Propagation of adipogenic signals through an epigenomic transition state

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The transcriptional mechanisms by which temporary exposure to developmental signals instigates adipocyte differentiation are unknown. During early adipogenesis, we find transient enrichment of the glucocorticoid receptor (GR), CCAAT/enhancer-binding protein \( \beta \) (CEBP\( \beta \)), p300, mediator subunit 1, and histone H3 acetylation near genes involved in cell proliferation, development, and differentiation, including the gene encoding the master regulator of adipocyte differentiation, peroxisome proliferator-activated receptor \( \gamma \) (PPAR\( \gamma \)). Occupancy and enhancer function are triggered by adipogenic signals, and diminish upon their removal. GR, which is important for adipogenesis but need not be active in the mature adipocyte, functions transiently with other enhancer proteins to propagate a new program of gene expression that includes induction of PPAR\( \gamma \), thereby providing a memory of the earlier adipogenic signal. Thus, the conversion of preadipocyte to adipocyte involves the formation of an epigenomic transition state that is not observed in cells at the beginning or end of the differentiation process.

[Keywords: PPAR\( \gamma \), adipogenesis, differentiation, enhancer, epigenomics]

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expression of the majority of genes determining the adipocyte phenotype [Lefterova et al. 2008]. Multiple binding sites for PPARγ and CEBPα are found at the Pparγ and Cebpα loci, consistent with the fact that each autoregulates its own expression as well as that of the other [Wu et al. 1999]. These observations suggest that PPARγ and CEBPα form a feed-forward circuit defined by a regulator that controls a second regulator, with the added feature that both regulators bind a common set of target genes [Alon 2007]. A feed-forward loop with positive regulators can provide consistent activity that is relatively insensitive to spurious changes in input [Mangan and Alon 2003]. That PPARγ and CEBPα are inextricably linked by interconnected autoregulatory loops may provide additional advantages for cell fate decisions. For example, a transient signal can cause the loop to lock irreversibly into the “on” state so that the circuit structure can provide memory of a developmental signal even after it is gone [Alon 2007].

The autoregulatory and feed-forward circuitry formed by PPARγ and CEBPα may explain how adipocyte cell identity is established and maintained upon induction of Pparγ and Cebpα expression. However, our understanding of the transcriptional mechanism initiating Pparγ and Cebpα expression during adipogenesis is incomplete. There is no disputing a role for CEBP proteins. CEBPβ and CEBPδ function in adipocyte differentiation before PPARγ and CEBPα [Cao et al. 1991], and ectopic expression of CEBPβ in nonadipogenic fibroblasts up-regulates Pparγ and Cebpα transcription [Farmer 2006]. Also, mouse embryonic fibroblasts (MEFs) obtained from mice lacking both CEBPβ and CEBPδ do not express Pparγ and Cebpα, and are unable to undergo adipogenesis in culture [Tanaka et al. 1997]. Yet, understanding of the precise role of CEBPβ and CEBPδ in regulating Pparγ and Cebpα is challenged by the finding that Pparγ and Cebpα are expressed in the poorly differentiated adipose tissue of neonatal mice lacking CEBPβ and CEBPα [Tanaka et al. 1997]. Thus, additional regulators may serve redundant and/or cooperative functions with CEBPβ and CEBPδ to ensure transcriptional activation of Pparγ and Cebpα. Identifying these should help to clarify the mechanisms regulating adipocyte differentiation.

We report here that glucocorticoid receptor (GR) and CEBPβ bind cooperatively near Pparγ and genes involved in cell proliferation during the beginning stages of 3T3-L1 adipogenesis. Colocalized and coincident with GR and CEBPβ binding are p300 and Med1 transcriptional coactivators and histone modification associated with transcriptional activation. The data indicate that GR–CEBPβ-bound regions serve as transcriptional enhancers, which is supported further by the finding that they can activate transcription from a heterologous promoter during the early stages of adipogenesis. Occupancy and enhancer function are triggered in response to temporary developmental signals provided by insulin, glucocorticoid, and an inducer of cAMP signaling, and diminish upon their removal. GR, which is required for adipogenesis but need not be active in the mature adipocyte, functions transiently with other enhancer proteins to propagate a new program of gene expression that includes induction of Pparγ. PPARγ regulates its own transcription as well as that of Cebpα to drive expression of adipocyte-specific genes, thereby providing a memory of the earlier adipogenic signal. Thus, the conversion of preadipocyte to adipocyte is directed by an epigenomic transition state that is not observed in cells at the beginning or end of the differentiation process.

Results

A transient peak of histone H3 acetylation appears early during adipogenesis at 10 kb upstream of Pparγ

Genome-wide mapping studies reveal that transcriptionally active genes are enriched for specific post-translational histone modifications, and that some of these can be used to identify cis-regulatory sequences [Wang et al. 2008; Heintzman et al. 2009]. Indeed, our previous studies mapping histone modifications in 3T3-L1-differentiated adipocytes identified intergenic regions enriched for histone marks that colocalize with PPARγ and CEBPα and confer enhancer activity [Steger et al. 2008; Tomaru et al. 2009]. Therefore, to identify potential cis-acting sequences controlling adipocyte differentiation, we combined chromatin immunoprecipitation with microarray analysis (ChIP–chip) to map histone modifications at high resolution during different stages of adipogenesis. 3T3-L1 cells were induced to differentiate by exposure to insulin, dexamethasone, and 3-isobutyl-1-methylxanthine in 10% fetal bovine serum for 2 d. Six days to 8 d are required to form lipid-storing mature adipocytes with this protocol, and histone modification profiles were generated at days 0, 1, and 10. Although the combinatorial patterns of histone modifications at active genes are complex [Wang et al. 2008], we chose to examine H3 Lys 4 trimethylation [H3K4me3] and dimethylation [H3K4me2] and H3K9 acetylation [H3K9ac]. Genomic regions with high H3K4me3 levels are strongly correlated with transcription start sites (TSSs), whereas regions enriched with H3K4me2 and H3 acetylation, but containing relatively low H3K4me3, are often found to be enhancers [Bernstein et al. 2005; Heintzman et al. 2007; Roh et al. 2007].

Of the 74 genes represented on the custom microarray, 20 had greater levels of H3K4me3, H3K4me2, and H3K9ac in day 10 adipocytes compared with day 0 undifferentiated cells, consistent with induced transcription from these genes during adipogenesis [Steger et al. 2008]. Interestingly, only Pparγ displayed increased levels for all three modifications at day 1 of differentiation. As shown in Figure 1, the TSS of Pparγ but not Pparδ contains a small peak of H3K4me3 on day 0, the Pparγ TSS is weakly marked on day 1, and both are strongly marked by day 10. Consistent with these data, Pparγ is expressed weakly in 3T3-L1-undifferentiated cells, Pparδ is induced ~24 h after the initiation of differentiation, and both isoforms are significantly up-regulated in mature adipocytes [Supplemental Fig. S1]. Profiles for H3K4me2 and H3K9ac show expected increases at the Pparγ TSSs for days 1 and 10. Outside of the TSSs, a notable region of
enrichment is located 10 kb upstream of Pparα2, where both H3K4me2 and H3K9ac levels increase from day 0 to day 1, then fall back to day 0 levels at day 10. A second region of interest is positioned 122 kb upstream of Pparα1. Here, H3K4me2 and H3K9ac levels increase at day 1, but remain high in mature adipocytes. Thus, regions with relatively low H3K4me3 and high H3K4me2 and H3K9ac are detected near Ppar during the time of adipogenesis when its expression is induced, suggesting that cis-regulatory sequences enhancing transcription reside there.

Identification of GR and CEBPβ at a subset of genomic regions acetylated transiently during adipocyte differentiation

Sequence-specific transcription factors can direct histone modifications to particular genomic loci by recruiting chromatin-modifying activities when bound to cellular DNA [Hassan et al. 2001]. Thus, we hypothesized that an activator binds a sequence motif located 10 kb upstream of Pparα2 to produce the transient acetylation, and its binding to additional sites in the genome would produce similarly marked regions. To identify the protein(s), we performed genome-wide mapping of H3K9ac during 3T3-L1 differentiation, identified regions with more acetylation at day 1 compared with days 0 and 10, and examined these for enriched sequence motifs. Using a peak-calling method we developed termed STAR (Significance Tester for Accumulation of Reads), 1629 regions located outside of TSSs were determined to be significantly enriched for H3K9ac at day 1 but not days 0 and 10 [Supplemental Table S1]. Gene ontology (GO) analysis of the closest genes determined that the most enriched biological processes were development [P = 3E-10] and cell proliferation and differentiation [P = 3E-10] [Supplemental Tables S2, S3]. These reflect the early events taking place in 3T3-L1 cell differentiation. At day 0, a confluent population of quiescent cells is induced to re-enter the cell cycle and carry out at least two rounds of cell division before giving rise to post-mitotic terminally differentiated adipocytes [Farmer 2006]. Thus, the GO analysis indicates that the transiently acetylated regions are likely functional.

Next, the Asap program [Marstrand et al. 2008] was used to determine whether transcription factor-binding sites were overrepresented in the transiently acetylated regions. Furthermore, the search was narrowed based on the observation that a CEBP-binding site colocalizes with the transient acetylation near Pparα2 (Fig. 1). Although CEBP proteins on their own are unlikely to cause the transient acetylation, because the vast majority of CEBP-binding sites at Ppar are not marked in this manner, we reasoned that the acetylation could result from the combined function of CEBP proteins and a specific activator. Intersection of the CEBPα-binding regions [Leferova et al. 2008] with the transiently acetylated regions uncovered 146 colocalizing regions. Within these, the top-scoring motifs from Asap were CEBPα and GR [P = 5E-05] and GR [P = 6E-05] [Supplemental Table S4].

To determine whether GR and CEBP proteins could occupy the motifs in vivo, multiple regions were examined by ChIP. Regions with a range of false discovery rate (FDR) values were selected [Supplemental Table S5], and H3K9ac ChIP validated the genome-wide findings for all (Fig. 2A). Each has increased acetylation at day 1 relative to day 0, and all but the region upstream of Pparα1 have more acetylation at day 1 compared with day 10. Acetylation remains high on day 10 upstream of Pparα1, with a level similar to the Retn enhancer [Tomaru et al. 2009], possibly because PPARγ and CEBPs occupy both regions in adipocytes. CEBPβ drives 3T3-L1 differentiation before CEBPα, and CEBPβ ChIP revealed variable levels of occupancy for each region at day 0 that increase at day 1 for all and decrease by day 10 for most [Fig. 2B]. CEBPβ, which plays a more limited role in adipogenesis, has a similar binding pattern, while CEBPs displays increased occupancy at day 10 relative to earlier times [Supplemental Fig. S2]. Importantly, GR was found to bind to all regions during the early stages of adipogenesis, with little or no binding detected at
days 0 and 10 (Fig. 2C). Note that GR is present in mature adipocytes (day 10), and thus its lack of binding is due to loss of the adipogenic signal conferred by dexamethasone. Indeed, GR binding at several of the transient sites could be restored by treatment of mature adipocytes with glucocorticoid (Supplemental Fig. S3).

The data suggest that GR colocalizes with CEBPβ at regions acetylated transiently during adipogenesis, with maximal binding coinciding temporally with maximal H3K9 acetylation. In addition, the data imply that GR regulates adipocyte differentiation. To directly assess its function in adipogenesis, we used siRNA to deplete GR in 3T3-L1 cells and examined their ability to differentiate. Cells with decreased GR levels were unable to efficiently store lipid and express adipocyte-selective proteins (Fig. 2D,E; Supplemental Fig. S4). Thus, although its occupancy at \( Ppary2 \) and other regions is transient, GR is required for the adipogenic differentiation of 3T3-L1 cells.

**GR and CEBPβ colocalize in differentiating 3T3-L1 cells**

Multiple transcription factors can coordinate complex cell behavior by targeting the same genes (Boyer et al. 2005). The data thus far suggest that GR and CEBPβ may act in concert to facilitate adipocyte differentiation. To determine the extent to which they function together, we performed genome-wide mapping of GR and CEBPβ during an early stage of 3T3-L1 differentiation. Because GR and CEBPβ appear to occupy their sites similarly at 6 and 24 h of differentiation, ChIP-seq was performed at 6 h so that factor binding would be examined independently of cell division, which occurs later.

Peak calling with GLITR (Tuteja et al. 2009) identified 20,746 and 4007 high-confidence binding regions for CEBPβ and GR, respectively. A search within 4107 of the most highly enriched CEBPβ-bound regions for both de novo and known sequence motifs identified a de novo sequence that strongly resembles the CEBP recognition element as the top-scoring motif (Fig. 3A). Consistent with this, the highest-scoring motifs from the TRANSFAC database are matrices for CEBP proteins (Supplemental Table S6). A similar search within all GR-bound regions revealed the top-scoring motif as a de novo sequence for the GR-binding element (Fig. 3B). Binding sites for the androgen receptor, progesterone receptor, and GR are the most enriched motifs from TRANSFAC (Supplemental Table S7), which is not surprising given that these factors have similar DNA-binding specificities. Interestingly, CEBP matrices are next on the list of enriched TRANSFAC motifs, suggesting that many of the GR-bound regions may also be occupied by CEBP proteins.
locations relative to GR- and CEBP
prior to differentiation (Fig. 2B), and examined their
CEBP in undifferentiated 3T3-L1 cells by ChIP-seq, because
interestingly, only 26% of the GR cistrome colocalizes with
the regions occupied at 0 h overlap those at 6 h. In-
ternal Table S8), agreeing with the finding that 71% of
enriched with the same CEBP motifs as for 6 h (Supple-
hmentary Table S8). Peak calling identified 10,359 regions
of differentiation. Peak calling identified 10,359 regions
bound in differentiating 3T3-L1 cells. Top-scoring se-
quence motifs at 6 h of 3T3-L1 differentiation for CEBP-bound
regions (A) or GR-bound regions (B). The number of binding
regions containing the motif is indicated relative to the number
examined. (C) Venn diagram representing the overlap of GR- and
CEBP-bound regions.

To determine the degree of GR and CEBP co-occupancy on a genome-wide scale, we intersected the GR and
CEBP cistromes to identify bound regions with any
overlap. Remarkably, 67% of GR-bound regions were
found to overlap with CEBP-bound regions (Fig. 3C),
revealing that a majority of GR-bound regions are posi-
tioned near CEBP-bound sites and implying that the
factors may influence each other’s binding in vivo. To
explore this further, we identified CEBP-bound regions
in undifferentiated 3T3-L1 cells by ChIP-seq, because
CEBP ChIP revealed robust occupancy at some sites
prior to differentiation [Fig. 2B]), and examined their
locations relative to GR- and CEBP-bound regions at 6 h
of differentiation. Peak calling identified 10,359 regions
enriched with the same CEBP motifs as for 6 h (Supple-
mental Table S8), agreeing with the finding that 71% of
the regions occupied at 0 h overlap those at 6 h. In-
terestingly, only 26% of the GR cistrome colocalizes with
the CEBP cistrome at 0 h, indicating that most regions
bound by both GR and CEBP are unoccupied in un-
derdifferentiated cells. In addition, nearly all (99%) of the 0 h
CEBP-bound regions that colocalized with GR-bound regions are also occupied at 6 h, and 57% of these show at
least a 1.5-fold increase in CEBP occupancy at 6 h based
on enrichment or stack height from peak calling. Thus,
where GR and CEBP colocalize at 6 h of differentiation,
induced binding by GR is associated with induced CEBP
binding at a majority of regions and increased CEBP
binding at most of the remaining regions bound with
CEBP in undifferentiated cells.

GR and CEBP facilitate each other’s occupancy

GR and CEBP may colocalize because the binding of one
factor facilitates binding of the other. To investigate this,
the ability of each factor to occupy native sites in the
absence of the other was evaluated by ChIP in 3T3-L1
cells treated with siRNAs to reduce GR or CEBP protein
levels. Because CEBP expression is increased during
the early stages of adipogenesis, a response counteracting
siRNA knockdown, we sought conditions that minimize
this up-regulation yet maintain robust GR and CEBP binding.
Without insulin and 3-isobutyl-1-methylxanthine,
dexamethasone treatment for 2 h induced GR and CEBP
binding at nearly all regions studied with little or no
change to CEBP level (Supplemental Fig. S5). Interest-
ingly, ChIP revealed decreased occupancy in cells deleted for GR [Fig. 4A]. As expected, GR binding in
these cells was decreased [Fig. 4B], as was GR, but not
CEBP protein [Fig. 4C]. Thus, at the regions tested, GR
binding is required for full dexamethasone-induced bind-
ing of CEBP. GR occupancy was significantly reduced at
six of eight regions in cells with reduced CEBP and
CEBP levels [Fig. 4D]. CEBP binding was also significa-
tantly reduced in these cells [Fig. 4E], although the fold
changes are moderate, likely because knockdown of
CEBP decreased its expression by only 50% [Fig. 4C;
Supplemental Fig. S6]. Consistent with this, more dra-
katic effects were observed in GR knockdown cells in
which GR was reduced to 10% of untreated cells. Taken
together, the data suggest that GR and CEBP facilitate
each other’s binding at colocalized regions responsive to
glucocorticoid.

Figure 3. A majority of GR-bound regions overlap with CEBP-bound regions in differentiating 3T3-L1 cells. Top-scoring se-
quence motifs at 6 h of 3T3-L1 differentiation for CEBP-bound
regions (A) or GR-bound regions (B). The number of binding
regions containing the motif is indicated relative to the number
examined. (C) Venn diagram representing the overlap of GR- and
CEBP-bound regions.

Transiently acetylated regions bound by GR and CEBP display enhancer properties

The coactivators p300 and Mediator commonly associate
with enhancers (Heintzman et al. 2009; Visel et al. 2009).
Therefore, to further assess the enhancer qualities of the
transiently acetylated regions targeted by GR and CEBP,
we performed ChIP for p300 and MED1. All regions show
increased occupancy of p300 and MED1 during the
beginning stages of adipocyte differentiation when com-
pared with day 0, and all but the region upstream of
Ppar have decreased occupancy at day 10 [Fig. 5A,B]. To
determine whether the regions can regulate transcription in
cis, we cloned them upstream of the luciferase gene
containing a minimal promoter. Clones were transfected
into 3T3-L1 cells and assayed for reporter activity during
the early stages of adipogenesis. With the exception of the
region located 122 kb upstream of Ppar, all confer
increased reporter activity at day 1 compared with days
0 and 4 [Fig. 5C]. It is possible that decreased reporter
activity after day 1 is due to plasmid dilution or loss
over time, because the cells undergo at least two cell
divisions by day 4. However, results from the Retn enh-
ancer and the region upstream of Ppar argue against
this because reporter activity is highest at day 4. Unlike
the other regions, these are targeted by PPARγ, which
may explain their ability to activate expression later,
given that PPARγ levels increase as differentiation pro-
cceeds. As a whole, the data demonstrate that the cloned
regions are sufficient to drive transcription when occupied
by GR, CEBP, p300, and MED1 during adipocyte
differentiation.

Epigenomic adipocytic transition state
A prediction from these results is that GR participates in the early activation of Ppar\(_g^2\). To test this, we examined Ppar\(_g^2\) induction in cells with a reduced GR level. Although considerably less than the level in mature adipocytes, Ppar\(_g^2\) expression increased dramatically from days 0 to 1, and this was markedly blunted by GR knockdown (Fig. 5D). Thus, GR is critical in initiating Ppar\(_g^2\) expression during the beginning stages of adipocyte differentiation.

**Discussion**

Our data show that adipogenic signals mobilize GR and CEBP\(\beta\) present in preadipocytic cells [Fig. 6A] to transiently assemble with p300 and MED1 and induce H3 acetylation at many enhancers throughout the genome. Colocalization of these enhancer activities for only a short time comprises an epigenomic transition state that launches adipogenesis by activating transcription of Ppar\(_g\) and potentially other genes involved in cell development, differentiation, and proliferation [Fig. 6B]. PPAR\(\gamma\) activates its own expression as well as that of CEBP\(\alpha\), and together they establish the adipocyte phenotype by driving transcription of adipocyte-specific genes [Fig. 6C]. Thus, the memory of temporary exposure to adipogenic signals is propagated by an epigenomic transition state during the conversion of preadipocytes to adipocytes.

The role we uncovered for GR in adipogenesis has important implications. The number of sequence-specific regulators impacting Ppar\(_g\) expression has proliferated in recent years [Farmer 2006], yet identification and functional analysis of binding sites for many of these factors have not been determined. Our work shows that GR is required for adipogenesis, and glucocorticoids act by inducing sequence-specific binding of GR to DNA, despite complicated models suggesting otherwise [Wiper-Bergeron et al. 2007]. Importantly, GR does not function in isolation, but cooperates with CEBP\(\beta\). An earlier study temporally uncoupling adipogenic signals found that dexamethasone treatment followed by IBMX, but not IBMX followed by dexamethasone, was sufficient for adipogenesis [Pantoja et al. 2008]. Interestingly, the non-differentiating IBMX-then-dexamethasone treatment transiently induced Cebp\(\beta\) and Cebp\(\delta\), but not Ppar\(_g\) and Cebp\(\alpha\), consistent with the idea that CEBP proteins must synergize with GR to activate target genes.
GR function is also likely related to metabolic physiology and pathophysiology. In humans, Cushing's syndrome due to endogenous or exogenous glucocorticoid excess leads to central adiposity (Peeke and Chrousos 1995; Pijl and Meinders 1996). A direct role for GR in mouse adipose physiology is unclear because GR-null mice die at birth (Cole et al. 1995). Mice carrying a targeted mutation that impairs GR dimerization in vitro are of normal weight (Reichardt et al. 1998); however, it has not been tested whether this GR mutant, which contains the wild-type DNA-binding domain, can occupy sites in native chromatin. Recent studies implicating PRDM16 and CEBPβ in brown fat differentiation from myoblastic precursors used a GR ligand for in vitro differentiation (Seale et al. 2008; Kajimura et al. 2009). Given the close similarity between brown and white fat, it is plausible that control of these cell fate decisions also involves a GR–CEBPβ transition state.

In general, understanding of cell development and differentiation requires understanding of the intermediate steps involved in the process. Importantly, as our study explicitly reveals, transcription factors, coactivators, and histone modifications can assemble simultaneously at specific sites throughout the genome in response to developmental signals, constituting a transition state that is required for differentiation. Although functioning for only a short time, identification of such transition states is critical, given that they can determine the outcome of developmentally induced cell fate decisions. The fact that the cellular transition state is critical but observed in neither the precursor nor the differentiated state is analogous to the transition state of a chemical reaction. Enzymes function by stabilizing the chemical transition state, and this forms the therapeutic basis for drugs that inhibit or activate enzymes (Berg et al. 2007). Likewise, the ability to facilitate or block the formation of cellular transition states based on an understanding of their molecular components could constitute novel modes of therapy for a wide range of diseases.

Materials and methods

Antibodies, ChIP, and Western analysis

The following antibodies were used in this study: histone H3 [Abcam, ab1791], H3K9ac [Upstate Biotechnologies, 06-942], H3K4me2 [Abcam, ab7766], H3K4me3 [Upstate Biotechnologies, 07-473], CEBPα [Santa Cruz Biotechnologies, sc-61], CEBPβ [Santa Cruz Biotechnologies, sc-150], CEBPδ [Santa Cruz Biotechnologies, sc-151 and sc-636], GR [Santa Cruz Biotechnologies, sc-1004, and Affinity BioReagents, PA1-511A], p300 [Santa Cruz Biotechnologies, sc-8998], HDAC2 [Santa Cruz Biotechnologies, sc-7899]. ChIP with 3T3-L1 cells was performed as described previously (Steger et al. 2008). Enrichments were plotted as a percentage of input. Primer sequences are provided in the Supplemental Material [Supplemental Table S9]. Western blots
Figure 6. Propagation of adipogenic signals by an epigenomic transition state converting preadipocyte to adipocyte. Schematic summarizing the transcriptional mechanisms at early and end stages of 3T3-L1 cell differentiation. (A) GR, CEBPβ, Ppar, and MED1 are present in the preadipocyte, but are not colocalized at specific sites throughout the genome. (B) Their assembly and H3 acetylation (purple star) is induced by many enhancers, including Ppary by treatment with insulin, CAMP inducers, and glucocorticoids (black squares). Binding of a transcription factor to a cis-regulatory sequence is indicated by a solid arrow. Genes encoding regulators are connected by dashed arrows. (C) Mature adipocytes emerge 4–6 d after removal of the adipogenic signals. In their absence, factor occupancy, enhancer function, and H3 acetylation associated with GR are abolished, yet Ppar enhancers, including Ppary, colocalize with CEBPα in mature adipocytes (Lefterova et al. 2008), suggesting that it also contributes to this process.

were exposed and quantified using the ChemiDoc XRS and Quantity One 1-D analysis software (Bio-Rad).

ChIP–chip and ChIP-seq
ChIP–chip was performed as described previously (Steger et al. 2008). In short, input and ChIP DNA were amplified linearly using in vitro transcription, and were hybridized to a custom DNA microarray tiled with 60-base-pair (bp) oligonucleotides centered every 25 bp at select genes. ChIP–chip signals [IP/input] were normalized to total histone H3 occupancy, and were plotted as a moving average of 19 contiguous probes spanning 510 bp.

For ChIP-seq, ~10 ng of ChIP DNA was prepared for sequencing according to the amplification protocol from Illumina. Sequence reads of 36 bp were obtained using the Solexa Analysis Pipeline, and were mapped to the mouse genome (mm8).

Uniquely matching reads were retained with the following exceptions: Reads mapping to regions enriched in input DNA were removed, and all but one of the identically aligning reads were removed to minimize PCR bias. MACS (Zhang et al. 2008) and CLIPTR (Tuteja et al. 2009) were used to identify enriched regions within the CEBPβ and GR ChIP-seq data sets. Transcription factor cistromes were intersected using the University of California at Santa Cruz Genome Browser, and sites were considered overlapping if at least 1 bp was shared between the regions.

In order to identify regions enriched for H3K9ac, we developed an algorithm that determines whether local accumulations of reads are statistically significant while controlling for false positives. The algorithm, STAR, takes as input the genomic locations and read lengths of all mapped reads. Sequence reads from control [i.e., nonimmunoprecipitated input DNA] samples are expected to be uniformly distributed; however, these samples tend to show a consistent pattern of bias in Illumina Analyzer data. These were characterized by comparing with simulated data sets of uniformly distributed reads, and regions of bias consistent across control samples were filtered out from all [noncontrol] samples. For a given [noncontrol] sample and a window W of L bases, we let N(W) be the number of reads overlapping the window. For each positive integer n we computed the number of windows of length L in uniformly randomly data set for which N(W) > n. This gives an estimation of the number of false-positive windows with N(W) > n. Using these estimates, we computed for each n the proportion F[n] of false-positive windows in the set of all windows for which N(W) > n. Each window, W, was given a score: S(W) = F[N(W)]. A minimum n0 was chosen so that F[n0] < 0.05. Peaks were then called by merging into spans all windows in the sample with S(W) > n0. Peaks were reported as genomic spans with a score given by the average S(W) across all windows, W, contributing to the peak. This score should decrease as the strength of evidence for the region to have been correctly determined increases. STAR is implemented in Java and is freely available as open source at http://www.cbil.upenn.edu/STAR. Detailed strategies for data analysis can be found in the Supplemental Material. The ChIP-seq data have been deposited in Gene Expression Omnibus.

GO analysis
For each enriched ChIP-seq region, the nearest gene was determined using CisGenome (Li et al. 2008). Only genes with an enriched region within 50 kb of the TSS were used for GO analysis in PANTHER (Thomas et al. 2003, 2006) to determine statistically significant biological processes. The entire mouse genome (NCBI: Mus musculus genes) was used as a reference list, and Bonferroni correction for multiple testing was applied.

Enriched motif analysis
The enrichment of JASPAR motifs within the H3K9ac ChIP-seq data was calculated by applying a Fisher’s exact test to Asap, a software package for searching with position weight matrices (Marstrand et al. 2008). A negative sequence file of matched controls was generated by CisGenome. The enrichment of TRANSFAC motifs within the GR and CEBPβ ChIP-seq data sets was calculated based on a test for positional bias relative to the center of the ChIP-enriched regions analogous to what has been described previously for FoxA1 genome-wide binding (Lupien et al. 2008). Sequence motifs were discovered de novo using MDscan (Liu et al. 2002), and were assessed for significance using the same positional bias statistic.
Transfection and reporter gene assay

Genomic regions examined for cis-acting activity were PCR-amplified and subcloned into pGL4.24 [Promega]. Amplification primers are listed in the Supplemental Material [Supplemental Table S9]. 3T3-L1 cells were transfected by electroporation as described previously (Tomaru et al. 2009). Luciferase activity was normalized to β-galactosidase activity. RNAi-mediated silencing of GR and CEBP proteins was performed by transfecting 3T3-L1 cells with 2–4 nmol of siRNA oligos (Dharmacon SMART pool) and growing the cells to confluency for differentiation studies.

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