Persistent Chromatin Modifications Induced by High Fat Diet**

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Obesity is a highly heritable complex disease that results from the interaction of multiple genetic and environmental factors. Formerly obese individuals are susceptible to metabolic disorders later in life, even after lifestyle changes are made to mitigate the obese state. This is reminiscent of the metabolic memory phenomenon originally observed for persistent complications in diabetic patients, despite subsequent glycemic control. Epigenetic modifications represent a potential mediator of this observed memory. We previously demonstrated that a high fat diet leads to changes in chromatin accessibility in the mouse liver. The regions of greatest chromatin changes in accessibility are largely strain-dependent, indicating a genetic component in diet-induced chromatin alterations. We have now examined the persistence of diet-induced chromatin accessibility changes upon diet reversal in two strains of mice. We find that a substantial fraction of loci that undergo chromatin accessibility changes with a high fat diet remain in the remodeled state after diet reversal in C57BL/6J mice. In contrast, the vast majority of diet-induced chromatin accessibility changes in A/J mice are transient. Our data also indicate that the persistent chromatin accessibility changes observed in C57BL/6J mice are associated with specific transcription factors and histone post-translational modifications. The persistent loci identified here are likely to be contributing to the overall phenotype and are attractive targets for therapeutic intervention.

Obesity and related metabolic diseases result from both genetic and environmental factors, such as exercise and diet. However, the molecular mechanisms that contribute to disease progression remain unclear. Intriguingly, previously obese individuals have increased mortality compared with normal individuals, despite therapeutic intervention (1). This is reminiscent of “metabolic memory,” a phenomenon originally described in diabetic patients in which micro- and macrovascular complications develop long after blood glucose is normalized (2–4). It has been hypothesized that epigenetic modifications, such as alterations to the chromatin and non-sequence changes to DNA, including DNA methylation, can contribute to this “metabolic memory” (5–7).

It is now well established that epigenetic modifications can contribute to disease progression (8). One manner by which external environmental factors can influence molecular pathways is through alterations to chromatin. It has been shown that high fat (HF)2 diet leads to chromatin accessibility changes in the liver tissue of mice (9). Intriguingly, the genomic loci with the greatest degree of diet-induced chromatin accessibility changes are largely strain-specific, indicating a role for genetics in this response (9). Previous studies examining diet-induced metabolic dysfunctions have shown that mice that transition from HF to diets that are low in fats do not completely revert to the same state as mice only maintained on low fat diets (10). The data indicate the presence of a “memory” in which previous metabolic states induced by HF diet persist even in the absence of the stimulus. Given the additional observation that metabolic signals can trigger epigenetic modifications, which can persist across cell division (11, 12), epigenetic modifications represent attractive candidates for mediating this metabolic memory. However, because epigenetic modifications can be dynamic and reversible, characterizing persistent versus transient chromatin changes in response to metabolic signaling is a major challenge for the field (13).

We investigated the extent of persistent chromatin accessibility changes induced by HF diet by comparing chromatin accessibility profiles from C57BL/6J (B6) mice fed a HF diet for 16 weeks, mice fed a control diet for 16 weeks, and mice fed a HF diet for 8 weeks and then put on a control diet for 8 weeks. Chromatin accessibility in liver tissue from these mice was assessed through formaldehyde-assisted isolation of regulatory elements (FAIRE) coupled with high throughput sequencing (FAIRE-seq). We show here that a subset of regions depicting chromatin accessibility changes induced by HF diet in the liver remain persistently remodeled despite the change of HF diet to control diet. We find that sites that are persistently more accessible in HF diet, as compared with control, are bound by HNF4α.
and that sites that are persistently less accessible are enriched for histone H3 lysine 9 dimethylation (H3K9me2), indicating a role for both transcription factors and histone post-translational modifications in mediating persistent chromatin accessibility changes. Furthermore, loci of persistent chromatin accessibility changes are associated with transcriptional regulation of important metabolic genes. We further investigated chromatin accessibility with the same diet scheme in A/J mice, which are known to display metabolic differences under HF diet as compared with B6 mice (14). Chromatin accessibility changes under HF diet feeding do occur in these mice, but the vast majority of the chromatin changes are reversible by the dietary changes. Taken together, these data indicate that HF diet-induced chromatin accessibility changes can persist even with reversal to control diet in a strain-dependent manner, and the changes in chromatin accessibility have the potential to contribute to the long term risk of metabolic disease.

**Experimental Procedures**

**Animals**—7–8-Week-old C57BL/6J and A/J mice were obtained from The Jackson Laboratory and fed a high fat diet (Research Diet D12266B) or control diet (Research Diets D12492B) for 8 or 16 weeks (Fig. 1A). Specifically, mice were placed on three diet regimens as follows: 1) control diet for 16 weeks; 2) HF diet for 16 weeks; or 3) HF diet for an initial 8 weeks followed by control diet for 8 weeks (diet reversal). Body weight was tracked weekly. After the feeding period, mice were humanely euthanized, and livers were collected for further analysis. Hematoxylin and eosin (H&E) staining was performed on liver sections by the Pathology Core at the City of Hope using standard procedures. Liver triglyceride levels were also analyzed (Abcam, ab65336).

*FAIRE-seq*—FAIRE-seq was performed on mouse liver tissue as described previously (9, 15). FAIRE DNA was sequenced from two biological replicates from each condition and aligned to the mouse genome (mm9) using bowtie2 (16). Aligned reads were filtered to remove improperly paired reads and PCR duplicates. F-seq (17) and irreproducible discovery rate (IDR) analysis (18) were utilized to discover reproducible peaks (open chromatin sites). Wiggle tracks were generated and viewed on the UCSC genome browser (19).

For B6 mice, a union set of 45,667 peaks was identified. To assess sample variability, read counts at each peak were obtained for each biological sample, and replicates within each group were analyzed for their correlation. To assess the relationship between the accessible chromatin sites across the three feeding groups, normalized read counts (20) for each site were obtained, and the conditions were hierarchically clustered with Cluster 3.0 (21) and viewed with TreeView (22). To find the top most variable sites, normalized read counts at each site were determined in control and HF livers and ranked comparing the density of read counts in HF to control (HF/control). The 1,000 most variable sites with HF diet greater than control (top1k) were assessed for overlap with HNF4α (23) and CTCF (24) ChIP-seq sites from B6 liver tissue. These top1k sites for 16 weeks of feeding were compared with those from 8 weeks of feeding (9). To determine the accessibility differences of transcription factor bound and non-bound sites for both HNF4α and CTCF, each open chromatin site was assessed for overlap with HNF4α and CTCF peaks (23, 24). Read densities comparing HF to control and reversal to control were generated for each site, and overall densities were compared.

For A/J, a union set of 40,548 sites was identified by our FAIRE-seq analysis. As with B6 sites, hierarchical clustering on the normalized read counts was used to identify the relationship between the chromatin accessibility and the diet. To identify the overlap between HNF4α-targeted sites and open chromatin sites in A/J, ChIP-seq data were utilized from Ref. 23. For comparison with chromatin accessibility changes that occur within 8 weeks of HF diet feeding in B6 mice (9), we utilized data from GSE55581.

*ChIP-Quantitative PCR*—Chromatin immunoprecipitation (ChIP) was performed with an anti-H3K9me2 antibody (Abcam, ab1220) and IgG control using standard ChIP protocols (25). Extracted DNA was assessed for enrichment of specific sites by quantitative PCR quantitation. The ΔΔCt method was utilized to evaluate enrichment of target DNA and normalized to input DNA.

*RNA-seq*—RNA was extracted from liver tissue using TRIzol (Qiagen). RNA was poly(A)-enriched (Illumina TrueSeq RNA Library Prep). Eluted RNA was sequenced with Illumina protocols on a HiSeq 2500. Sequenced reads were aligned to the mm9 genome with TopHat2 (26) with the RefSeq gene annotation as a reference. Transcript expression was quantified, and DESeq2 (27) was utilized to identify differentially expressed genes. Hierarchical clustering (Cluster 3.0) assessing average linkage of correlation was used to group genes that are similarly expressed across the diet groups (21). DAVID (28) was used to identify the enriched KEGG pathway for the 56 genes that are persistently up-regulated in C57BL/6J mice and for the 30 genes that are persistently up-regulated in A/J mice.

**Results**

To understand the extent and functions of persistent diet-induced changes in chromatin accessibility, C57BL/6J (B6) mice were placed on one of three diet regimens (see “Experimental Procedures”; Fig. 1A). During the initial 8 weeks, all mice placed on HF diet displayed accelerated weight gain compared with control mice, as expected (Fig. 1B). However, the reversal diet mice displayed attenuated weight gain when switched to a control diet. Mice on both HF and reversal diets showed elevated levels of lipid storage (Fig. 1C) and triglyceride levels (Fig. 1D). Although weight gain was mitigated by diet reversal, lipid accumulation and triglyceride levels were resistant to change upon diet reversal. We next sought to investigate the molecular mechanisms associated with these phenotypes.

We profiled accessible chromatin across the genome in liver tissue of biological replicates from each group of mice with FAIRE-seq (see “Experimental Procedures”) (15). This methodology is one of many (including DNase-seq and ATAC-seq (29)) used to identify regions of accessible chromatin. Although there are differences with these methods (30), generally, they all are able to identify active promoters and enhancers. For this study we chose FAIRE-seq, a method that does not rely on enzymatic reactions and therefore is more reproducible across conditions with low technical variability (31), and it has been pre-
Previously used to identify open chromatin sites in mouse liver (9). Regions of open chromatin were identified by first using F-seq (17) to identify FAIRE-enriched sites and then IDR analysis, a method suggested by the guidelines set by the ENCODE consortium (32), to set thresholds. Following this methodology, we first identified reproducible regions of open chromatin between the replicates using IDR analysis (IDR threshold $<0.005$) for each condition. We then merged the open chromatin sites across the conditions for a union set of 45,667 open chromatin sites throughout the genome (see “Experimental Procedures”). To address variability across our biological samples from the same condition, we assessed the correlation of read counts at each open chromatin site and found high correlation among the samples (Fig. 2A). To evaluate the potential relationships between chromatin accessibility and diet, we performed hierarchical clustering (see “Experimental Procedures”) on the union set of sites across the three conditions (Fig. 2B). Overall, the clustering analysis indicated that the chromatin accessibility profiles of the mice that were put on the reversal diet regimen were more similar to mice on a HF diet than the control mice. To classify regions with changes in chromatin accessibility as persistent or reversible, we focused on regions with an absolute fold change between HF and control groups of greater than 1.5, leading to 9,343 regions (5,211 more accessible, 4,132 less accessible) that are changed with the HF diet (Fig. 2C). This group was then stratified into those that had the same or greater change in the reversal group (persistent, 2,058 sites; red dots in Fig. 2C) and those that had less change in the reversal group (reversible, 7,285 sites; blue dots in Fig. 2C). Overall, more than 15% (1,493) of the sites of chromatin accessibility changes induced by HF diet were persistently more accessible, and 6% (565) were persistently less accessible. An example of a persistently more accessible region is shown in Fig. 2D at the Serpina6 locus.

We have previously demonstrated that the regions of greatest diet-induced chromatin accessibility changes are liver regulatory regions, bound by transcription factors such as HNF4α (9). We first assessed the extent of chromatin accessibility changes at 16 weeks of HF diet that are observed after 8 weeks of HF diet by comparing the chromatin changes to our previous data (9). Out of the 5,211 sites that are more accessible after 16 weeks of HF diet about 23% (1,203 sites) were changed after 8 weeks of HF diet (Fig. 3B). Furthermore, 751 of those sites remained more accessible in the reversal group, indicating more than half ($\sim 62\%$) of the persistently accessible sites were initially changed after 8 weeks of HF diet and remained so even after diet reversal. Interestingly, 733 ($\sim 97.6\%$) of these sites are targeted by HNF4α (23). Because we previously had demonstrated that sites targeted by HNF4α have increased accessibility after 8 weeks of HF diet, we assessed whether the regions that have increased chromatin accessibility at 16 weeks are targeted by this transcription factor. As with the 8-week HF feeding regimen (74%) (9), the overwhelming majority of the top1k sites at 16 weeks were bound by HNF4α (881/1000). To evaluate the overall trend of chromatin changes at all HNF4α-binding sites at 16 weeks of HF feeding, we calculated the fold change of chromatin accessibility for HF and control at all FAIRE sites bound by HNF4α and those FAIRE sites not bound by HNF4α. Overall, sites bound by HNF4α were more open in HF as compared with control, and this trend remained in the
reversal diet condition (Fig. 3C, left). Remarkably, over 93% (1,396 out of 1,493) of the persistently more accessible sites, as classified in Fig. 2C, are bound by HNF4α/H9251. As a comparison, sites targeted by CTCF, a known ubiquitous transcription factor, do not display the same behavior (Fig. 3C, right). Taken together, these results indicate that HNF4α/H9251 binding, in response to HF diet, contributes to the persistent accessibility of chromatin after reversal of diet.

In addition to considering regions that are persistently more accessible under reversal diet, we also considered regions that were persistently less accessible (565 sites in Fig. 2C). Inaccessible chromatin is associated with specific repressive histone modifications, including H3K9me2 and H3K9me3 (33). We have previously demonstrated that H3K9me2 leads to reduced chromatin accessibility at specific punctate loci (34). We therefore hypothesized that the persistently less accessible regions would be marked with H3K9me2 for both the HF and reversal mice. We randomly chose three persistently less accessible sites and evaluated the enrichment of H3K9me2 at those loci. At each of the loci investigated, we detected a marked increase in H3K9me2 associated with condensed chromatin, and furthermore, the H3K9me2 remained elevated under the reversal diet (Fig. 3D). These data indicate that histone modifications, including H3K9 methylation, are involved in diet-induced chromatin accessibility changes that can persist upon diet reversal.

It is well established that chromatin remodeling can affect the regulation of transcription and downstream gene expression. We next evaluated whether changes in gene expression under HF diet are persistent upon diet reversal. We performed RNA-seq, as described previously (9), on the same set of B6 mouse liver tissues. In total, 680 genes were detected as differentially expressed across the three conditions (see under “Experimental Procedures”; false discovery rate <0.05). To characterize the gene expression changes that occur in response to diet, differentially expressed genes were clustered across the three conditions (control, HF, and reversal). This analysis revealed 44 unique genes (with a total of 56 isoforms)
that are up-regulated under HF diet and also continue to remain up-regulated in the reversal diet as compared with control (Fig. 4A, red bar). One persistently up-regulated gene, Cyp2b9, displays the most dramatic increase in response to HF diet. This gene encodes for a cytochrome P450, which previously has been shown to be strongly up-regulated by HF diet and important for regulating lipid homeostasis (35, 36).

In contrast to these 44 genes, 122 of the genes differentially up-regulated in HF diet as compared with control were reversible in expression with diet reversal (Fig. 4A, blue bar). To further evaluate the dynamics of gene expression changes across conditions, we plotted the relative expression of persistently up-regulated (Fig. 4A, red bar) and reversible (Fig. 4A, blue bar) genes. This analysis revealed that the reversible genes had a relatively greater increase in transcription under HF diet as compared with the persistently up-regulated genes (Fig. 4B). Notably, there was greater variation in gene expression across these two groups than within the groups.

One potential mechanism by which persistently up-regulated genes remain in the up-regulated state is through regulation at regions of persistent chromatin alterations. To test this, we evaluated the likelihood that local chromatin changes are
associated with gene expression changes. Specifically, we assessed the genomic location of the persistently accessible sites relative to the transcriptional start site of the persistently up-regulated genes. We found that compared with randomly selected sites, the persistently up-regulated genes were more likely to be in the vicinity (within 100 kb) of a persistently accessible site (Fig. 4C; 15 genes, empirical \( p < 0.02 \)).

To better understand the role of the persistently up-regulated genes in the liver, we examined enrichment of KEGG pathways using DAVID (28). The only enriched pathway was the alanine, aspartate, and glutamate metabolism pathway \((p < 0.05)\). We confirmed the expression of several genes in this pathway, including Gpt, Abat, and Agxt, with quantitative PCR (Fig. 4D). Gpt (glutamate pyruvate transaminase 1) is the mouse homolog of the human Alt1, which encodes for glutamate pyruvate transaminase proteins. Alt1/Gpt is responsible for conversions of glutamate to alanine, which is critical for production of pyruvate (37). We hypothesized that the chromatin in the Gpt locus is persistently accessible, allowing for increased transcription of Gpt. Indeed, we found a 1.5-kb region upstream of the Gpt transcriptional start site with an open chromatin site that remains persistently accessible in the reversal group (Fig. 4E). Other persistently up-regulated genes of note are Fam73b and Acot11. Fam73 knock-out mice are lean and have increased macroH2A1.1 expression, a histone variant associated with regulation of lipogenic gene expression (38, 39). Acot11 encodes an enzyme that catalyzes the hydrolysis of fatty acyl-CoAs, protecting against diet-induced obesity. However, it is not known how these two genes function in the context of the liver metabolism. These data indicate they may be important in the long term impact of obesity in the liver.

The gene expression analysis also revealed a set of genes that are up-regulated only in the livers of the reversal group. This group of genes is enriched for mitochondrial genes (DAVID analysis; \( p = 5.6e-5 \)). These results indicate that the dietary changes have potentially long lasting effects, even upon later diet reversal.

We previously demonstrated that the regions of the genome with the greatest degree of diet-induced chromatin alterations are largely strain-specific, indicating a genetic component to the response (9). To investigate the genetic component of the persistence of diet-induced changes in chromatin accessibility, we chose to examine A/J mice, a strain of mice known to display differences in metabolic dysfunction under HF diet as compared with B6 mice (14). A/J mice placed on a HF diet for 16 weeks or those fed 8 weeks of HF diet followed by 8 weeks of control diet (reversal) do not gain more weight than those fed a control diet for 16 weeks (Fig. 5A). In contrast to B6 mice fed a HF diet, A/J mice do not display elevated levels of lipid accumulation in the livers (Fig. 5B). We explored the gene expression changes in A/J and found 699 genes that are differentially expressed across the diets (false discovery rate \(< 0.05\)). 115 (16%) of these genes are also differentially expressed in B6. KEGG enrichment pathway analysis for the overlapping gene set shows that alanine, aspartate, and glutamate metabolism \((p < 0.05)\) is the top pathway commonly affected by diet changes. We further performed hierarchical clustering, as with B6 gene expression data, and observed 298 genes that are induced with HF diet with 30 genes that are persistently up-regulated even with dietary changes (red bar, Fig. 5C). These persistently up-regulated genes are unique from those persistently up-regulated in B6 mice and are enriched for retinol metabo-
lism pathways ($p < 0.05$). Overall, these data indicate that although there is overlap between gene expression changes in B6 and A/J, the majority of genes that change in HF diet are unique to each strain.

To understand the potential chromatin remodeling that occurs in the A/J liver, we performed FAIRE-seq with the livers from each A/J group and generated chromatin profiles as with the B6 mice. We performed hierarchical clustering to assess the effect of diet on chromatin accessibility changes (Fig. 6A). In contrast to the B6 analysis, the control diet and reversal diet groups are more similar in terms of chromatin accessibility profiles, indicating that the majority of diet-induced chromatin remodeling is reversible for A/J mice. Similarly, scatterplot analysis revealed that although 16 weeks of HF diet does induce chromatin remodeling in A/J, the magnitude of change is clearly smaller than that for B6, and the majority of chromatin changes do not persist after diet reversal (Fig. 6B; compare with Fig. 2B). Using the same thresholds as for B6, HF diet alters chromatin at 5,825 sites across the A/J genome. However, only a small fraction of the sites displaying chromatin accessibility changes in HF diet remain altered in the reversal diet group (220/5825 sites, 4%). In B6 liver, HNF4α-targeted sites are more accessible in both HF diet and reversal livers (Fig. 3A), suggesting this transcription factor contributes to the chromatin remodeling in this strain. To assess whether HNF4α also contributes to the remodeling in A/J, we assessed the number of HF-induced sites and the persistently accessible sites for HNF4α association (23). For B6, the vast majority of the HF-induced reversible and persistently accessible sites, 86.9% (4,604/5,298) and 93.5% (1,396/1,493), respectively, are targeted by HNF4α (Fig. 6C). In contrast, only a small fraction, 9.5% (289/3034), of the HF-induced open chromatin sites in A/J livers are targeted by HNF4α. Of the persistently accessible sites in A/J, 29.5% (68/162) are targeted by HNF4α. Altogether, these data suggest that HNF4α functions to a lesser extent in the chromatin accessibility changes in A/J.

**Discussion**

Clinical studies examining blood glucose control for diabetic patients have demonstrated that vascular complications can continue to develop long after blood glucose normalization, a phenomenon that was originally termed metabolic memory (2, 3). Similarly, many obese people find it difficult to maintain weight loss (40, 41), with long term physiological changes contributing to weight regain (42, 43). It has also been shown that formerly obese individuals continue to have increased mortality rates compared with the rest of the population (1). Our previous work indicated that a HF diet was associated with alterations to chromatin in the liver (9). To further understand the potential long term role of the chromatin alterations, this study extends the duration of the feeding scheme and includes a diet reversal group to examine whether the changes in chromatin accessibility can be reverted. We have now shown for the first time that these changes in chromatin accessibility can persist
upon diet reversal, suggesting they are involved in a similar metabolic memory-like phenomenon. These data further indicate that transcription factors such as HNF4α and histone post-translational modifications may play a key role in maintaining this persistence of chromatin alterations. We observed that HNF4α sites remain more accessible with long term (16 weeks versus 8 weeks) HF feeding. Interestingly, these sites are also persistently accessible even with reversal of diet, indicating that there is specificity to the persistence of diet-induced changes in chromatin accessibility. We further observed that H3K9me2 is increased at persistently inaccessible sites. The gene expression data in B6 livers showed that Kdm3a is misregulated with HF diet. Further investigations into the role of Kdm3a in the liver and whether it impacts diet-induced chromatin modifications is warranted. It is also likely that other chromatin regulatory complexes play a role in this phenomenon. Recently, it has been reported that Baf60a, a member of the SWI/SNF chromatin remodeling complex, is involved in the regulation of cholesterol homeostasis in response to a cholesterol-rich diet (44). We also observed mitochondrial genes to be up-regulated only in the livers of the mice in the reversal group. Mitochondrial dysfunction has been observed in obese and diabetic individuals and in HF diet-fed mice (45–47). The reversion of HFD to low fat diets in mice can result in reversion of some mitochondrial defects observed in heart cells (48). We speculate that the up-regulation of mitochondrial genes in the liver may be important for the mitochondrial recovery after HF diet.

Interestingly, in conjunction with the chromatin and gene expression changes, we also found that the mice in the reversal group still have slightly higher body weight and elevated triglyceride levels. Whether chromatin alterations are a cause or effect of these persistent physiological alterations is not clear from our data, but future studies examining this will be important.

Compared with B6, HF diet-induced chromatin alterations in A/J are overwhelmingly reversible. These results indicate that chromatin accessibility changes, and the reversibility of these events, depend on genetic factors. We further observed a strain-specific effect when we examined gene expression changes. Although ~15% of the differentially expressed genes are common between A/J and B6, the vast majority are strain-specific. These results also underscore the importance of jointly considering genetic and environmental factors when investigating complex diseases such as metabolic disease. Further
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studies investigating the differences between strains of mice will be useful to understand the variation that exists in human population in terms of weight gain, weight loss, and response to different diets.

Our results indicate that HF diet leads to persistent alterations of chromatin accessibility that are partially mediated by transcription factors and histone post-translational modifications. These chromatin alterations are furthermore strain-specific, indicating a genetic component to the response. These results suggest that persistent epigenetic modifications induced by HF diet have the potential to impact the long term risk for metabolic diseases.

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