Liver-Specific Deletion of Mouse CTCF Leads to Hepatic Steatosis via Augmented PPARγ Signaling

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SUMMARY
Liver-specific CCCTC-binding factor-deficient mice develop hepatic steatosis in a normal chow diet condition primarily through augmented peroxisome proliferator-activated receptor (PPARγ) activity. Activated PPARγ up-regulates its downstream target genes associated with lipid metabolic processes. Hepatic steatosis developed upon CCCTC-binding factor depletion is ameliorated by repression of PPARγ.

BACKGROUND & AIMS: The liver is the major organ for metabolizing lipids, and malfunction of the liver leads to various diseases. Nonalcoholic fatty liver disease is rapidly becoming a major health concern worldwide and is characterized by abnormal retention of excess lipids in the liver. CCCTC-binding factor (CTCF) is a highly conserved zinc finger protein that regulates higher-order chromatin organization and is involved in various gene regulation processes. Here, we sought to determine the physiological role of CTCF in hepatic lipid metabolism.

METHODS: We generated liver-specific, CTCF-ablated and/or CD36 whole-body knockout mice. Overexpression or knockdown of peroxisome proliferator-activated receptor (PPARγ) in the liver was achieved using adenovirus. Mice were examined for development of hepatic steatosis and inflammation. RNA sequencing was performed to identify genes affected by CTCF depletion. Genome-wide occupancy of H3K27 acetylation, PPARγ, and CTCF were analyzed by chromatin immunoprecipitation sequencing. Genome-wide chromatin interactions were analyzed by in situ Hi-C.

RESULTS: Liver-specific, CTCF-deficient mice developed hepatic steatosis and inflammation when fed a standard chow diet. Global analysis of the transcriptome and enhancer landscape revealed that CTCF-depleted liver showed enhanced accumulation of PPARγ in the nucleus, which leads to increased expression of its downstream target genes, including fat storage-related gene CD36, which is involved in the lipid metabolic process. Hepatic steatosis developed in liver-specific, CTCF-deficient mice was ameliorated by repression of PPARγ via pharmacologic blockade or adenovirus-mediated knockdown, but hardly rescued by additional knockout of CD36.

CONCLUSIONS: Our data indicate that liver-specific deletion of CTCF leads to hepatosteatosis through augmented PPARγ DNA-binding activity, which up-regulates its downstream target genes associated with the lipid metabolic process. (Cell Mol Gastroenterol Hepatol 2021;12:1761–1787; https://doi.org/10.1016/j.jcmgh.2021.07.016)
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The liver is the body’s central hub for lipogenesis, fatty acid β-oxidation, and lipoprotein uptake and secretion. Dysregulation of these processes leads to various diseases, including fatty liver disease and liver cancer. Nonalcoholic fatty liver disease (NAFLD), characterized as an abnormal retention of excess lipid within hepatocytes (steatosis), is rapidly becoming a major health concern, with obesity and other metabolic syndromes increasing in prevalence worldwide. Although simple steatosis is relatively benign, it can develop into nonalcoholic steatohepatitis, along with hepatocyte injury, liver inflammation, and fibrosis. The physiologic and molecular machineries that cause NAFLD and trigger its progression to nonalcoholic steatohepatitis, however, remain poorly understood.1

CTCF is a DNA binding protein with highly conserved 11 zinc finger domains that controls various aspects of gene expression, including transcription activation and repression, RNA splicing, chromatin insulation, genomic imprinting, and Variable(V), Diversity (D), and Joining (J) recombination.2 Recent evidence has suggested that CTCF, along with cohesin proteins, mediates long-range chromatin interactions that contribute to the establishment of 3-dimensional genome organization. Research has shown that topological remodeling of the genome by CTCF can affect the expression of genes necessary for survival and differentiation in mammals and that CTCF mutations in human beings are associated with microcephaly and intellectual disability.3 Meanwhile, numerous studies have reported that genetically inducible CTCF knockout in specific cell types, including oocytes, lymphocytes, neurons, cardiomyocytes, and Langerhans cells, results in tissue-specific failure characterized by aberrant transcriptional dysregulation.4,5 However, the precise role of CTCF in controlling liver metabolism in vivo remains poorly understood.

In this study, we used a conditional knockout (cKO) mouse system to show the role of CTCF in liver metabolism. Our results highlight an essential association between CTCF and liver steatosis and showed CTCF to be a crucial regulator of hepatic lipid homeostasis.

Results
Liver-Specific Deletion of CTCF Leads to Liver Steatosis

To investigate the physiological role of CTCF in the liver, we generated liver-specific CTCF cKO mice by crossing mice bearing floxed CTCF alleles (Ctf1f/f) with mice in which Cre expression was under the control of albumin promoter. Depletion of endogenous CTCF in the liver was confirmed at the messenger RNA (mRNA) and protein levels (Figure 1A–C). At 8 weeks of age, the livers of cKO mice appeared paler than those of WT mice (Figure 2A), although body weight, liver weight, and epididymal fat weight were not significantly different between wild-type (WT) and cKO mice (Figure 1D–F). Oil Red O staining showed that all of the cKO mice (6 of 6) experienced accumulation of lipid droplets in the liver, whereas none (0 of 6) of the WT mice did (Figure 2B). Consistent with this observation, hepatic total cholesterol (TC) and triglyceride (TG) levels were increased markedly in the cKO mice (Figure 2C). Meanwhile, significant decreases in serum TC and TG were noted in cKO mice (Figure 2D), suggesting that the uptake of lipids into the liver was increased owing to the loss of CTCF. However, hepatic secretion rates of TG were similar between WT and cKO mice administered injections of tyloxapol to inhibit lipoprotein lipase activity (Figure 2E). Further examination of lipid metabolism by quantifying individual fatty acids with mass spectrometry showed that levels of fatty acids, whether saturated, monounsaturated, or polyunsaturated, were increased significantly in cKO mice, compared with WT mice (Figure 2F). These data indicate that ablation of CTCF significantly accelerates hepatic fat accumulation and facilitates the development of liver steatosis at 8 weeks of age, even on a normal chow diet.

We also examined whether hepatic fat accumulation elicited by the loss of CTCF affects glucose metabolism. In doing so, we found that fasting glucose levels in the blood, as well as in the liver, were unaffected by CTCF deficiency (Figure 3A). In addition, glucose tolerance, insulin tolerance, and pyruvate tolerance test results showed no differences between cKO and control mice (Figure 3B–D). These data show that liver steatosis caused by CTCF deficiency does not lead to insulin resistance or glucose metabolic imbalance.

Hepatic CTCF Deficiency Causes Enhanced Hepatocellular Injury and Liver Inflammation

Next, we examined the extent of liver injury in cKO mice given that hepatic accumulation of fatty acids is known to be harmful to hepatocytes.13 H&E staining of liver sections showed significant increases in the number of hepatocytes with karyomegaloy and binucleation in cKO livers (Figure 4A). Increased serum levels of alanine aminotransferase and aspartate aminotransferase in the cKO mice showed that CTCF depletion induced hepatotoxicity in the liver (Figure 4B). Furthermore, the number of apoptotic and cycling cells was increased significantly in the livers of cKO mice when assessed by cleaved caspase 3 (Figure 4C) and bromodeoxyuridine (BrdU) incorporation (Figure 4D), consistent with the observed pathological changes.

Abbreviations used in this paper: Ad-PPARγ:2, adenosaral peroxisome proliferator-activated receptor γ2; BrdU, bromodeoxyuridine; ChIP-seq, chromatin immunoprecipitation sequencing; cKO, conditional knockout mouse; CTCF, CCCTC-binding factor; Ctcflox/lox, floxed CCCTC-binding factor alleles; HNF-4α, hepatocyte nuclear factor-4α; H3K27ac, H3K27 acetylation; IL, interleukin; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; P, promoter; PPAR, peroxisome proliferator-activated receptor; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; RNA-seq, RNA sequencing; ROS, reactive oxygen species; TAD, topologically associating domain; TC, total cholesterol; TG, triglyceride; TNF, tumor necrosis factor; WT, wild-type.

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respectively. Because increasing evidence has shown that reactive oxygen species (ROS) may be critical mediators of liver damage, we examined intracellular ROS production by flow cytometric analysis using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA). The primary hepatocytes isolated from CTCF-depleted livers showed markedly higher ROS production than primary hepatocytes isolated from WT livers (Figure 5A). The hepatic population of F4/80high Kupffer cells, which release proinflammatory cytokines and ROS, also were higher in CTCF-depleted livers (Figure 5B and C). In parallel with these results, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analyses showed that the expression of proinflammatory cytokines tumor necrosis factor (TNFα) and interleukin (IL)1β was increased significantly in CTCF-depleted livers, compared with WT livers (Figure 5D). In contrast, protein levels of TNF and IL1β present in serum were very low and unaffected by CTCF deficiency (Figure 5E). Collectively, these data suggest that hepatic CTCF is essential to maintaining low levels of ROS and inflammation and that CTCF deficiency results in liver injury.

Increased Accumulation of PPARγ Protein in the Nuclei of CTCF-Deficient Livers

To investigate the molecular mechanism of how CTCF deficiency affects lipid metabolism, we examined genome-wide gene expression patterns by performing RNA sequencing (RNA-seq) analysis (Figure 6A). Differential RNA expression analysis showed 970 deregulated genes (adjusted P < .05; fold change, >2.0), 652 up-regulated and 318 down-regulated, upon CTCF depletion (Figure 6A). Gene ontology analysis indicated that the genes up-regulated by CTCF depletion were principally enriched for cell adhesion and many other development-related pathways, while the down-regulated genes were mostly associated with negative regulation of gluconeogenesis (Figure 7).

H3K27 acetylation (H3K27ac) is tightly coupled to epigenetic regulation of gene expression and marks both active promoters and enhancers. To investigate whether hepatic CTCF deficiency affects liver function via enhancer deregulation, we performed chromatin immunoprecipitation sequencing (ChIP-seq) analysis to compare genome-wide H3K27ac occupancies between WT and cKO livers (n = 3 per group) (Figure 6B). Differential H3K27ac regions were identified, with 1686 regions being hyperacetylated and 1917 regions being hypoacetylated, in cKO livers, compared with WT livers. De novo transcription factor binding motif analysis showed that hepatocyte nuclear factor-4α (HNF4α) motifs were enriched at hypoacetylated regions (P = 10^-16), while peroxisome proliferator-activated receptor (PPAR) motifs were enriched at hyperacetylated regions in CTCF-depleted livers (P = 10^-14) among others (Figure 6C). HNF4α is

Figure 1. Depletion of endogenous CTCF in the livers of cKO mice. (A) qRT-PCR analysis was performed with total RNA isolated from livers from WT and cKO mice (n = 6 per group). (B and C) Immunoblotting was performed using total protein lysates extracted from (B) liver tissue or (C) primary hepatocytes using antibodies as indicated. (D) Body weight, (E) liver weight, and (F) epididymal fat weight of WT and cKO mice. ***P < .001, unpaired 2-tailed Student t test. HSP90, Heat Shock Protein 90.
known as a master regulator of hepatocyte-specific gene expression and to be essential for liver function. PPAR belongs to a class of nuclear receptors and plays a key role in the transcriptional regulation of lipid metabolism in multiple tissues, including the liver. To further explore the cause of CTCF deficiency-induced hepatic fat...

Figure 2. Mice with liver-specific CTCF knockout are susceptible to hepatic steatosis. (A) Macroscopic view of livers from WT and cKO mice at 8 weeks. (B) Representative images of histologic liver sections of WT and cKO mice stained with Oil Red O. Scale bar: 100 μm. (C and D) TC and TG levels in (C) liver and (D) serum from WT and cKO mice at 8 weeks (n = 4–6 per group). (E) Plasma TG levels at the indicated time points after injection of tyloxapol (n = 5 per group). The data are presented as the means ± SD. (F) Mass spectrometric analysis of fatty acids from WT and cKO livers. The heat maps show fold differences between WT and cKO livers for saturated free fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) (n = 7 per group). *P < .05, **P < .01, unpaired 2-tailed Student t test.
accumulation, we examined the expression of HNF4α and PPAR transcription factors in cKO livers. qRT-PCR showed that CTCF depletion in the liver did not affect the expression of HNF4α, PPARα, or PPARγ at the mRNA level (Figure 6D). However, PPARγ protein levels were significantly higher in cKO livers than in WT livers, while protein levels of HNF4α and PPARα were similar (Figure 6E). We then examined subcellular distributions thereof and found that these transcription factors were mostly enriched in nuclear fractions (Figure 6F). Of note, nuclear enrichment of PPARγ was significantly greater in CTCF-depleted livers than in WT livers (Figure 6F). These results suggest that CTCF deficiency in the liver is accompanied by enhanced accumulation of PPARγ protein in the nucleus, which may affect global gene expression profiles by controlling enhancer landscapes.

Identification of PPARγ Target Genes in CTCF-Deficient Livers

To gain insight into whether and how PPARγ affects CTCF deficiency-induced liver steatosis, we explored genome-wide PPARγ recruitment and its effect on gene expression. We first performed ChIP-seq to analyze the distribution of binding sites for PPARγ in WT vs cKO livers. Consistent with enhanced accumulation of PPARγ in the nuclei of cKO livers, the number of PPARγ binding sites, as well as the levels of PPARγ occupancy, were increased markedly in CTCF-depleted livers, compared with WT livers (Figure 8A and B). Gene ontology analysis showed that the genes associated with PPARγ peaks in both WT and cKO livers were mostly enriched in metabolism-related pathways (Figure 9). In addition, we analyzed differential enrichment of PPARγ peaks and found that many more specific PPARγ peaks were gained in cKO livers, while only a few peaks were lost in cKO livers, compared with WT livers (n = 12,283 vs 341, respectively) (Figure 8C). We then investigated genes associated with PPARγ peaks gained in cKO livers (n = 5683), and differential RNA expression analysis for these genes showed 244 deregulated genes (adjusted P < .05; fold change, >2.0), 174 up-regulated and 70 down-regulated, upon CTCF depletion (Figure 8D). Gene set enrichment analysis showed that some of the differentially expressed PPARγ target genes were enriched in cellular lipid metabolic processes (Figures 8E and F and 10). Among PPARγ target genes, we found that the expression of CD36 was increased significantly in CTCF-depleted livers (Figure 8F and G). CD36 is a member of the class B scavenger-receptor family and shows fatty acid translocase activity. High expression of CD36 on the surface of a number
of cell types, including muscle, adipose, and intestinal cells, is essential for lipid uptake, energy storage, and fat absorption, respectively. Basal expression of CD36 in normal hepatocytes is low, and overexpression of CD36 elicits hepatic fatty acid uptake and mediates liver steatosis. WT livers showed little enrichment for PPARγ and H3K27ac in the CD36 locus, which contains 3 promoters (P1–P3) (Figure 8G). In contrast, a regulatory region located between P1 and P2 showed enhanced PPARγ occupancy, as well as increased H3K27ac levels, in cKO livers (Figure 8G), consistent with increased expression of CD36 in cKO livers. Moreover, we detected marked enrichment of H3K4 tri-methylation (H3K4me3), the most frequent histone modification found at active promoters (Figure 11A and C), at P3, suggesting that the majority of CD36 transcripts in cKO livers originated from P3 (Figure 8G). There also was marked enrichment of H3K27ac near the P3 region and a corresponding depletion of the repressive histone mark H3K27 tri-methylation (H3K27me3) throughout the gene body region in cKO livers (Figures 8G and 11B and 11D). PDK4 and NEU3 genes, known to be involved in lipid metabolic processes and to be up-regulated in cKO livers, also were shown to be associated with enhanced PPARγ occupancies and deregulated enhancer activity (Figure 12). These data show that enhanced PPARγ activity mediated by CTCF depletion drives up-regulation of genes associated with lipid metabolic process, possibly via PPARγ-mediated histone modifications.

Figure 4. Enhanced injury, apoptosis, and proliferation in CTCF-deficient livers. (A) Representative images of histologic liver sections of WT and cKO mice at 8 weeks stained with H&E. Scale bar: 40 μm. (B) Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in WT and cKO mice. (C) Extent of hepatocyte apoptosis determined by staining of cleaved caspase-3 in WT and cKO mice. Scale bar: 40 μm. Box plot indicates the percentages of cleaved caspase 3-positive cells. (D) Extent of cellular proliferation determined by BrdU labeling in WT and cKO mice. The arrows designated the location of BrdU+ cells. Scale bar: 40 μm. Box plot indicates the ratio of BrdU-positive cells normalized to total hepatocytes in WT and cKO mice. n = 4–6 per group. *P < .05, **P < .01, ***P < .001, unpaired 2-tailed Student t test.
Further experiments with qRT-PCR showed that the mRNA levels of CD36 were increased in CTCF-depleted livers (Figure 13A). Increased levels of CD36 protein also were shown in cKO livers by Western blot and immunofluorescence (Figure 13B and C). Flow cytometric analysis also showed that primary hepatocytes isolated from cKO livers contained higher levels of surface CD36 protein.
(Figure 13D), as well as increased lipid accumulation, which was abrogated by pretreating the hepatocytes with sulfo-N-succinimidyl oleate, a chemical that binds irreversibly to CD36 and inhibits fatty acid uptake (Figure 13E).

To examine the contribution of PPARγ to CTCF deficiency–induced increases in CD36, we treated primary hepatocytes with the PPARγ inhibitor GW9662. Upon doing so, increased surface expression of CD36 protein, as

| Direction | Motif | Best match | P-value |
|-----------|-------|-------------|---------|
| High in cKO | CAGATTCGCC | BMYB | 1e-15 |
| Low in cKO | CAGATTCGCC | ZNF519 | 1e-15 |
| Low in cKO | CTTCTTTGGCT | PPAR | 1e-14 |
| Low in cKO | CGCTGCTCT | Foxq1 | 1e-13 |
| Low in cKO | CGCTGCTCT | AR-halfsite | 1e-17 |
| Low in cKO | TTTTTATCACAG | HNF4a | 1e-16 |
| Low in cKO | TTCCATGGAAA | FOXM1 | 1e-13 |
| Low in cKO | X-box | 1e-12 |
well as enhanced fatty acid uptake, in primary hepatocytes isolated from cKO livers were diminished by treatment with GW9662 in a dose-dependent manner (Figure 14A and B). To further examine the role of PPARγ in liver steatosis induced by CTCF depletion, we injected the PPARγ inhibitor GW9662 into 4-week-old male mice daily for 2 weeks. Higher expression of CD36 in cKO livers was reduced markedly by PPARγ inhibition at mRNA and protein levels (Figure 14C and D). Moreover, we detected a decrease in hepatic TC and TG levels in cKO mice treated with GW9662 (Figure 14E and F). Thus, inhibition of PPARγ by GW9662 partially reversed upregulation of CD36 and lipid accumulation in CTCF-depleted livers.

**Effects of the Overexpression and Knockdown of PPARγ in Hepatic Steatosis**

Next, we investigated whether overexpression of PPARγ can be a direct cause of liver steatosis by administering

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**Figure 7.** Bar plot of $-\log_{10}$ (adjusted $P$ value) showing enrichment of gene ontology terms (biological process) associated with genes whose expressions were up-regulated (top) or down-regulated (bottom) by CTCF depletion with more than 2-fold changes. Only the top 20 terms are shown.

**Figure 6.** (See previous page). Enhanced accumulation of PPARγ protein in the nuclei of CTCF-deficient livers. (A) Volcano plot showing significant changes in genome-wide RNA expression between WT and cKO livers. The number of genes that showed greater than 2.0-fold increases in WT (blue) or cKO (red) livers with an adjusted $P$ value of less than .05 is indicated. (B) MA plot showing differential enrichment of H3K27ac between WT and cKO livers. Differentially acetylated regions that showed greater than 1.3-fold increases in WT (lower) and cKO (upper) livers with an adjusted $P$ value of less than .05 are indicated. (C) Enriched de novo motifs found by hypergeometric optimization of motif enrichment among differential H3K27ac regions. (D) Expression of PPARγ, PPARα, and HNF4α mRNA was determined by real-time qRT-PCR ($n = 11–12$ per group). (E) Expression of CTCF, PPARγ, PPARα, HNF4α, and HSP90 protein was determined by Western blot. (E) Right: The band densities of the proteins were quantified with HSP90 as the loading control and presented as box plots ($n = 7$ per group). (F) Western blot analysis of cytoplasmic and nuclear fractions of PPARγ, PPARα, and HNF4α from WT and cKO livers. (F) The band densities of the nuclear proteins were quantified with Lamin B as the loading control and presented as box plots in the right panel ($n = 5–9$ per group). *$P < .05$, **$P < .01$, ***$P < .001$, unpaired 2-tailed Student $t$ test.
adenoviral PPARγ2 (Ad-PPARγ2) into wild-type mice via tail vein injections (Figure 15A–E). Ad-PPARγ2 injection induced the expression of PPARγ2 and its downstream target gene CD36 (Figure 15A and B), with higher levels of hepatic TG and higher liver weights than control Ad-GFP mice (Figures 15C and 16A–C). Moreover, histologic analysis by H&E staining and Oil Red O staining showed the presence of numerous lipid droplets in the livers of Ad-
PPARγ2–injected mice (Figure 15D and E). These results suggest that hepatic overexpression of PPARγ2 results in severe hepatic steatosis, consistent with a previous report.15

To examine whether suppression of PPARγ can reduce hepatic steatosis, liver-specific CTCF-deficient mice were administered adenovirus expressing either control (Ad-unspecified [Ad-US]) or PPARγ short hairpin RNA via tail vein injections (Figure 15F–J). Expression of PPARγ short hairpin RNA in the liver significantly reduced protein levels of PPARγ, as well as CD36 (Figure 15F and G). Furthermore, cKO mice injected with Ad-shPPARγ showed lower levels of hepatic TG (Figure H) and fewer lipid droplets (Figure 15I and J) than those injected with control Ad-US. Meanwhile, knockdown of PPARγ in cKO livers did not change body or liver weights (Figure 16D–F). Taken together, these results indicate that activated PPARγ signaling in CTCF-deficient livers plays a crucial role in the development of liver steatosis.

Figure 8. (See previous page). Identification of PPARγ-regulated genes in CTCF-deficient livers. (A) Pie charts showing the total numbers and genomic distributions of PPARγ ChIP-seq peaks identified from WT and cKO livers. (B) Histogram showing the average tag density of PPARγ ChIP-seq peaks. (C) Volcano plot showing significant changes in PPARγ enrichment between WT and cKO livers. The number of peaks that showed greater than 2.0-fold increases in WT (blue) or cKO (red) livers with an adjusted P value of less than .05 is indicated. (D) RNA-seq MA plot for genes (n = 5683) associated with PPARγ peaks (n = 12,283) gained in cKO livers. Mean abundance is plotted on the x-axis, and enrichment is plotted on the y-axis. The number of genes that showed more than 2.0-fold increases in WT (blue) or cKO (red) livers with an adjusted P value of less than .05 is indicated. (E) GSEA plot illustrating the enrichment of gene signatures for cellular lipid metabolic process. (F) Heatmap of RNA-seq signals for genes enriched in the cellular lipid metabolic pathway as shown in panel E. (G) Genomic snapshot of the CD36 locus. Densities of ChIP-seq reads for PPARγ, H3K27ac, H3K4me3, and H3K27me3 are shown. Densities of RNA-seq reads also are shown. Three known promoters (P1–P3) of CD36 are indicated by arrows. TPM, transcripts per million; UTR, untranslated region.
CD36 Knockout Is Not Enough to Rescue Hepatic Steatosis in CTCF-Deficient Mice

CD36 overexpression is well known to elicit hepatic fatty acid uptake and mediate liver steatosis. Given that CD36 expression is under the control of PPARγ signaling, we examined whether CTCF deficiency–induced hepatic fat accumulation is attributable primarily to CD36 overexpression. We therefore crossed liver-specific CTCF conditional knockout (CTCF cKO) mice with CD36 knockout (CD36 KO) mice to generate double-knockout mice (CTCF cKO/CD36 KO). CD36 protein, overexpressed in CTCF-deficient (CTCF cKO/CD36 WT) mice livers, clearly disappeared in double-knockout (CTCF cKO/CD36 KO) mice livers (Figure 17A). Hepatic TG levels, which were up-regulated markedly in CTCF-deficient (CTCF cKO/CD36 WT) mice livers, clearly disappeared in double-knockout (CTCF cKO/CD36 KO) mice livers (Figure 17B). Histologic analysis by H&E staining and Oil Red O staining showed that the lipid accumulation seen in CTCF-deficient livers (CTCF cKO/CD36 WT) was not diminished by additional depletion of CD36 (CTCF cKO/CD36 KO) (Figure 17C and D). These results showed that knockout of CD36 alone is not enough to rescue hepatic steatosis in CTCF-deficient mice and that several PPARγ downstream target genes may work together to contribute to the development of hepatic steatosis in a pleiotropic manner. Indeed, several PPARγ target genes, such as PDK4, NEU13, and CIDEC, which have been reported to be associated with lipid metabolism, showed increased mRNA expression in CTCF-deficient livers, as well as CTCF/CD36 double-knockout livers, compared with WT livers (Figure 17E–G), further suggesting the coordinated contribution of multiple target genes.

Genome-Wide CTCF Occupancy and Higher-Order Chromatin Structure in cKO Livers

Given that CTCF is a well-known chromatin architectural protein and essential to organizing mammalian genomes, we examined genome-wide CTCF binding patterns by performing ChIP-seq (Figure 18A). CTCF occupancy was preferentially mapped at intergenic regions, as well as promoters and introns, in both WT and cKO livers (Figure 18B). Relatively modest levels of reduced CTCF enrichment was observed in cKO livers (Figure 18C and D), indicating that a significant amount of CTCF protein still was left in cKO livers (Figure 18B and C). Differential CTCF binding regions were identified, with 1178 regions being significantly lost in cKO livers, compared with WT livers (Figure 18E). The low-affinity binding sites were more sensitive to CTCF depletion in the decrease of CTCF occupancy than the high-affinity binding sites (Figure 18E). Integrated analysis of ChIP-seq for CTCF and H3K27ac showed little change in the enrichment of H3K27ac levels at the CTCF binding sites lost in cKO livers, indicating that loss of CTCF occupancy has little effect on enhancer activity at binding sites (Figure 18F).

We additionally performed in situ Hi-C to analyze higher-order chromatin structures in cKO livers (Figure 18G–N). Contact maps (Figure 18G) and compartment signals (Figure 18H–J) showed that segregation of active and inactive chromosome domains into A and B compartments was not altered significantly with CTCF depletion. We also examined topologically associating domains (TADs) using insulation scores and found that TAD numbers were quite similar between WT and cKO livers (Figure 18K). Moreover, the capacity of preventing inter-TAD interactions was decreased only slightly in cKO livers.
livers (Figure 18L and M), and we noted little changes in genome-wide intra-TAD chromatin interactions (Figure 18N). These data suggest that 3-dimensional genome organization, as reflected in compartments and TADs, still was preserved in cKO livers, possibly owing to significant amounts of residual CTCF protein, most of
which is thought to bind preferentially to high-affinity binding sites forming TAD boundary elements.

**Discussion**

CTCF is a versatile transcriptional regulator and chromatin architectural protein required for mammalian development and for cellular differentiation of various lineages; however, its function in the liver is poorly understood.\(^\text{4–12}\) Our genetic analysis of CTCF in a liver-specific knockout mouse model indicated that it is essential for maintaining homeostasis of hepatic lipid metabolism.

PPAR\(\gamma\), which is implicated in the development of many metabolic diseases, can bind various free fatty acids to control the transcription of many genes governing lipid metabolism.\(^\text{16}\) Although PPAR\(\gamma\) is found at low levels in normal liver, increased expression of PPAR\(\gamma\) is a general property of steatotic liver.\(^\text{17–20}\) Consistent with these previous reports, our H3K27ac ChIP-seq analysis, followed by de novo transcription factor binding motif analysis, highlighted the PPAR binding motif as being highly enriched in activated enhancer regions in CTCF-depleted livers. Moreover, we observed increased accumulation of PPAR\(\gamma\) in the nuclei of CTCF-deficient livers that seemed to be regulated by post-translational modifications, such as phosphorylation, ubiquitination, and small ubiquitin-like modifier (SUMO) conjugation (or SUMOylation),\(^\text{21,22}\) given that mRNA levels of PPAR\(\gamma\) were comparable between WT and cKO livers. Augmented PPAR\(\gamma\) activity in cKO livers also was indicated by significant increases in PPAR\(\gamma\) DNA binding in vivo, leading to increased expression of its downstream target genes involved in lipid metabolic processes. The crucial role of PPAR\(\gamma\) in the development of hepatic steatosis in CTCF-depleted mice was validated further by gain-of-function and loss-of-function experiments. Adenoviral overexpression of PPAR\(\gamma\)2 in WT mice resulted in accumulation of hepatic TG and lipid droplets. In contrast, repression of PPAR\(\gamma\) by both pharmacologic inhibition and adenovirus-mediated knockdown, significantly decreased liver TG contents with a lessening of hepatic steatosis developed in CTCF-deficient mice.

One of the major PPAR\(\gamma\) target genes up-regulated in CTCF-deficient livers is CD36, which facilitates fatty acid transport, allowing for the accumulation of lipids in the liver. CD36 has been reported previously to have a role in intestinal fat absorption, lipid storage in adipose tissue, and lipid utilization by cardiac and skeletal muscle.\(^\text{16–28}\) Although basal expression of CD36 in the liver is very low and although CD36 does not play a major role in fatty acid uptake in normal liver, recent findings have suggested a causative role for CD36 in the pathogenesis of hepatic steatosis. For example, increased hepatic CD36 expression has been observed in various pathologic conditions, including NAFLD and type 2 diabetes mellitus.\(^\text{29–31}\) Moreover, forced expression of CD36 by adenovirus-mediated infection was found to increase hepatic fatty acid uptake and triglyceride storage, and disruption of hepatic CD36 was shown to attenuate fatty liver in high-fat-diet–fed mice.\(^\text{32–35}\)

Consistent with these previous reports, we also discovered that hepatic fatty acid uptake enhanced in CTCF-null primary hepatocytes was down-regulated after treatment with a CD36 inhibitor, sulfo-N-succinimidyl olate.

Many studies have shown that PPAR\(\gamma\) regulates CD36 expression via binding to a PPAR\(\gamma\)-responsive element in the proximal region of the CD36 promoter and that both natural and synthetic PPAR\(\gamma\) ligands, including prostaglandin J2 and thiazolidinediones, can increase CD36 expression.\(^\text{36–38}\) In WT livers in which expression of CD36 is very low, we found near-baseline levels of H3K27ac and PPAR\(\gamma\) at the CD36 locus. However, significant increases in CD36 expression induced by loss of CTCF was accompanied by increased binding of PPAR\(\gamma\) to several enhancer regions of the CD36 locus, with increased occupancies of active histone marks H3K27ac and H3K4me3, but decreased levels of the repressive histone mark H3K27me3. CD36 may exploit a feed-forward cycle to positively influence its own de novo synthesis by facilitating the entry of fatty acids, which can function as a ligand of PPAR\(\gamma\) to activate PPAR\(\gamma\)-mediated transcription of CD36.

The essential role of PPAR\(\gamma\) in CD36 expression was verified by experiments showing that treatment of the PPAR\(\gamma\) antagonist GW9662, as well as adenovirus-mediated PPAR\(\gamma\) knockdown, in the CTCF-deficient mice significantly
Figure 13. Increased CD36 expression and fatty acid uptake in CTCF-deficient liver. (A and B) Expression of CD36 (A) mRNA (n = 13–14 per group) and (B) protein (n = 15 per group) in livers from WT and cKO mice was determined by real-time qRT-PCR and Western blot, respectively. Right: The band densities of the proteins were quantified and presented as box plots. (C) Representative immunofluorescence staining for CD36 (red) and 4’,6-diamidino-2-phenylindole (DAPI) (blue) in livers from WT and cKO mice. Scale bar: 50 μm. (D) CD36 expression levels in primary hepatocytes from WT and cKO mice were assessed by flow cytometry (n = 3 per group). (E) Uptake of fatty acid by primary hepatocytes was determined by flow cytometry in the absence or presence of sulfo-N-succinimidyl oleate (SSO) at the indicated concentrations. Cells were gated for fluorescence-tagged fatty acid and indicated in the box plots (n = 3 per group). *P < .05, **P < .01, ***P < .001, unpaired 2-tailed Student t test. FITC, Fluorescein; HSP90, Heat shock protein 90; MFI, Mean Fluorescent Intensity.
decreased hepatic CD36 expression. Because GW9662 was clearly able to repress fatty acid transport activity in CTCF-deficient primary hepatocytes, we wondered whether blocking CD36 could ameliorate hepatic steatosis developed as a result of CTCF depletion. However, introduction of CD36 deletion to liver-specific CTCF knockout mice elicited only modest changes in hepatic accumulation of lipid droplets, suggesting additional gene sets regulated by PPARγ may pleiotropically contribute to the development of hepatic steatosis. Indeed, the PPARγ target genes differentially expressed between WT and cKO livers regulate various aspects of lipid metabolism: For example, cell death–inducing DFFA-like effector c, a lipid droplet-associated protein that promotes intracellular triglyceride death may pleiotropically contribute to the development of hepatic steatosis.

In summary, we discovered that liver-specific CTCF-deficient mice develop hepatic steatosis in a normal chow-diet condition primarily through augmented PPARγ activity that modulates genome-wide enhancer landscapes to up-regulate target genes associated with hepatic lipid metabolism.

Methods

Mice

Mice carrying a conditional CTCF allele (Ctcffl/fl) were described in our previous study. To generate mice with conditional deletion of CTCF in the liver, Ctcffl/fl mice were bred with mice expressing Cre recombinase driven by an albumin promoter (Alb-cre; Jackson Laboratory, Bar Harbor, ME). Alb-cre mice were kindly provided by Han-Woong Lee (Yonsei University, Seoul, Korea). Ctcffl/fl littermate mice were used as WT controls throughout the study. CD36 knockout mice were purchased from Jackson Laboratory and bred with liver-specific conditional CTCF knockout mice (Alb-cre/Ctcffl/fl) to generate double-knockout mice for CTCF and CD36 (Alb-cre/ Ctcffl/fl/CD36KO). All experiments were performed on 8- to 20-week-old male mice fed with a standard chow diet after an overnight fast. All mouse experimental procedures were approved by the Department of Laboratory Animal Resources Committee of Yonsei University College of Medicine.

Chemical Inhibitors

Sulfo succinimidyl oleate (Cayman Chemical, Ann Arbor, MI) stock was prepared in 100% dimethyl sulfoxide, and GW9662 (Sigma-Aldrich, St.Louis, MO) stock was prepared in corn oil and diluted to indicated concentrations for in vitro and in vivo experiments.

Preparation of Recombinant Adenovirus

Adenovirus-expressing PPARγ2 was generated through homologous recombination between a linearized transfer vector pAD-Track and the adenoviral backbone vector pAD-Easy as described previously. Adenovirus for Green fluorescent protein (GFP) only was described previously. Adenovirus expressing unspecific RNA interference (US) and PPARγ RNA interference were described previously.

Histologic Analysis

A cross-section of the left lateral lobe of the liver was fixed in 4% paraformaldehyde at room temperature. The

![Figure 14](See previous page). Pharmacologic inhibition of PPARγ decreases hepatic lipid levels in CTCF-deficient mice. (A and B) Primary hepatocytes were treated with dimethyl sulfoxide or GW9662 for 24 hours, and (A) levels of CD36 expression (n = 3 per group) and (B) uptake of fatty acid (n = 3 per group) were quantified by flow cytometry. (C–F) Mice were injected with GW9662 (100 μM) daily for 2 weeks. Expression of CD36 (C) mRNA (n = 13–14 per group) and (D) protein (n = 4–10 per group) were determined by real-time qRT-PCR and Western blot, respectively. (E) TC and (F) TG levels in livers (n = 3 per group). *P < .05, **P < .01, ***P < .001, unpaired 2-tailed Student t test. FITC, Fluorescein; MFI, Mean Fluorescent Intensity.
Figure 15. Effect of overexpression and knockdown of PPARγ in hepatic steatosis. (A–E) WT mice were injected with adenovirus expressing control (Ad-GFP) or PPARγ2 (Ad-PPARγ2) 7 days before death (n = 4 per group). (A) Western blot analysis of PPARγ, CD36, and HSP90 proteins. (B) The band densities of the proteins in panel A were quantified and presented as box plots (n = 4 per group). (C) TG levels in livers (n = 4 per group). (D and E) Representative images of histologic liver sections stained with (D) H&E and (E) Oil Red O. Scale bars: 50 μm (D) and 200 μm (E). (F–J) CTCF cKO mice were injected with adenovirus expressing control (Ad-US) or PPARγ short hairpin RNA (Ad-shPPARγ) 7 days before death (n = 4–5 per group). (F) Western blot analysis of PPARγ, CD36, and HSP90 proteins. (G) The band densities of the proteins in panel F were quantified and presented as box plots (n = 4–5 per group). (H) TG levels in livers (n = 4 per group). (I and J) Representative images of histologic liver sections stained with (I) H&E and (J) Oil Red O. Scale bar: 50 μm (I) and 200 μm (J). *P < .05, **P < .01, ***P < .001, unpaired 2-tailed Student t test.
liver tissues were dehydrated, embedded in paraffin, sectioned at 5 μm, and stained with H&E. For visualizing lipid deposits, liver tissues were embedded in optimal cutting temperature compound (OCT) (Sakura Finetek, Torrance, CA) and frozen on dry ice, after which 10-μm sections were stained with Oil Red O (Sigma-Aldrich, St. Louis, MO).

**Metabolic Analysis**

Serum levels of alanine aminotransferase, aspartate aminotransferase, total cholesterol, and triglycerides were monitored by standard clinical chemistry assays on an automatic chemistry analyzer (7020; Hitachi, Tokyo, Japan). Hepatic lipids were extracted using a chloroform/methanol mix (2:1, vol/vol), as described previously, and total cholesterol and triglyceride levels in the liver were measured using cholesterol/cholesteryl ester assay kits (Abcam, Cambridge, United Kingdom) and triglyceride assay kits (Abcam, Cambridge, United Kingdom), respectively. The levels of free fatty acids in the liver were analyzed with gas chromatography–triple quadrupole tandem mass spectrometry. To measure Very-low-density lipoprotein (VLDL)-TG secretion rates, mice were fasted for 16 hours, pre-bled by retro-orbital bleeding, and administered intravenous injections of 10% tyloxapol (Triton WR-1339, 500 mg/kg body weight; Sigma-Aldrich, St. Louis, MO). Plasma samples were drawn serially at 0, 60, and 120 minutes after injection, and plasma TG levels were measured using triglyceride assay kits (Abcam, Cambridge, United Kingdom).

**Tolerance tests**

Glucose, insulin and pyruvate tolerance tests were performed as described previously. Mice fasted for either 18 or 6 hours before the tolerance tests, respectively. Mice were injected intraperitoneally with either 1.5 g/kg of glucose (Sigma-Aldrich, St. Louis, MO, USA), 0.75 U/kg of insulin (regular humulin; Eli Lilly and Company,
Figure 17. CD36 knockout is not enough to rescue hepatic steatosis in CTCF-deficient mice. (A) Western blot analysis of CTCF, CD36, and HSP90 proteins. (B) TG levels in livers (n = 4 per group). (C and D) Representative images of histologic liver sections stained with (C) H&E and (D) Oil Red O. Scale bar: 50 μm. (E–G) Expression of (E) PDK4, (F) NEU3, and (G) cell death–inducing DFFA-like effector c (CIDEC) mRNA (n = 4 per group) in the livers from mice with the indicated genotype was determined by real-time qRT-PCR. *P < .05, **P < .01, ***P < .001, unpaired 2-tailed Student t test.
Indianapolis, IN) or 1.5g/kg of pyruvate (Sigma-Aldrich, St. Louis, MO, USA), respectively. Glucose measurements were taken up to 4 to 6 hours after injection using a glucometer (ACCU-CHEK, Active/Blood Glucose Meter/Blood Glucose Monitors; Roche Applied Science, Grenzach-Wyhlen, Basel, Switzerland).

**Immunohistochemistry and Immunofluorescence**

Liver samples were fixed in 4% paraformaldehyde at room temperature, dehydrated, and embedded in paraffin. After blocking with Protein Block (Agilent Technologies DAKO, Santa Clara, CA), the sections (5 μm) were labeled with corresponding primary antibody at 4°C overnight and horseradish-peroxidase-conjugated secondary antibody (Agilent Technologies DAKO, Santa Clara, CA) for 15 minutes at room temperature. For immunohistochemistry, detection was developed with 3,3′-diaminobenzidine substrate (Agilent Technologies DAKO, Santa Clara, CA). For detection of apoptotic cells, sections were stained with anti–cleaved caspase-3 antibodies. Cycling cells were detected by injecting the mice intraperitoneally with 200 μg/g of BrdU in phosphate-buffered saline 4 hours before they were killed. BrdU was detected using anti-BrdU antibodies. For immunofluorescence, Alexa fluoroscently labeled secondary antibodies were used at a 1:300 dilution. Nuclear DNA was counterstained with VECTASHIELD Hardset Antifade Mounting Medium (Vector Laboratories, Burlingame, CA) with 4′,6-diamidino-2-phenylindole. Immunofluorescence images were generated with a LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany) and analyzed with ZEN software (Carl Zeiss, Oberkochen, Germany). The antibodies and reagents used are listed in Table 1.

**Flow Cytometry**

For determination of intracellular ROS, primary hepatocytes were isolated using the collagenase perfusion method as described previously and were incubated with 1 μmol/LM 5-(and-6)-carboxy-2′,7′-dichlorofluorescin diacetate (Molecular Probes, Eugene, OR) diluted in phosphate-buffered saline for 30 minutes at 37°C in the dark, followed by extensive washing with phosphate-buffered saline. For measuring Kupffer cells, stained cells with anti-F4/80 antibodies were acquired using a FACSVerse or LSRFortessa flow cytometer (BD Biosciences, Franklin Lakes, NJ). TNF and IL1β in plasma were quantified using Cytoometric Bead Array Flex Sets (BD Biosciences, Franklin Lakes, NJ). All flow cytometry data were analyzed with FlowJo software (Tree Star, Woodburn, OR).

**Fluorescent Dyes**

Flow cytometry was performed using Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647 fluorescent dyes for detecting intracellular fatty acid were measured using flow cytometry.

**RNA-Seq**

RNA-Seq libraries were prepared using TruSeq RNA Sample Prep Kits (Illumina, San Diego, CA). Library quality and quantity were assessed on a bioanalyzer (Agilent Technologies DAKO) and Qubit (Invitrogen, Waltham, MA), respectively. Libraries were sequenced on the Illumina HiSeq 2500 system, generating 100-bp, paired-end reads. Reads were quality-trimmed and filtered using the NGS QC Toolkit (version 2.3.58; National Institute of Plant Genome Research, New Delhi, India) to remove reads with low-quality bases (quality score, >20). High-quality reads were aligned to the mm10 mouse genome using STAR (version 2.5.3a; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) with the following parameters: --sjdbOverhang 100 -twopassMode Basic -outSAMtype BAM SortedByCoordinate Unsorted --chimSegmentMin 20, with gene annotation from Ensembl (version 90; European Molecular Biology Laboratory’s European Bioinformatics Institute, Hinxton, Cambridge, United Kingdom). Gene expression was quantified using RSEM (version 1.2.31; University of Wisconsin-Madison, Madison, WI) and differentially expressed genes were identified using DESeq2 (version 1.28.1; Law et al., 2014).
Gene ontology and pathway enrichment analyses were performed using the DAVID database (Frederick National Laboratory, Frederick, MD). Gene set enrichment analysis was performed with the Broad Institute GSEA software (v4.1.0; Broad Institute, Cambridge, MA).
ChIP-seq

ChIP assays were performed as described previously with minor modifications. Chromatin samples were immunoprecipitated with antibodies listed in Table 1. ChIP-seq libraries were prepared using a Nextera DNA Sample Prep Kit (Illumina, San Diego, CA) as described previously. Libraries were sequenced on an Illumina HiSeq 2500 system, generating 101-bp, paired-end reads. Reads were quality-trimmed and filtered using Trim Galore (version 0.5.0; Babraham Bioinformatics, Cambridge, UK) to remove reads with low-quality bases (quality score, >5). High-quality reads were aligned to the mm10 mouse genome using BWA-mem (version 0.7.21; Wellcome Sanger Institute, Cambridge, MA) (http://broadinstitute.github.io/picard). The aligned libraries were processed using HiC-Pro (version 2.11.4; Mines ParisTech, Waltham, MA). Normalized signals for ChIP-seq data were visualized using the Integrative Genomics Viewer (Broad Institute, Cambridge, MA).

Differential enrichment of ChIP-seq peaks for H3K27ac, PPARγ, and CTCF was analyzed using DiffBind (version 2.6.6; Cambridge Research Institute, Cambridge, United Kingdom). Normalized signals for ChIP-seq data were visualized using the Integrative Genomics Viewer (Broad Institute, Cambridge, MA).

In Situ Hi-C

In situ Hi-C was performed as previously described. In brief, liver samples were harvested, flash-frozen in liquid nitrogen, and pulverized before 1% formaldehyde cross-linking for 20 minutes and subsequent quenching with 0.125 mol/L glycine. Liver nuclei were isolated using a gentleMACS Tissue Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Chromatin was digested using DpnII restriction enzyme (New England Biolabs, Ipswich, MA). A total of 150 ng was used for capture with Dynabeads MyOne Streptavidin C-1 (Thermo Fisher Scientific, Waltham, MA).

Table 1. Antibodies and Reagents Used in the Study

| Reagent or resource | Source | Identifier |
|---------------------|--------|------------|
| BrdU                | Invitrogen | #033900 |
| Cleaved caspase-3   | Cell Signaling Technology | #9664 |
| F4/80               | Abcam   | Ab6640     |
| F4/80               | eBioscience (San Diego, CA) | #51-4801-80 |
| HNF4x               | Abcam   | Ab41898    |
| PPARα              | Santa Cruz Biotechnology | sc-398394 |
| PPARγ              | Cell Signaling Technology | #2443 |
| CD36               | Novus Biologicals (Centennial, CO) | NB400-144 |
| CTCF               | Cell Signaling Technology | #2899 |
| α-tubulin           | Santa Cruz Biotechnology | sc-32293 |
| HSP 90α/β          | Santa Cruz Biotechnology | sc-13119 |
| Lamin B1           | Abcam   | Ab133741   |
| DAPI                | Vector Laboratories (Burlingame, CA) | H-1500 |
| DCFDA              | Invitrogen | C6827 |
| CD36               | BioLegend (San Diego, CA) | #102612 |
| CD45.2             | eBioscience | #47-0454-82 |
| DAPI, 4',6-diamidino-2-phenylindole; DCFDA, 2',7'-dichlorodihydrofluorescein diacetate. | |

Figure 18. (See previous page). Higher-order chromatin structures in WT and CTCF-deficient liver. (A) Snapshot of ChIP-seq signal tracks for CTCF. (B) Pie charts showing the total numbers and genomic distributions of CTCF peaks identified from WT and cKO livers. (C) Histogram showing the average tag density of CTCF ChIP-seq peaks. (D) Heatmap of ChIP-Seq signals called for CTCF. (E) MA plot showing differential enrichment of CTCF between WT and cKO livers. (F) Histogram showing the average tag density of H3K27ac ChIP-seq signals at CTCF peaks lost in cKO, compared with WT (n = 1178). (G) In situ Hi-C contact maps at 100-kb and 10-kb resolution. ChIP-seq signal tracks for CTCF were aligned below the contact maps at 10-kb resolution. (H) Distributions of cis eigenvector 1 values across the entirety of chromosome 5. (I) Scatterplot shows that CTCF depletion does not affect genome-wide cis eigenvector 1 values. (J) Saddle plots representing compartmentalization strength in WT and cKO livers. (K) Number of TAD boundaries obtained with in situ Hi-C data. (L) Genome-wide averaged insulation scores plotted vs distance around insulation centers at WT TAD boundaries. (M) Boxplot shows the TAD boundary strength in WT and cKO livers. (N) Heatmaps show the average observed/expected Hi-C interactions in TAD regions. UTR, untranslated region. ****P < .0001, unpaired 2-tailed Student t test.
settings were used to align reads to the mm10 mouse genome, remove duplicate reads, assign reads to DpnII restriction fragments, filter for valid interactions, and generate binned interaction matrices. After confirmation of good reproducibility between biological replicates using HiC-Spector, the replicate data were merged for reprocessing as combined results. The validated contact pairs were transformed to Juicer hic files with hicpro2juicebox (Mines ParisTech, PSL-Research University, Paris, France). To segregate A and B compartments, the eigenvector of each chromosome of each sample was generated from the Hi-C data using the Juicer tool eigenvector command with Knight-Ruiz (KR) normalization at 100-kb resolution (version 1.22.01; (Aiden Lab, Houston, TX)). The Juicer hic files were converted to .cool files using hic2cool with default parameters. Saddle plots were yielded at 100-kb resolution using cooltools (version 0.3.2; University of Edinburgh, Edinburgh, United Kingdom), and the strength of compartmentalization was defined as a ratio of (A–A+B)–B/(A–B+B–A) interactions. To identify topological domain boundaries following an insulation square analysis, contact matrix files were generated from Hi-C data and subjected to calculation of insulation scores using matrix2insulation.pl (DEKKER LAB, Worcester, MA) with parameters -b 500,000 -ids 200,000 -im mean -bmode 3 -nt 0.1. Intra-TAD DNA interactions represented as TAD strengths were determined using FAN-C with parameters –tad-strength (version 0.9.10; Max Planck Institute for Molecular Biomedicine, Muenster, Germany).

Data Access
Raw and processed sequencing data were deposited with Gene Expression Omnibus under the accession number GSE151503.

Statistical Analysis
Data were analyzed with an unpaired Student 2-tailed t-test using Prism software (GraphPad Software, Inc, San Diego, CA). Errors bars plotted on graphs are presented as the means ± SD. The upper and lower hinges of the box-and-whisker plots represent the 75th and 25th percentile, respectively. All P values less than .05 were considered statistically significant.

References
1. Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, George J, Bugianesi E. Global burden of NAFLD and NASH: trends, predictions, risks factors and prevention. Nat Rev Gastroenterol Hepatol 2018; 15:11–20.
2. Merkenschlager M, Nora EP. CTCF and cohesin in genome folding and transcriptional gene regulation. Annu Rev Genomics Hum Genet 2016;17:17–43.
3. Rowley MJ, Corces VG. Organizational principles of 3D genome architecture. Nat Rev Genet 2018;19:789–800.
4. Kim TG, Kim M, Lee JJ, Kim SH, Je JH, Lee Y, Song MJ, Choi Y, Chung YW, Park CG, Cho JW, Lee MG, Lee YS, Kim HP. CCCTC-binding factor controls the homeostatic maintenance and migration of Langerhans cells. J Allergy Clin Immunol 2015;136:713–724.
5. Gomez-Velazquez M, Badia-Careaga C, Lechuga-Vieco AV, Nieto-Arellano R, Tena JJ, Rollan I, Alvarez A, Torroja C, Caceres EF, Roy AR, Galjart N, Delgado-Olguin P, Sanchez-Cabo F, Enriquez JA, Gomez-Skarmeta JL, Manzanares M. CTCF counter-regulates cardiomyocyte development and maturation programs in the embryonic heart. PLoS Genet 2017;13:e1006985.
6. Shin JO, Lee JJ, Kim M, Chung YW, Min H, Kim JY, Kim HP, Bok J. CTCF regulates otic neurogenesis via histone modification in the neurog1 locus. Mol Cells 2018;41:695–702.
7. Soshnikova N, Montavon T, Leleu M, Galjart N, Duboule D. Functional analysis of CTCF during mammalian limb development. Dev Cell 2010; 19:819–830.
8. Chistov M, Clark AR, Corbin B, Hakroush S, Rhee EP, Saito H, Brooks D, Hesse E, Bouxsein M, Galjart N,
Jung JY, Mundel P, Juppner H, Weins A, Greka A. Inducible podocyte-specific deletion of CTCF drives progressive kidney disease and bone abnormalities. JCI Insight 2018;3:e95091.

Wan LB, Han H, Hannenhalli S, Cheng Y, Ma J, Fedorow A, Lobanenko V, Latham KE, Schultz RM, Bartolomei MS. Maternal depletion of CTCF in Drosophila reveals multiple functions during oocyte and preimplantation embryo development. Development 2008; 135:2729–2738.

Sams DS, Nardone S, Getselter D, Raz D, Tai M, Rayi PR, Kaphzan H, Hakim O, Elliott E. Neuronal CTCF is necessary for basal and experience-dependent gene regulation, memory formation, and genomic structure of BDNF. Proc Natl Acad Sci U S A 2012;109:13656–13661.

Lee YJ, Ko EH, Kim JE, Kim E, Lee H, Choi H, Yu JH, Kim HJ, Seong JK, Kim KS, Kim JW. Nuclear receptor PPARgamma-regulated monoacylglycerol O-acyltransferase 1 (MGAT1) expression is responsible for lipids scavenging and mitochondrial integrity. Cell Death Dis 2017;8:e3158.

Liu J, Li D, Zhang T, Tong Q, Ye RD, Lin L. SIRT3 protects hepatocytes from oxidative injury by enhancing ROS scavenging and mitochondrial integrity. J Biol Chem 2003;278:1449–1456.

Mara F, Svegliati-Baroni G. Lipotoxicity and the gut-liver axis in NASH pathogenesis. J Hepatol 2018;68:280–295.

Li T, Lu Z, Lu L. Regulation of eye development by transcription control of CCCTC binding factor (CTCF). J Biol Chem 2004;279:27583–27588.

Beagan JA, Duong MT, Titus KR, Zhou L, Cao Z, Ma J, Lachanski CV, Gillis DR, Phillips-Cremins JE. YY1 and CTCF orchestrate a 3D chromatin looping switch during early neural lineage commitment. Genome Res 2017;27:1139–1152.

Marra F, Svegliati-Baroni G. Lipotoxicity and the gut-liver axis in NASH pathogenesis. J Hepatol 2018;68:280–295.

Mougiou V, Patel K, Matsakas A. Loss of CD36 protects against diet-induced obesity but results in impaired muscle stem cell function, delayed muscle regeneration and hepatic steatosis. Acta Physiol (Oxf) 2020;228:1–12.

Abumrad NA, Goldberg IJ. CD36 actions in the heart: Lipids, calcium, inflammation, repair and more? Biochim Biophys Acta 2016;1861:1442–1449.

Taylor J, Fischer A. Endothelial cells dictate cardiac fuel source. Aging (Albany NY) 2019;11:1083–1084.

Verpoorten S, Syri P, Scully D, Mitchell R, Tzimou A, Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Taylor J, Fischer A. Endothelial cells dictate cardiac fuel source. Aging (Albany NY) 2019;11:1083–1084.

CD36 in obesity-induced hepatic steatosis as determined by hepatocyte- and macrophage-specific conditional knockouts. FASEB J 2011;25:2538–2550.

Chen Z, Luo X, Chen J, Zhou B, Yang M, Liu R, Liu D, Gu HF, Zhu Z, Zheng H, Li L, Yang G. Osteoprotegerin promotes liver steatosis by targeting the ERK-PPAR-gamma-CD36 pathway. Diabetes 2019;68:1902–1914.

Abumrad NA, Goldberg IJ. CD36 actions in the heart: Lipids, calcium, inflammation, repair and more? Biochim Biophys Acta 2016;1861:1442–1449.

Aouadi M, Vangala P, Yawe JC, Tencerova M, Nicoloro SM, Cohen JL, Shen Y, Czech MP. Lipid storage by adipose tissue macrophages regulates systemic glucose tolerance. Am J Physiol Endocrinol Metab 2014;307:E374–E383.

Cifarelli V, Abumrad NA. Intestinal CD36 and other key proteins of lipid utilization: role in absorption and gut homeostasis. Compr Physiol 2018;8:493–507.

Alkhatatbeh MJ, Enjeti AK, Acharya S, Thorne RF, Lincz LF. The origin of circulating CD36 in type 2 diabetes. Nutr Diabetes 2013;3:e59.

Miquilena-Colina ME, Lima-Cabello E, Sanchez-Campos S, Garcia-Mediavilla MV, Fernandez-Bermejo M, Lozano-Rodriguez T, Vargas-Castrillon J, Buque X, Ochoa B, Aspichueta P, Gonzalez-Gallego J, Garcia-Monzon C. Hepatic fatty acid translocase CD36 upregulation is associated with insulin resistance, hyperinsulinaemia and increased steatosis in non-alcoholic steatohepatitis and chronic hepatitis C. Gut 2011;60:1394–1402.

Samovski D, Abumrad NA. Regulation of lipophagy in NAFLD by cellular metabolism and CD36. J Lipid Res 2019;60:755–757.

Ferriero M, Guy E, Coburn C, Knapp FF Jr, Beets AL, Abumrad NA, Silverstein RL. The impact of overexpression and deficiency of fatty acid translocase (FAT)/CD36. Mol Cell Biochem 2002;239:193–197.

Koonen DP, Jacobs RL, Febbraio M, Young ME, SoltyS CL, Ong H, Vance DE, Dyck JR. Increased hepatic CD36 expression contributes to dyslipidemia associated with diet-induced obesity. Diabetes 2007;56:2863–2871.

Wilson CG, Tran JL, Erion DM, Vera NB, Febbraio M, Weiss EJ. Hepatocyte-specific disruption of CD36

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21. Hauser S, Adelman G, Sarraf P, Wright HM, Mueller E, Spiegelman BM. Degradation of the peroxisome proliferator-activated receptor gamma is linked to ligand-dependent activation. J Biol Chem 2000; 275:18527–18533.

22. Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Perissi V, Rose DW, Willson TM, Rosenfeld MG, Glass CK. A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. Nature 2005;437:759–763.

23. Taylor J, Fischer A. Endothelial cells dictate cardiac fuel source. Aging (Albany NY) 2019;11:1083–1084.

24. Verpoorten S, Syri P, Scully D, Mitchell R, Tzimou A, Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Taylor J, Fischer A. Endothelial cells dictate cardiac fuel source. Aging (Albany NY) 2019;11:1083–1084.
35. Brundert M, Heeren J, Merkel M, Carmambia A, Herkel J, Grott P, Dobner T, Ramakrishnan R, Moore KJ, Rinninger F. Scavenger receptor CD36 mediates uptake of high density lipoproteins in mice and by cultured cells. J Lipid Res 2012;5:743–758.

36. Bujold K, Rhainds D, Jossart C, Febbraio M, Marleau S, Ong H. CD36-mediated cholesterol efflux is associated with PPARgamma activation via a MAPK-dependent COX-2 pathway in macrophages. Cardiovasc Res 2009;83:457–464.

37. Zhou J, Febbraio M, Wada T, Zhai Y, Kuruba R, He J, Lee JH, Khadem S, Ren S, Li S, Silverstein RL, Xie W. Hepatic fatty acid transporter Cd36 is a common target of LXR, PXR, and PPARgamma in promoting steatosis. Gastroenterology 2008;134:556–567.

38. Wilmsen HM, Ciaraldi TP, Carter L, Reehman N, Langhi C, Baldan A. CIDEC/FSP27 is regulated by SREBP-1c and is associated with fat accumulation in skeletal muscle of type 2 diabetic subjects. Am J Physiol Endocrinol Metab 2003;285:E354–E362.

39. Langhi C, Baldan A. CIDEC/FSP27 is regulated by peroxisome proliferator-activated receptor alpha and plays a critical role in fasting- and diet-induced hepatic steatosis. Hepatology 2015;61:1227–1238.

40. Zhang M, Zhao Y, Li Z, Wang C. Pyruvate dehydrogenase kinase 4 mediates lipogenesis and contributes to the pathogenesis of nonalcoholic steatohepatitis. Biochem Biophys Res Commun 2018;495:582–586.

41. Yoshizumi S, Suzuki S, Hirai M, Hinokio Y, Yamada T, Yamada T, Tsunoda U, Aburatani H, Yamaguchi K, Miyagi T, Oka Y. Increased hepatic expression of ganglioside-specific sialidase, NEU3, improves insulin sensitivity and glucose tolerance in mice. Metabolism 2007;56:420–429.

42. Zuin J, Dixon JR, van der Reijden MI, Ye Z, Kolovos P, Brouwer RW, van de Corput MP, van de Werken HJ, Knoch TA, van IW, Grosveld FG, Ren B, Wendt KS. Cohesin and CTFC differently affect chromatin architecture and gene expression in human cells. Proc Natl Acad Sci U S A 2014;111:996–1001.

43. Nora EP, Goloborodko A, Valton AL, Gibcus JH, Bujold K, Rhainds D, Jossart C, Febbraio M, Marleau S, Ong H. CD36-mediated cholesterol efflux is associated with PPARgamma activation via a MAPK-dependent COX-2 pathway in macrophages. Cardiovasc Res 2009;83:457–464.

44. Feldstein AE, Canbay A, Guicciardi ME, Higuchi H, Bronk SF, Gores GJ. Diet-associated hepatic steatosis sensitizes to Fas-mediated liver injury in mice. J Hepatol 2003;39:978–983.

45. Cheung O, Sanyal AJ. Abnormalities of lipid metabolism in nonalcoholic fatty liver disease. Semin Liver Dis 2008;28:351–359.

46. Nolan CJ, Larter CZ. Lipotoxicity: why do saturated fatty acids cause and monounsaturates protect against it? J Gastroenterol Hepatol 2009;24:703–706.

47. Anderson N, Borlak J. Molecular mechanisms and therapeutic targets in steatosis and steatohepatitis. Pharmacol Rev 2008;60:311–357.

48. Garbarino J, Sturley SL. Saturated with fat: new perspectives on lipotoxicity. Curr Opin Clin Nutr Metab Care 2009;12:110–116.

49. Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, Shelton KD, Lindner J, Cherrington AD, Magnuson MA. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. J Biol Chem 1999;274:305–315.

50. Febbraio M, Abumrad NA, Hajjar DP, Sharma K, Cheng W, Pearce SF, Silverstein RL. A null mutation in murine CD36 reveals an important role in fatty acid and lipoprotein metabolism. J Biol Chem 1999;274:19055–19062.

51. Luo J, Deng ZL, Luo X, Tang N, Song WX, Chen J, Sharff KA, Luu HH, Haydon RC, Kinzler KW, Vogelstein B, He TC. A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. Nat Protoc 2007;2:1236–1247.

52. Koo SH, Flechner L, Qi L, Zhang X, Sereaton RA, Jeffries S, Hedrick S, Xu W, Boussonf A, Brindle P, Takemori H, Montmyny M. The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. Nature 2005;437:1109–1111.

53. Herzig S, Hedrick S, Morantte I, Koo SH, Galimi F, Montmyny M. CREB controls hepatic lipid metabolism through nuclear hormone receptor PPAR-gamma. Nature 2003;426:190–193.

54. Park S, Oh TS, Kim S, Kim EK. Palmitate-induced autophagy liberates monounsaturated fatty acids and increases Agrp expression in hypothalamic cells. Ani Cyst Syst (Suol) 2019;23:384–391.

55. Ferron M, Wei J, Yoshizawa T, Del Fattore A, DePinho RA, Teti A, Ducy P, Karsenty G. Insulin signaling in osteoblasts integrates bone remodeling and energy metabolism. Cell 2010;142:296–308.

56. Kim SY, Kim HI, Kim TH, Im SS, Park SK, Lee IK, Kim KS, Ahn YH. SREBP-1c mediates the insulin-dependent hepatic glucokinase expression. J Biol Chem 2004;279:30823–30829.

57. Patel RK, Jain M. NGS QC Toolkit: a toolkit for quality control of next generation sequencing data. PLoS One 2012;7:e30619.

58. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR, STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15–21.

59. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 2011;12:323.

60. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2. Genome Biol 2014;15:550.

61. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009;4:44–57.

62. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 2009;37:1–13.
63. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Poteroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545–15550.

64. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 2003;34:267–273.

65. Park JH, Choi Y, Song MJ, Park K, Lee JJ, Kim HP. Dynamic long-range chromatin interaction controls expression of IL-21 in CD4+ T cells. J Immunol 2016;196:4378–4389.

66. Schmidl C, Rendeiro AF, Shef

67. Rao SS, Huntley MH, Durand NC, Stamenova EK, Bochkov IK, Robinson JT, Sanborn AL, Machol I, Omer AD, Lander ES, Aiden EL. A 3D map of the human genome at kilobase resolution reveals principles of chromatin looping. Cell 2014;159:1665–1680.

68. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W, Liu XS. Model-based analysis of ChIP-Seq (MACS). Genome Biol 2008;9:R137.

69. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, Cheng JX, Murre C, Singh H, Glass CK. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell 2010;38:576–589.

70. Ross-Innes CS, Stark R, Teschendorff AE, Holmes KA, Ali HR, Dunning MJ, Brown GD, Gojis O, Ellis IO, Green AR, Ali S, Chin SF, Palmieri C, Caldas C, Carroll JS. Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. Nature 2012;481:389–393.

71. Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP. Integrative genomics viewer. Nat Biotechnol 2011;29:24–26.

72. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, Cheng JX, Murre C, Singh H, Glass CK. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell 2010;38:576–589.

73. Servant N, Varoquaux N, Lajoie BR, Viara E, Chen CJ, Vert JP, Heard E, Dekker J, Barillot E. HiC-Pro: an optimized and flexible pipeline for Hi-C data processing. Genome Biol 2015;16:259.

74. Yan KK, Yardimci GG, Yan C, Noble WS, Gerstein M. HiC-spector: a matrix library for spectral and reproducibility analysis of Hi-C contact maps. Bioinformatics 2017;33:2199–2201.

75. Durand NC, Shamim MS, Machol I, Rao SS, Huntley MH, Lander ES, Aiden EL. Juicer provides a one-click system for analyzing loop-resolution Hi-C experiments. Cell Syst 2016;3:95–98.

76. Crane E, Bian Q, McCord RP, Lajoie BR, Wheeler BS, Raiston EJ, Uzawa S, Dekker J, Meyer BJ. Condensin-driven remodelling of X chromosome topology during dosage compensation. Nature 2015;523:240–244.

77. Kruse K, Hug CB, Vaquerizas JM, FAN –C. a feature-rich framework for the analysis and visualisation of chromsome conformation capture data. Genome Biol 2020;21:303.