous genes are expressed specifically or predominantly in fat cells (5). They include transcription factors, metabolic enzymes, structural proteins, and secreted proteins. Transcription factors implicated in adipogenesis include basic leucine zipper-containing C/EBP<sup>1</sup> family members and the nuclear receptor peroxisome proliferator activated receptor  $\gamma$  (PPAR $\gamma$ ) (6, 7). C/EBP $\beta$  and C/EBP $\delta$  are transiently induced during adipogenesis (8) and are involved in the up-regulation of C/EBP $\alpha$  and PPAR $\gamma$  (9, 10), which remain expressed in mature adipocytes. C/EBP $\alpha$  and PPAR $\gamma$  are both able to induce adipogenesis in part by inducing each other's expression (11). Recent studies indicate that PPAR $\gamma$  can induce adipogenesis in cells lacking C/EBP $\alpha$  (12, 13), whereas C/EBP $\alpha$  is insufficient for adipogenesis in the absence of PPAR $\gamma$  (14). Nevertheless, numerous adipocyte-specific genes contain binding sites for C/EBP $\alpha$  as well as PPAR $\gamma$  (15).

Resistin, also known as Adipocyte Secreted Factor (ADSF) and Found in Inflammatory Zone 3 (FIZZ3), is a recently described protein whose expression is adipocyte-specific in the mouse (16–18). Resistin belongs to a family that in the mouse includes two other members called Resistin-Like Molecules (RELMs) and FIZZ proteins (18, 19). Although other RELM/FIZZ family members exhibit tissue-specific expression, resistin is the only one of these family members to be expressed in adipocytes. The function of resistin is not well understood, but there is evidence that it plays a role in obesity-related insulin resistance as well as in adipocyte differentiation (16, 17).

Little is known about the adipocyte-specific determinants of resistin gene expression. Here we show that the mouse resistin promoter contains a C/EBP $\alpha$  binding site that is necessary and sufficient for expression. The binding of C/EBP $\alpha$  in adipocytes is associated with the recruitment of coactivators CBP and p300 and abundant acetylation of histones at the resistin promoter. Multiple classes of PPAR $\gamma$  ligands as well as RXR ligands down-regulate resistin gene expression. This is associated with reduced histone acetylation without change in C/EBP $\alpha$  or coactivator recruitment. These results suggest that positive regulation of resistin regulation is because of C/EBP $\alpha$ , whereas negative regulation by PPAR $\gamma$  ligands involves a different mechanism converging on histone acetylation at the resistin promoter.

Adipose tissue is increasingly recognized as a dynamic tissue that serves functions other than storage of energy in the form of triglycerides (1). A lack of adipose tissue causes hyperlipidemia, insulin resistance, and type 2 diabetes (2). Excess adipose tissue is more common among humans in industrialized

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<sup>1</sup> The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; PPAR $\gamma$ , peroxisome proliferator activated receptor  $\gamma$ ; FIZZ, Found in Inflammatory Zone 3; RELM, Resistin-Like Molecules; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; FMO, N-(9-fluorenyl)methoxycarbonyl; RXR, retinoid x receptor; RAR, retinoid acid receptor; aP2, adipocyte-specific fatty acid-binding protein; AP-1, activator protein-1.

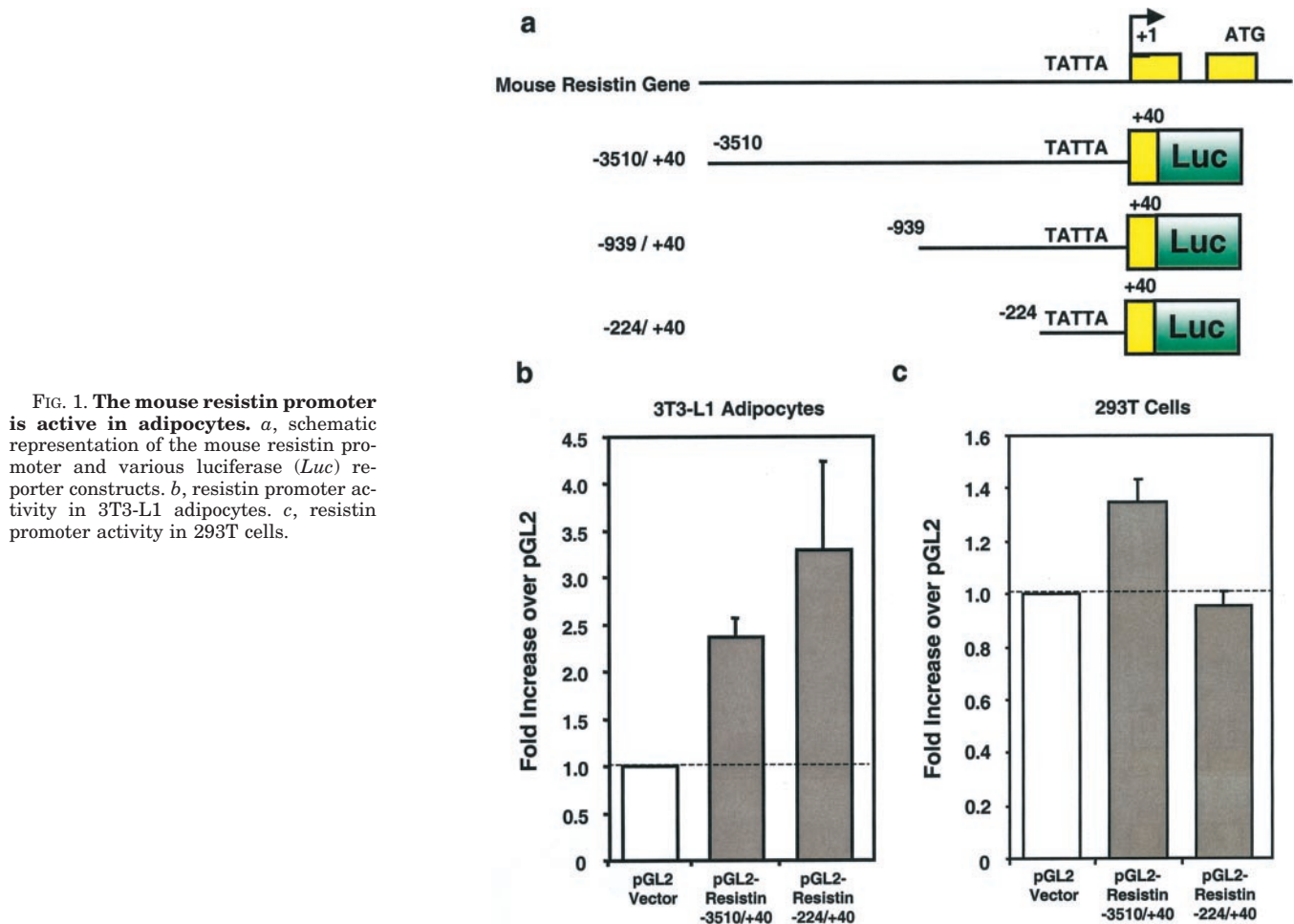


FIG. 1. The mouse resistin promoter is active in adipocytes. *a*, schematic representation of the mouse resistin promoter and various luciferase (*Luc*) reporter constructs. *b*, resistin promoter activity in 3T3-L1 adipocytes. *c*, resistin promoter activity in 293T cells.

#### MATERIALS AND METHODS

**Isolation of the Resistin Gene**—Resistin promoter fragments were isolated from a BAC clone by PCR and subcloned into pGL2-enhancer vector.

**Transfection Studies**—Transient transfection of 293T cells using LipofectAMINE and luciferase reporter assays were performed as described previously (20). 3T3-L1 adipocytes were transfected by electroporation. Day 5 3T3-L1 adipocytes ( $10^7$  cells) were trypsinized and resuspended in media without serum and electroporated at 960 microfarads, 150 V. After electroporation, the cells were then replated. Ligands were added to cells after adherence. Luciferase and  $\beta$ -galactosidase reporter assays were done after 48-h treatment.

**Electrophoretic Mobility Shift Assays**—Gel shift assays were performed as described previously (21).

**Chromatin Immunoprecipitation (ChIP) Assays**—The method of Shang *et al.* (22) was modified as follows. 3T3L1 cells were grown to confluency in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Two days post confluency, cells were either collected as described below (as preadipocytes) or incubated with differentiation media (dexamethasone, isobutylmethylxanthine, and insulin) for 48 h as described previously (11). Day 7 adipocytes were treated with 1  $\mu$ M rosiglitazone or Me<sub>2</sub>SO vehicle and collected at various time points after treatment. Cells were collected by washing twice with phosphate-buffered saline and cross-linking with 1% formaldehyde in phosphate-buffered saline at 37 °C for 10 min. Cells were then rinsed twice with ice-cold phosphate-buffered saline, centrifuged for 4 min at 700  $\times$  *g* and resuspended in lysis buffer (1% SDS, 5 mM EDTA, 50 mM Tris-HCl, pH 8.1). Following a 20-min incubation on ice, samples were sonicated at 15-s pulses three times on ice. The lysates were centrifuged at 14,000  $\times$  *g* for 10 min, and the collected supernatant was diluted in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) with protease inhibitors (Roche Molecular Biochemicals). Samples were precleared with 2  $\mu$ g of sheared salmon sperm DNA and 45  $\mu$ l of protein A-Sepharose beads for 2 h. Immunoprecipitation with the following antibodies was performed overnight: C/EBP $\alpha$ , p300, normal rabbit IgG, CBP (Santa Cruz

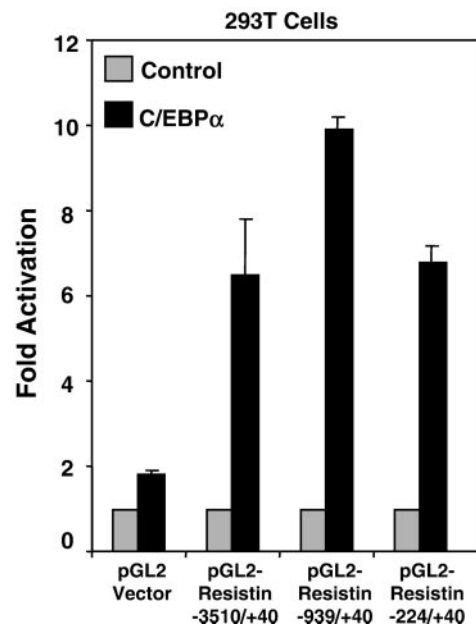
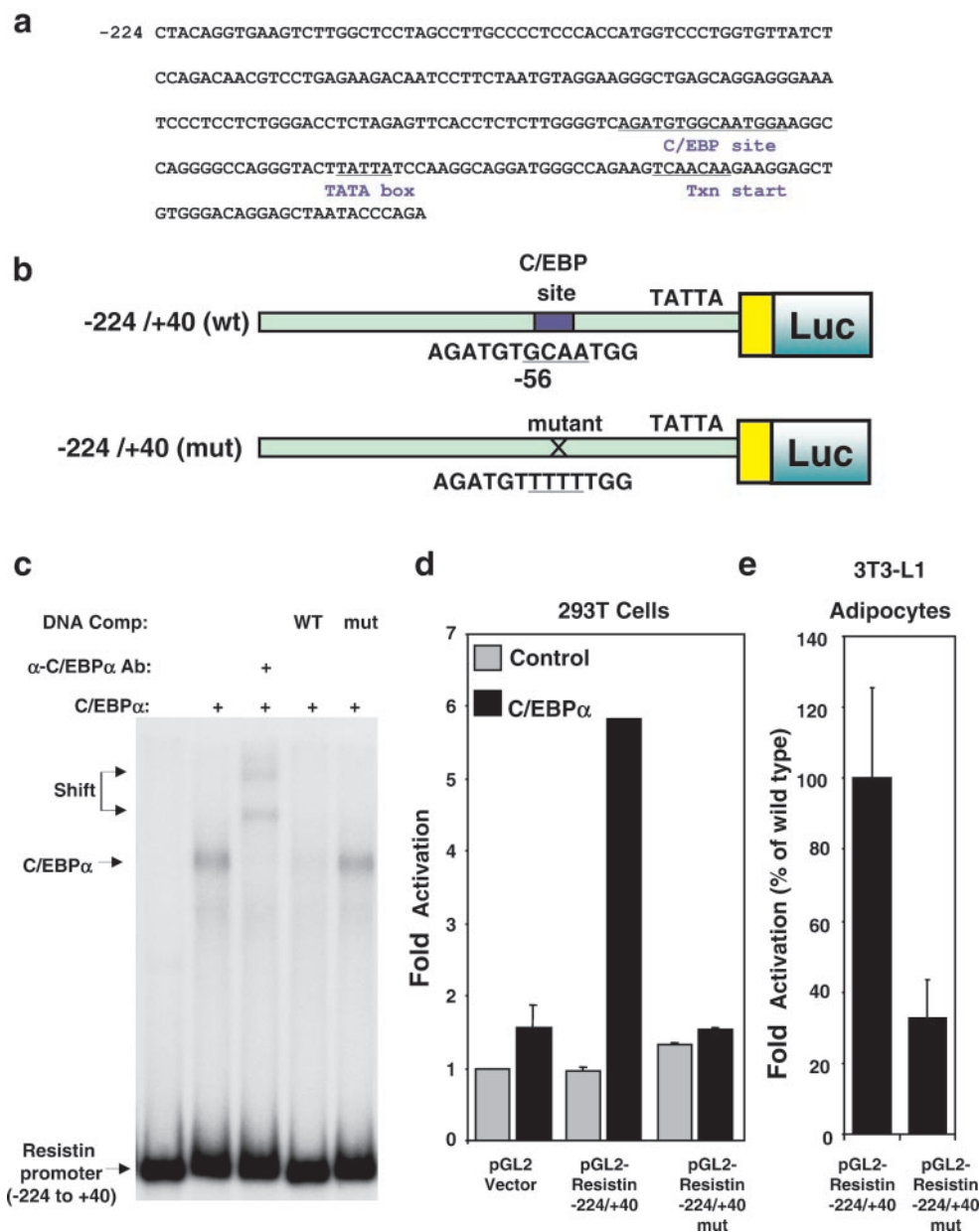


FIG. 2. The mouse resistin promoter is activated by C/EBP $\alpha$  in 293T cells. C/EBP $\alpha$  was co-transfected with luciferase reporter constructs into 293T cells.

Biotechnology, Santa Cruz, CA), acetylated histone H3, acetylated histone H4, acetylated histone H3 (lysine 9), and acetylated histone H4 (lysine 8) (Upstate Biotechnology, Inc., Lake Placid, NY). Samples were then incubated with 45  $\mu$ l of protein A-Sepharose beads for 1 h followed by 10-min sequential washes in TSE I (0.1% SDS, 1% Triton



**FIG. 3. Transcription from the resistin promoter required a C/EBP binding site in the proximal promoter.** *a*, sequence of the mouse resistin promoter near the transcription start site. The C/EBP binding site, TATA box, and transcription start site are underlined and labeled. *b*, schematic representation of the wild type (*wt*) and C/EBP binding site mutation (*mut*) reporter constructs. *c*, C/EBP $\alpha$  binds to the resistin promoter. Electrophoretic mobility shift assay using  $^{32}$ P-labeled fragment of resistin promoter. *Shift* denotes migration of fragment supershifted by C/EBP antibody. *d*, C/EBP $\alpha$  increases the transcription of the wild type but not the mutant promoter reporter in transiently transfected 293T cells. *e*, wild type but not mutant resistin promoter is active in transiently transfected 3T3-L1 adipocytes.

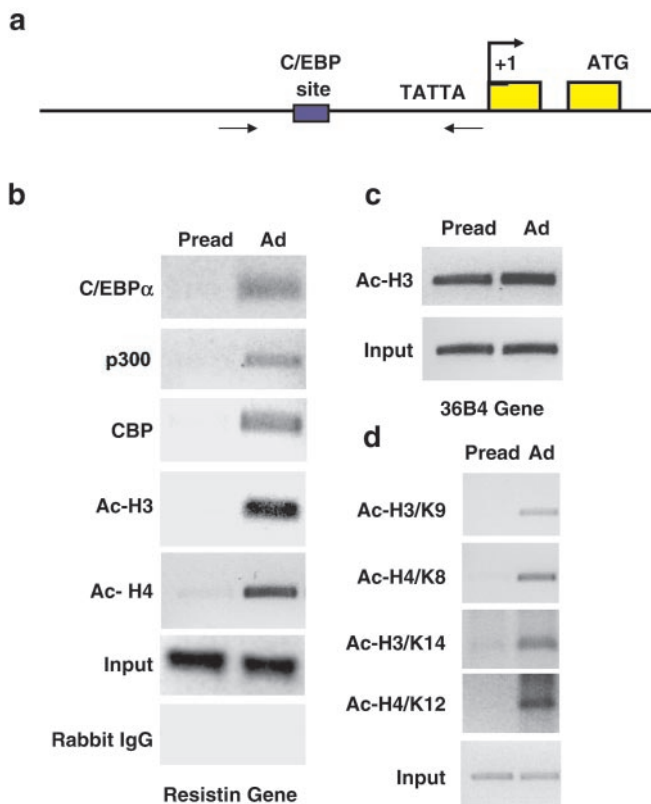
X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl), buffer III (0.25% M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl), and Tris-EDTA buffer. Precipitates were then extracted by incubating with elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>) at 65 °C for 6 h or overnight. DNA fragments were purified with Qiagen PCR purification kit. 2 to 10  $\mu$ l of purified sample were used in 29 cycles of PCR. Primers surrounding the resistin transcription start site had sequences 5'-gtc ttg gct cct agc ctt gc-3' and 5'-gtt gac ttc tgg ccc atc c-3'. Primers for the 36B4 control gene were 5'-cct cgt tgg agt gac atc g-3' and 5'-ggt gtt ctt gcc cat cag c-3'.

## RESULTS

**The Proximal Resistin Promoter Is Sufficient for Adipocyte-specific Expression**—To understand the regulation of mouse resistin expression, the mouse resistin gene was isolated, and its 5'-flanking region plus 40 base pairs of the first exon were fused to a luciferase reporter gene (Fig. 1*a*). A luciferase re-

porter containing 3510 base pairs of 5'-flanking DNA supported the expression in 3T3-L1 adipocytes (Fig. 1*b*) but not in non-adipocytic 293T embryonal kidney cells (Fig. 1*c*). Moreover, a construct containing only the most proximal 224 base pairs of 5'-flanking sequence was sufficient for adipocyte expression.

**The Adipogenic Transcription Factor C/EBP $\alpha$  Induces Expression of the Resistin Promoter in Non-adipocytic Cells**—C/EBP $\alpha$  and PPAR $\gamma$  are adipogenic transcription factors that frequently transactivate adipocyte-specific genes. Therefore, we tested the ability of these factors to stimulate expression from the resistin promoter in 293T cells. The expression of PPAR $\gamma$  did not increase the expression of any of the resistin-luciferase reporter genes in the presence or absence of the thiazolidinedione ligand rosiglitazone (data not shown). By contrast, the expression of C/EBP $\alpha$  led to a robust increase in



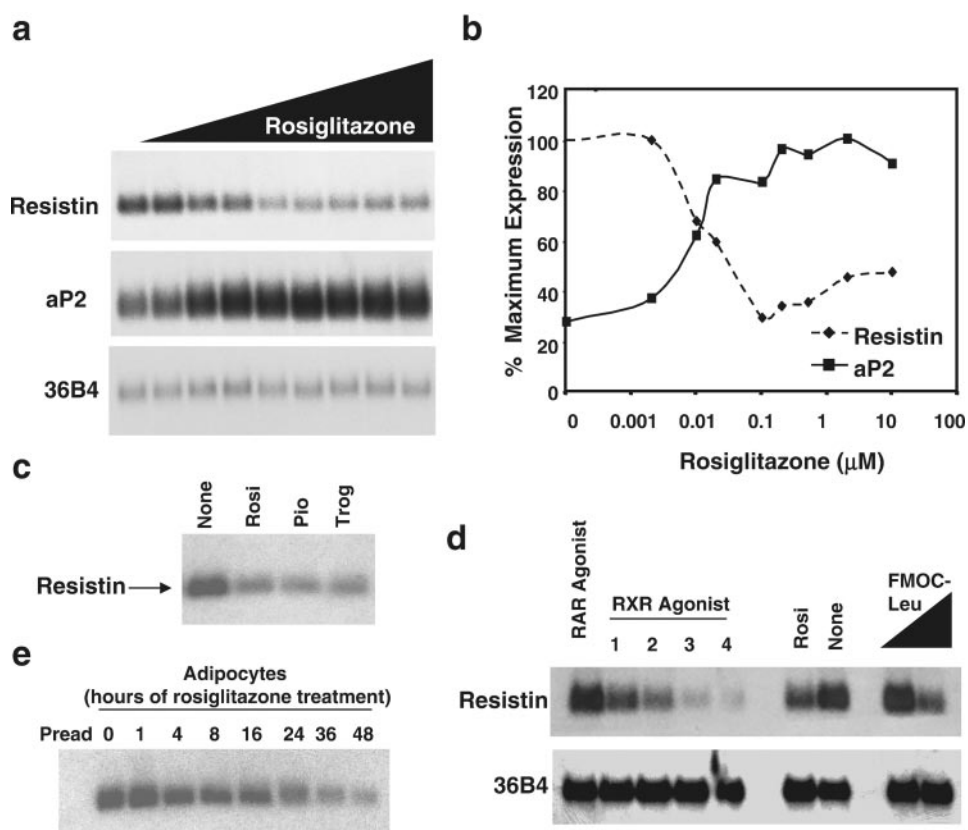
**FIG. 4. Chromatin immunoprecipitation assays of resistin promoter in preadipocytes and adipocytes.** *a*, schematic of resistin promoter. Arrows indicate the PCR primers used to evaluate ChIP samples. *b*, ChIP analysis for C/EBP $\alpha$ , p300, CBP, and acetylated histone H3 (Ac-H3) and H4 (Ac-H4) at the resistin promoter. ChIP protocol was performed on preadipocytes and adipocytes as described under "Materials and Methods." *c*, ChIP analysis for acetylated histone H3 at the 36B4 promoter. *d*, ChIP analysis for acetylated lysines 9 and 14 of histone H3 (Ac-H3/K9 and Ac-H3/K14, respectively) and acetylated lysines 8 and 12 of histone H4 (Ac-H4/K8 and Ac-H4/K12, respectively).

the activity of the resistin promoter (Fig. 2). The magnitude of the stimulation of transcription by C/EBP $\alpha$  was similar for all constructs tested. Thus, a construct containing 5'-flanking 224 base pairs of the resistin transcriptional start site was sufficient for C/EBP $\alpha$ -induced expression.

**The Proximal Resistin Promoter Contains a Functional C/EBP Binding Site**—The sequence of the proximal resistin promoter is shown in Fig. 3*a*. The inspection of the sequence identified a putative C/EBP binding site centered 56 base pairs from the transcriptional start site of the resistin mRNA. We studied the properties of the wild type sequence as well as one containing a 4-bp substitution in the middle of the putative C/EBP binding site (Fig. 3*b*). Electrophoretic mobility shift analysis showed that C/EBP $\alpha$  bound to the wild type resistin promoter (Fig. 3*c*). This binding could be supershifted by anti-C/EBP $\alpha$  antiserum and competed by cold competitor DNA containing the wild type but not a mutated sequence (Fig. 3*c*). The importance of this C/EBP $\alpha$  binding site for resistin expression was tested by comparing the transcriptional activity of the wild type resistin promoter with that of a promoter bearing the mutation in the C/EBP $\alpha$  binding site. In contrast to the wild type promoter, the mutant promoter was inactive in 293T cells cotransfected with C/EBP $\alpha$  (Fig. 3*d*) and also was inactive in 3T3-L1 adipocytes expressing endogenous C/EBP $\alpha$  (Fig. 3*e*). Together, these results implicate C/EBP $\alpha$  as necessary and sufficient for the stimulation of transcription from the resistin promoter.

**Endogenous C/EBP $\alpha$  Is Bound to the Resistin Promoter in Adipocytes in Association with Coactivators and Local Histone Hyperacetylation**—The transfection and gel shift studies used to implicate C/EBP $\alpha$  in the regulation of resistin expression use recombinant overexpressed C/EBP $\alpha$  with an artificial reporter gene. The role of endogenous C/EBP $\alpha$  was investigated by ChIP analysis of the resistin promoter in 3T3-L1 preadipocytes and adipocytes. In this procedure, chromatin is isolated and subjected to cross-linking and shearing of the DNA prior to immunoprecipitation with antibodies against specific proteins. The association of the protein of interest with the resistin promoter was assessed by PCR using primers specific for the resistin promoter (Fig. 4*a*) after the reversal of cross-linking. By this analysis, endogenous C/EBP $\alpha$  was clearly associated with the resistin promoter in adipocytes but not preadipocytes (Fig. 4*b*). p300 has been shown to function as a potent coactivator of C/EBP $\alpha$  (23). Indeed, p300 as well as the closely related coactivator CBP were found to be recruited to the resistin promoter specifically in adipocytes (Fig. 4*b*). We next investigated histone acetylation, because p300 and CBP both contain intrinsic histone acetyltransferase activity, which is critical to their coactivation function (24, 25). The acetylation of histones H3 and H4 was also found to be dramatically increased in the region of the resistin promoter (Fig. 4*b*). By contrast, nonspecific antibodies did not precipitate the resistin promoter sequences in preadipocytes or in adipocytes. Moreover, the increased histone acetylation was specific to the resistin gene because acetylation of histone H3 (as well as H4, data not shown) in the vicinity of the constitutively active 36B4 promoter was indistinguishable in preadipocytes compared with adipocytes (Fig. 4*c*). Using specific antibodies, we found that the robust increase in acetylation of H3 and H4 was because of acetylation of multiple lysine residues including lysines 9 and 14 of H3 and lysines 8 and 12 of H4 (Fig. 4*d*). Together, these results suggest that the functional C/EBP $\alpha$  binding site identified in transfection and gel shift studies is an endogenous binding site for C/EBP $\alpha$ , which recruits histone acetyltransferase-containing coactivators, leading to hyperacetylation and activation of the resistin gene promoter.

**Resistin Gene Expression Is Down-regulated by Multiple Thiazolidinedione (TZD) and non-TZD PPAR $\gamma$  Ligands as well as RXR ligands**—We next explored the mechanism by which rosiglitazone down-regulates resistin gene expression. Many of the effects of rosiglitazone are mediated by PPAR $\gamma$ , but unfortunately fat cells lacking PPAR $\gamma$  are not available and might be impossible to generate given the requirement of PPAR $\gamma$  for adipogenesis (14, 26–28). Therefore, we addressed the potential role of PPAR $\gamma$  in other ways. First, we compared the ED<sub>50</sub> for down-regulation of resistin gene expression by rosiglitazone in 3T3-L1 cells with the  $K_d$  of PPAR $\gamma$  for rosiglitazone binding. The ED<sub>50</sub> for rosiglitazone down-regulation was ~50 nM, very similar to that for the up-regulation of the substantiated PPAR $\gamma$  target aP2 (Fig. 5, *a* and *b*). This is similar to the  $K_d$  of rosiglitazone binding to PPAR $\gamma$  (29, 30). In addition, TZDs other than rosiglitazone, such as pioglitazone and troglitazone, were effective at down-regulating resistin gene expression (Fig. 5*c*). Moreover, FMOCL-leucine, a PPAR $\gamma$  ligand, which is structurally unrelated to TZDs (31), also markedly down-regulated resistin expression in 3T3-L1 cells (Fig. 5*d*). We explored the effects of RXR agonists, which activate the PPAR $\gamma$ /RXR heterodimer (32), and multiple RXR agonists down-regulated resistin gene expression, whereas an RAR-specific ligand had little effect (Fig. 5*d*). All of these data are consistent with the possibility that PPAR $\gamma$  mediates the effect of rosiglitazone. This effect could be direct, *i.e.* because of PPAR $\gamma$  binding to the resistin promoter, or could



**FIG. 5. Down-regulation of resistin expression by ligands for PPAR $\gamma$  and RXR.** *a*, dose-dependent down-regulation of resistin and up-regulation of aP2 by rosiglitazone. Northern analysis of resistin, aP2, and 36B4 loading control. The rosiglitazone concentrations are (from left to right) 0, 0.002, 0.01, 0.02, 0.1, 0.2, 0.5, 2, 10, and 100  $\mu\text{M}$ . *b*, quantitative analysis of the results shown in *a* by phosphorimaging. *c*, multiple TZDs down-regulate resistin expression. The concentration of each ligand is 1  $\mu\text{M}$ . *Rosi*, rosiglitazone; *Pio*, pioglitazone; *Trog*, troglitazone. *d*, the non-TZD PPAR $\gamma$  ligand FMOCL-leucine (*FMOCL-Leu*) and RXR ligands down-regulate resistin expression. The concentrations for FMOCL-Leu are 10 and 100  $\mu\text{M}$ . RAR ligand is BMS453 (66). RXR ligands: 1 = BMS649 (identical to SR11237 (67, 68)); 2 = BMS749 (69); 3 = HX630 (70); and 4 = HX600 (70). RAR and RXR ligands were used at a concentration of 10  $\mu\text{M}$ . *e*, time course of resistin down-regulation by rosiglitazone.

also be indirect, involving the induction of a protein or proteins that repress resistin expression. Indeed, the latter possibility is suggested by the relatively lengthy time course of resistin down-regulation with a half-maximal reduction of resistin mRNA levels observed after  $\sim 24$  h of exposure of 3T3-L1 cells to rosiglitazone (Fig. 5*e*).

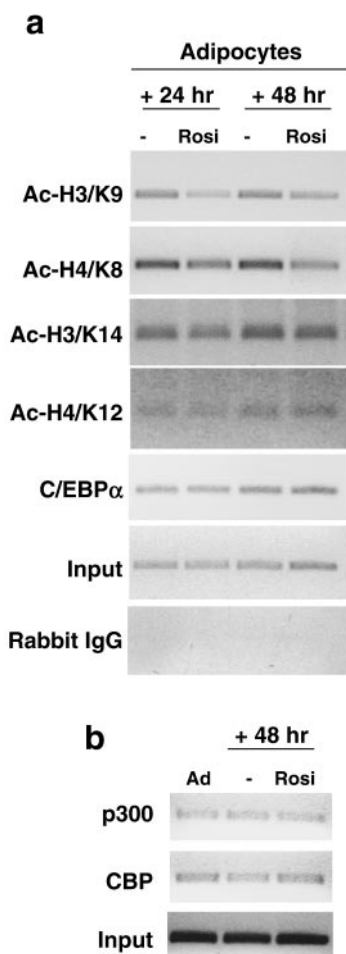
**Rosiglitazone Treatment Reduces Histone Acetylation at the Resistin Promoter**—Given the ability of C/EBP $\alpha$  to activate the resistin promoter in transiently transfected 293T cells, we investigated the ability of rosiglitazone to regulate the resistin promoter. However, using the constructs shown in Fig. 1*a* as well as another construct containing 6.2 kb of flanking sequence, only a modest decrease in transcription from the resistin promoter, never more than 30%, was observed in the presence of rosiglitazone and transfected PPAR $\gamma$  ( $\pm$  C/EBP $\alpha$ , data not shown). By contrast, rosiglitazone down-regulates resistin gene expression by >50%, generally 80–90% as judged by Northern analysis (see Fig. 5) (17, 33). Therefore, we evaluated the effects of rosiglitazone on the resistin promoter in 3T3-L1 adipocytes using the ChIP assay.

Up to 48 h of rosiglitazone treatment had little effect on total histone H3 and H4 acetylation in the vicinity of the resistin promoter (data not shown). Remarkably, rosiglitazone treatment reduced the acetylation of Lys 9 of histone H3 and Lys 8 of histone H4 at the resistin promoter without significantly altering acetylation of Lys 14 of H3 or Lys 12 of H4 (Fig. 6*a*). Consistent with the time course of the rosiglitazone-induced reduction in gene expression, this effect was observed at 24 and 48 h of rosiglitazone treatment (Fig. 6*a*) but not at 5 h (data not shown). C/EBP $\alpha$  binding to the resistin promoter was unaf-

fected by rosiglitazone (Fig. 6*a*). We considered the possibility that ligand binding to PPAR $\gamma$  could “squench” transcription by recruiting p300 and CBP away from the promoter as has been suggested to explain negative regulation of AP-1 activity by nuclear receptor ligands (34). However, we observed no change in CBP or p300 recruitment to the resistin promoter in the presence of rosiglitazone (Fig. 6*b*).

#### DISCUSSION

**Adipocyte Specificity of Resistin Gene Expression**—Adipocyte specificity is a hallmark of resistin gene expression in the mouse. C/EBP $\alpha$  and PPAR $\gamma$  are the two major adipogenic factors that have been implicated as direct regulators of numerous adipocyte-specific genes. Recent studies have demonstrated that PPAR $\gamma$  can induce adipogenesis in the absence of C/EBP $\alpha$ , whereas C/EBP $\alpha$  is unable to induce adipogenesis in the absence of PPAR $\gamma$ , thereby establishing PPAR $\gamma$  as a master regulator of adipogenesis (14). The present studies clearly suggest that C/EBP $\alpha$  binds to and activates the proximal resistin promoter *in vitro* in transfected cells and most importantly in the endogenous situation in fat cells. A 224-bp segment of the resistin promoter contains the C/EBP $\alpha$  binding site and is sufficient for expression in adipocytes. By contrast, PPAR $\gamma$  does not directly activate resistin gene constructs containing up to 6.2 kb of 5'-flanking sequence. Thus, PPAR $\gamma$  appears not to be directly required for the expression of the resistin promoter, although it is possible that the resistin gene contains a PPAR $\gamma$ -responsive element either further 5' or in an intron that was not included in the promoter constructs analyzed here. Even if PPAR $\gamma$  does not directly activate the resistin gene, it is likely that PPAR $\gamma$  pro-



**FIG. 6. Effect of rosiglitazone on histone acetylation and factor association with the resistin promoter.** *a*, Differentiated adipocytes were treated with rosiglitazone for 24 and 48 h, and then ChIP analysis was performed for acetylated lysines 9 and 14 of histone H3 (*Ac-H3/K9* and *Ac-H3/K14*) and acetylated lysines 8 and 12 of histone H4 (*Ac-H4/K8* and *Ac-H4/K12*) as well as C/EBP $\alpha$ . *b*, ChIP analysis for CBP and p300 in differentiated adipocytes and after 48h treatment with rosiglitazone or vehicle.

notes resistin expression by inducing C/EBP $\alpha$  during normal adipocyte differentiation. Indeed, constitutively active PPAR $\gamma$  induces both C/EBP $\alpha$  and resistin expression in the course of adipocyte differentiation (35).

Although it is unusual for adipocyte-specific genes not to be directly up-regulated by liganded PPAR $\gamma$ , there does appear to be a subset of adipocyte genes that rely on C/EBP $\alpha$  for expression. Interestingly, none of these is required for the adipocyte phenotype, but rather all play a role in adipocyte function. These functions include the genes involved in adipocyte insulin sensitivity such as insulin receptor and insulin-receptor substrate 1 (12), the insulin-responsive glucose transporter 4 (36), and adipocyte-secreted factors, leptin (37–39) and adipocyte complement-related protein 30/adiponectin (40). Unlike resistin, leptin, and adipocyte complement-related protein 30/adiponectin, C/EBP $\alpha$  expression is not restricted to adipocytes. Thus, it will be important for future studies to address the mechanisms restricting the expression of resistin as well as other C/EBP $\alpha$ -dependent adipocyte transcripts. Interestingly, like resistin, leptin gene expression in adipocytes is dependent upon C/EBP $\alpha$  (37–39) and is down-regulated by TZDs (39, 41–43) despite the apparent lack of a PPAR $\gamma$  binding site in the leptin promoter (39). The fact that TZDs down-regulate both

resistin and leptin gene expression provides further evidence against a direct role of PPAR $\gamma$  in the up-regulation of both of these genes during adipogenesis.

The putative human homologue of mouse resistin is only 53% identical at the amino acid level, and although it is expressed in human adipose tissue, the level of its expression has been noted to be considerably less than that observed for the gene encoding mouse resistin in mouse adipose tissue (44–47). Consistent with this finding, the promoter sequence of the putative human resistin gene is remarkably divergent from that of the mouse gene reported here with little or no similarity.<sup>2</sup> Thus, the gene regulatory sequences have diverged tremendously. Because there are two other genes related to resistin (*i.e.* three resistin/RELM/FIZZ family genes) in the mouse and only two members of this family have been identified thus far in humans, it is possible that there exists a closer relative to resistin in the human genome that has yet to be discovered. In any case, the differing primary amino acid sequences and expression patterns of the mouse and human genes explained by the divergent regulatory sequences are suggestive of different functions for these proteins.

**Coactivators and Histone Hyperacetylation of the Resistin Gene Promoter**—Our studies have demonstrated that the tails of histones H3 and H4 are hyperacetylated in the region of the resistin promoter. Histone hyperacetylation is recognized as a mechanism of gene activation in many cell types (48, 49). This histone hyperacetylation is most probably mediated at least in part by the p300 and CBP coactivators that we have shown to associate with the resistin promoter in adipocytes. Importantly, these molecules have intrinsic histone acetyltransferase activity (24, 25) and are validated coactivators of C/EBP $\alpha$  (23). Further analysis with antibodies specific for different acetylation sites revealed that lysine sites 8, 9, 12, 14 were all acetylated upon gene activation. These data agree with *in vitro* studies showing that CBP/p300 can acetylate these sites (25, 50, 51), which have been specifically implicated in transcriptional activation (52–56). To our knowledge, this is the first example of specific histone hyperacetylation accompanying gene activation in adipocytes.

**Down-regulation of Resistin Gene Expression by TZDs**—Resistin was identified as a gene whose expression was down-regulated by TZDs in 3T3-L1 adipocytes (17). A number of observations presented herein suggest that TZD down-regulation of resistin expression is mediated by PPAR $\gamma$ . 1) The ED<sub>50</sub> for down-regulation of resistin expression by rosiglitazone is similar to the ED<sub>50</sub> for the induction of aP2 by rosiglitazone. 2) The ED<sub>50</sub> for down-regulation of resistin expression by rosiglitazone is on the order of 100 nM, similar to the *K<sub>d</sub>* of rosiglitazone binding to PPAR $\gamma$  (29, 30). 3) Multiple TZDs down-regulate resistin expression. 4) The non-TZD PPAR $\gamma$  ligand FMOC-L-leucine also down-regulates resistin expression. However, rosiglitazone and PPAR $\gamma$  separately or together did not markedly down-regulate the expression of the resistin promoter in 293T cells, and we have not been able to demonstrate PPAR $\gamma$  binding to the proximal resistin promoter (data not shown). It is possible that the putative PPAR $\gamma$ -negative response element in the resistin gene is outside of the region contained in the promoter constructs we have studied, or that another adipocyte factor (perhaps induced by PPAR $\gamma$ ) represses resistin transcription. Also, TZDs have been shown to have cellular targets other than PPAR $\gamma$  (57–59), and it remains possible that PPAR $\gamma$  is not involved. In this context, it is noteworthy that recent studies of PPAR $\gamma$  null macrophages suggest that PPAR $\gamma$  is not

<sup>2</sup> K. Tyler, X. Hu, H. B. Hartman, and M. A. Lazar, unpublished observations.

required for TZD-dependent down-regulation of cytokine expression (60). Unfortunately, TZD down-regulation of resistin cannot be assessed directly in knock-out models, because PPAR $\gamma$  is required for adipogenesis.

As for other nuclear receptor ligands, the mechanism of negative regulation of gene expression by TZDs is not as straightforward as transcriptional activation (61). TZDs interfere with the transcriptional activity of C/EBP proteins as well as another basic leucine zipper transcription factor, AP-1, on other target genes (62, 63). The ability of nuclear receptor ligands to inhibit the activity of AP-1 has been previously suggested to be attributed to the sequestration of CBP and/or p300 (34). However, our results indicate that this is unlikely to be the mechanism by which TZDs reduce resistin gene expression, because we observe that occupancy of the resistin promoter by CBP and p300 is unchanged by rosiglitazone treatment.

Although the mechanism by which TZDs down-regulate resistin expression is elusive, ChIP analysis of the endogenous resistin promoter demonstrated reduced histone acetylation at a subset of lysines in the tails of histones H3 and H4. The reduced acetylation of lysine 9 in histone H3 is consistent with recent observations correlating deacetylation of lysine 9 with transcriptional repression (52). Interestingly, acetylation at sites lysine 14 of histone H3 and lysine 12 of histone H4 were unchanged by rosiglitazone treatment. The histone code theory postulates that the pattern of modifications of specific residues in histone tails serves as a code to determine the recruitment of different cofactors (56). Thus, it is likely that the specific differences in acetylation fine-tune the level of resistin expression. Although CBP and p300 have not been shown to preferentially acetylate specific lysine residues, it is possible that one or more histone deacetylases might target lysine 9 of histone H3 and lysine 8 of histone H4. Thus far, we have been unable to identify specific histone deacetylases recruited to the resistin promoter by rosiglitazone (data not shown), but this remains a possibility as novel histone deacetylases continue to be discovered (64, 65).

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