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Obesity increases histone H3 lysine 9 and 18 acetylation at *Tnfa* and *Ccl2* genes in mouse liver

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Abstract. Obesity contributes to the development of non-alcoholic fatty liver disease (NAFLD), which is characterized by the upregulated expression of two key inflammatory mediators: tumor necrosis factor (Tnfa) and monocyte chemotactic protein 1 (Mcp1; also known as Ccl2). However, the chromatin make-up at these genes in the liver in obese individuals has not been explored. In this study, to identify obesity-mediated epigenetic changes at *Tnfa* and *Ccl2*, we used a murine model of obesity induced by a high-fat diet (HFD) and hyperphagic (ob/ob) mice. Chromatin immunoprecipitation (ChIP) assay was used to determine the abundance of permissive histone marks, namely histone H3 lysine 9 and 18 acetylation (H3K9/K18Ac), H3 lysine 4 trimethylation (H3K4me3) and H3 lysine 36 trimethylation (H3K36me3), in conjunction with polymerase 2 RNA (Pol2) and nuclear factor (NF)-κB recruitment in the liver. Additionally, to correlate the liver tissue-derived ChIP measurements with a robust *in vitro* transcriptional response at the *Tnfa* and *Ccl2* genes, we used lipopolysaccharide (LPS) treatment to induce an inflammatory response in Hepa1-6 cells, a cell line derived from murine hepatocytes. ChIP revealed increased H3K9/K18Ac at *Tnfa* and *Ccl2* in the obese mice, although the differences were only statistically significant for *Tnfa* ($P<0.05$). Unexpectedly, the levels of H3K4me3 and H3K36me3 marks, as well as Pol2 and NF-κB recruitment, did not correspond with the increased expression of these two genes in the obese mice. By contrast, the acute treatment of Hepa1-6 cells with LPS significantly increased the H3K9/K18Ac marks, as well as Pol2 and NF-κB recruitment at both genes, while the levels of H3K4me3 and H3K36me3 marks remained unaltered. These results demonstrate that increased Tnfa and Ccl2 expression in fatty liver at the chromatin level corresponds to changes in the level of histone H3 acetylation.

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Introduction

Obesity is the consequence of an imbalance between energy intake and energy utilization, and animal models of obesity reflect the behavioural and metabolic features that predispose humans to chronic caloric overconsumption. The overnutrition of animals can be achieved by forced feeding, the administration of high calorie diets, the genetic modification of feeding behaviour or a combination of these approaches (1).

Obesity is tightly connected with chronic, low-grade systemic inflammation (2). In the liver, this complex process begins with the migration of monocytes across the endothelium, followed by their differentiation into liver macrophages (Kupffer cells) (3). Kupffer cells secrete tumor necrosis factor (Tnfa), interleukin (Il)-6 and other inflammatory mediators and are thus highly pro-inflammatory. Monocyte migration is dependent on different chemotactic signals, such as monocyte chemotactic protein 1 (Mcp1; also known as Ccl2), which is predominant in the liver (4). The increased synthesis of these mediators reflects obesity-induced transcriptional upregulation of inflammatory genes.

Although the links between gut microbiota and obesity remain unclear, chronic low-grade endotoxemia has been suggested as one of the mechanisms involved in the development of obesity-related disorders (5). Lipopolysaccharides (LPS) are endotoxins that act on Toll-like receptor 4 (TLR4), induce mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB expression, and subsequently induce the release of pro-inflammatory molecules (6). An excess calorie intake increases the LPS concentration in serum as a consequence of a change in the proportion of Gram-negative bacteria in the gut or the permeability of the gut to bacteria (7). Obesity-induced endotoxemia and related systemic and liver inflammation can be lowered by treatment with prebiotics (6). Thus, chronic low-grade inflammation, a characteristic of obesity, can be indicative of the response to LPS released by the gut microflora. The results of our recently published transcriptome-based analyses are in agreement with this finding (8,9).

The genetic program is directed by epigenetic information and accomplished by cell-specific, developmental stage-specific

and metabolism-related changes in gene expression. Epigenetic changes modulate the accessibility of genes and their *cis*-regulatory elements to transcriptional complexes, predominantly through histone post-translational modifications (PTMs). PTMs are highly dynamic, may appear and disappear within only a few minutes in response to stimuli, and are likely to be common to each cell type in an organism (10). The acetylation of lysines is an ubiquitous PTM and is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), two families of enzymes with opposing activities (11). Acetylation neutralises the positive charge on the lysine side-chain, relaxes the chromatin structure and generates docking sites for bromodomain (BRD)-containing proteins (12).

Improvements in the sensitivity of chromatin immunoprecipitation (ChIP) assays have allowed the study of transcriptional and epigenetic changes in tissue (13-15). Using a multiplex Matrix-ChIP platform (14), in this study, we simultaneously analyzed the selected chromatin modifications and chromatin-bound factors in liver samples obtained from obese mice and cultured hepatocytes treated with LPS. We found that the increased expression of *Tnfa* and *Ccl2* was marked by acetylated histone H3.

Materials and methods

Tissue samples. For chromatin isolation, we used liver samples collected in our previous studies (8,9). Briefly, 5-week-old male wild-type (wt) C57BL/6J mice and mice that were homozygous for the leptin gene mutation B6.V-Lep^{ob}/J (ob/ob) were purchased from the Jackson Laboratory (Raritan, NJ, USA). During 1 week of adaptation, all mice were fed a normal diet (ND) with 10% of calories from fat (D12450B; Research Diets, New Brunswick, NJ, USA). At 6 weeks of age, 12 wt C57BL/6J (control group) and 12 ob/ob mice were further fed a ND, while another 12 wt C57BL/6J mice were fed a high-fat diet (HFD) with 60% of calories from fat (D12492; Research Diets). At either 16 or 48 weeks of age, 6 mice from each group were sacrificed and liver tissues collected. The experimental protocols were approved by the 2nd Local Ethics Committee for Animal Research in Warsaw, Poland. The experimental design and results of liver histopathological examination and serum biochemical analyses were described in our previous study (9).

Cell line. The mouse hepatocyte cell line, Hepa1-6, was delivered from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were grown for 24 h in 6-well culture plates to 40-50% confluence, then treated with either DMSO (0.01%) or LPS at the final concentration of 10 μg/ml for 30, 60 and 90 or 120 min and harvested for downstream analyses.

ChIP assay. Tissue (50 mg) was immersed in 2 ml of hypotonic buffer A (10mM HEPES pH 7.9, 2 mM $MgCl_2$, 2 mM KCl) supplemented with protease and phosphatase inhibitors (78441; Thermo Scientific, Rockford, IL, USA) and homogenized by 10 strokes with a loose pestle and 30 strokes with a tight pestle in a Dounce homogenizer (Wheaton, Millville, NJ, USA) followed by centrifugation at 2,000 rpm for 5 min in 4˚C. The nuclei pellet was cross-linked in 1 ml 1% formaldehyde/PBS for 10 min at room temperature and then formaldehyde was quenched at room temperature for 5 min by the addition of 2 M glycine to a 125 mM final concentration. Following 5-min centrifugation at 2,000 rpm at room temperature, the nuclei were washed once with 1 ml of PBS and stored at -80˚C.

The nuclei pellet was resuspended in lysis buffer (12.5 mM Tris-HCl, pH 8.0; 2.5 mM EDTA; 0.25% SDS) containing protease and phosphatase inhibitors (Thermo; 78441). Chromatin was sheared in a Bioruptor® Plus [Diagenode, Seraing (Ougrée), Belgium] using 30 sec on-off cycles for 15 min at high intensity according to the protocol. ChIP assays were performed using Matrix-ChIP on polypropylene plates (BioExpress, Kaysville, UT, USA), as previously described (16). The ChIP DNA data were expressed as the percentage of input DNA or as the ratio of modified histone to total histone H3, as previously described (14). The following antibodies were used in ChIP assay: non-specific rabbit IgG (I-1,000; Vector Laboratories, Inc., Burlingame, CA, USA), polymerase 2 RNA (Pol2; 4H8) (sc-47701; Santa Cruz Biotechnology, Santa Cruz, CA, USA), NF-κB p65 (ab7970; Abcam, Cambridge, MA, USA), histone H3 (ab1791; Abcam), histone H3 lysine 9 and 18 acetylation (H3K9/K18Ac, 07-593; Millipore, Billerica, MA, USA), H3 lysine 4 trimethylation (H3K4me3, pAb-003-050; Diagenode), H3 lysine 36 trimethylation (H3K36me3, ab9050; Abcam).

Total RNA extraction and RT reaction. Total RNA was extracted from either cells or liver tissue using the TRIzol® Plus RNA Purification kit (Invitrogen, Carlsbad, CA, USA) with On-column DNase I treatment. Total RNA (1 μg) and random hexamers were used in cDNA synthesis with SuperScript III (Invitrogen) according to the manufacturer's instructions.

Quantitative PCR (qPCR). qPCR analysis of the cDNA and ChIP samples was carried out using an Applied Biosystems 7900HT Fast Real-Time PCR System with a SensiMix SYBR kit (Bioline, Tautan, MA, USA). The expression of Mrpl36, Hmbs and Mcoln1 was used to generate a normalizing factor with geNorm software, as previously described (17). The sequences of all primers used are listed in Table I. Differences were evaluated using the Mann-Whitney U test with GraphPad Prism 5 software. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Animal experiments. Altered gene expression leading to the the activation of pro-inflammatory signaling and the deregulation of metabolic pathways is characteristic of obesity-induced hepatic inflammation (18). In this study, we performed experiments using 16- and 48-week-old HFD-fed and ND-fed wild-type C57BL/6J mice and hyperphagic (ob/ob) obese mice, as previously described (9). Six mice per group of each given genotype, age and diet were used.

The expression of *Tnfa* and *Ccl2* was analyzed in the liver samples by qPCR. As shown in Fig. 1, the mRNA levels of both transcripts were higher in the 16-week-old (young) and the 48-week-old (old) ob/ob mice and the old HFD-fed mice than in the control and young wt HFD-fed mice. As was observed in a recent study of ours (9), young and old ob/ob mice developed steatosis, which was accompanied by moderate inflammation in the older mice. The livers of both the 16- and 48-week-old control mice, as well as of the young HFD-fed mice, were histologically normal, while the livers of the old

Table I. List of primers used in qPCR.

Gene	Orientation	Sequence (5'→3')	Assay
Gapdh	Forward	CCCATCACGTCCTCCATC	
	Reverse	TGGGCACTGTACGGGTCT	ChIP
Ccl2-ex1	Forward	GCCAACACGTGGATGCTC	
	Reverse	AGCCAACTCTCACTGAAGCC	ChIP
Ccl2-ex3	Forward	TTAAGGCATCACAGTCCGAG	
	Reverse	TTGAATGTGAAGTTGACCCG	ChIP
Tnfa-ex1	Forward	GCAGGTTCTGTCCCTTTCAC	
	Reverse	AGTGCCTCTTCTGCCAGTTC	ChIP
Tnfa-ex4	Forward	TATGGCTCAGGGTCCAACTC	
	Reverse	GCTCCAGTGAATTCGGAAAG	ChIP
Tnfa	Forward	GCTACGACGTGGGCTACAG	
	Reverse	CCCTCACACTCAGATCATCTTCT	RT
Ccl2	Forward	ATGTCTGGACCCATTCCTTCT	
	Reverse	AGGTGTCCCAAAGAAGCTGTA	RT
Mrpl36	Forward	CTCAGGTGCAGGAACGGGTC	
	Reverse	CCCTTCCCAGGTCTGGGCTC	RT
Hmbs	Forward	AAGGGTTTTCCCGTTTGC	
	Reverse	TCCCTGAAGGATGTGCCTA	RT
Mcoln1	Forward	CCACCACGGACATAGGCATAC	
	Reverse	GCTGGGTTACTCTGATGGGTC	RT

ChIP, chromatin immunoprecipitation; RT, reverse transcriptase; ex, exon.

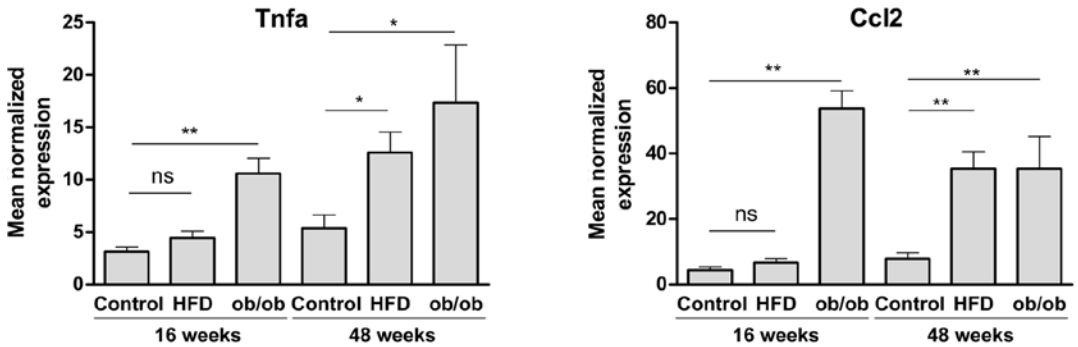

Figure 1. Analysis of tumor necrosis factor (*Tnfa*) and monocyte chemotactic protein 1 (*Ccl2*) mRNA expression in the livers of normal diet-fed (control), high-fat diet (HFD)-fed and ob/ob mice. Livers were collected at the age of either 16 or 48 weeks. One microgram of total RNA was reverse-transcribed with random hexamers to generate cDNA, and qPCR was performed with specific primers (Table I) using SYBR-Green I chemistry. Geometric mean expression of Mrpl36, Hmbs and Mcoln1 was used for normalization. Data are presented as the means ± SEM, n=6 mice in each group. Statistical significance was assessed by the Mann-Whitney U test. *P<0.05, **P<0.001 indicate statistical significance; ns, not significant.

HFD-fed mice displayed steatosis with mild inflammation. Hyperinsulinemia, hyperglycemia and hypercholesterolemia were observed in the ob/ob and old HFD-fed mice (9). Thus, animals exhibiting increased expression of both pro-inflammatory cytokines related to fatty liver were combined into a single group henceforth referred to as the obese group. The control group comprised of 16-week old HFD-fed mice and ND-fed control mice of both age groups.

The distribution of histone PTMs and nuclear proteins along chromatin, including Pol2 and transcription factors, were analyzed using ChIP assays. To estimate the contribution of Pol2 and NF-κB recruitment to changes in Tnfa and Ccl2 transcript levels, Pol2 and NF-κB levels were determined by ChIP assay at the first exon near the transcription start site and at one site within the last exon of each gene (Fig. 2). NF-κB plays a central role in the induction of pro-inflammatory gene

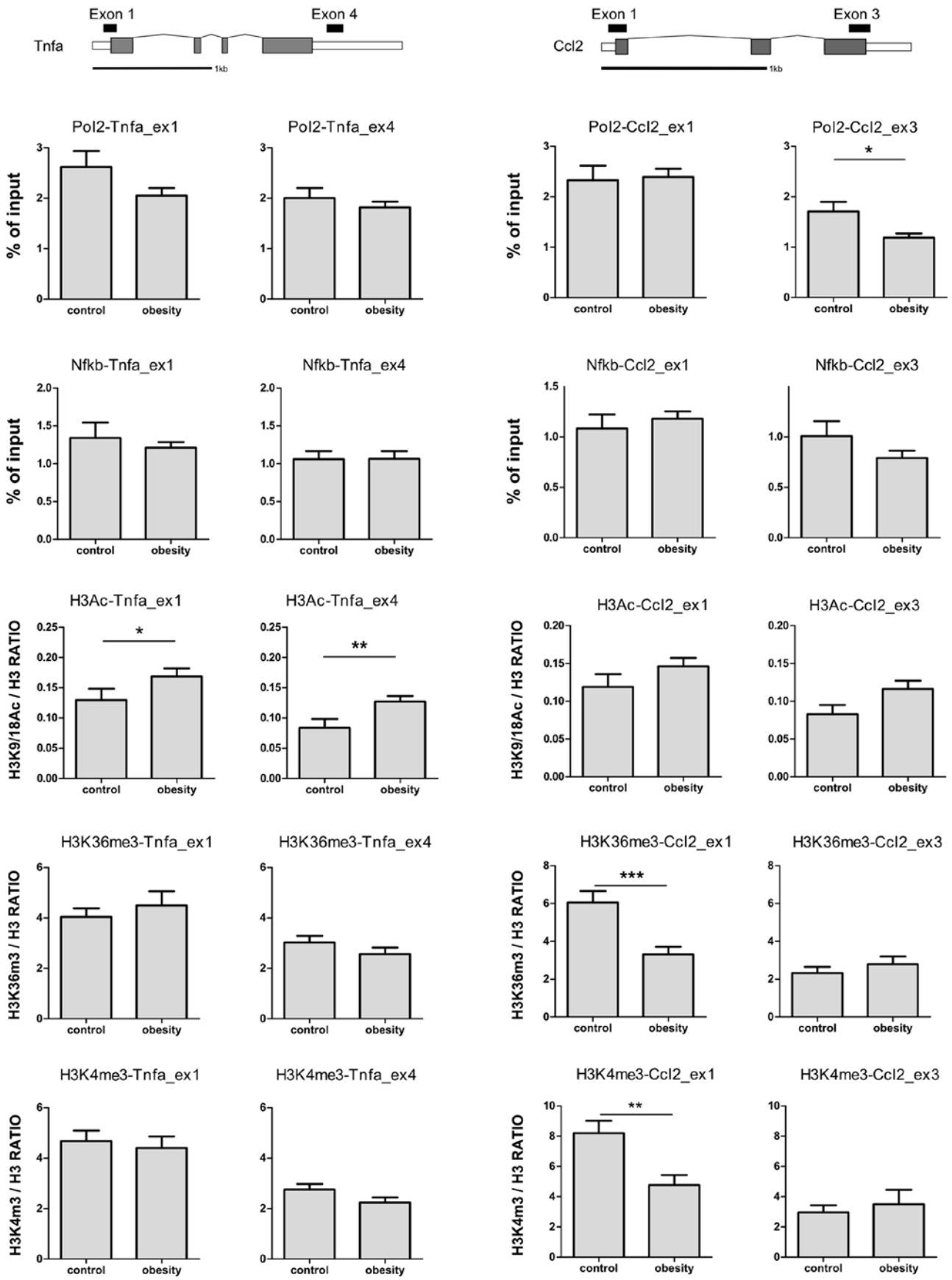

Figure 2. ChIP assay of RNA polymerase II (Pol 2), nuclear factor (NF)-κB and histone modifications at tumor necrosis factor (*Tnfa*) and monocyte chemotactic protein 1 (*Ccl2*) in obese and lean livers. Sheared cross-linked chromatin was prepared from frozen livers. ChIP assay was performed using a Matrix-ChIP platform. ChIP DNA were analyzed at the first and last exons in qPCR. The control group comprised of 16-week old high-fat diet (HFD)-fed mice and normal diet (ND)-fed control mice of both age groups, while the obese group contained 48-week old HFD-fed and ob/ob mice of both age groups. Data are presented as the means ± SEM (n=18 animals from each group), expressed either as the percentage (%) of input DNA or as the ratio of modified histone to total histone H3. Statistical significance was assessed by the Mann-Whitney U test. *P<0.05, **P<0.001, ***P<0.0001 indicate statistical significance; ns, not significant; ex, exon.

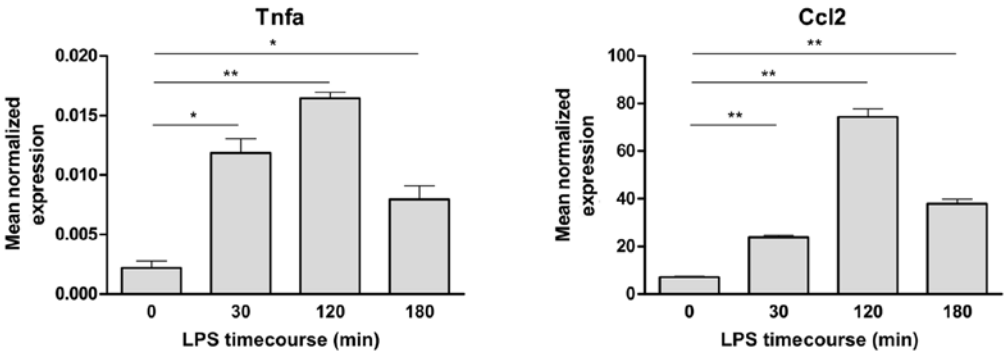

Figure 3. Analysis of tumor necrosis factor (*Tnfa*) and monocyte chemotactic protein 1 (*Ccl2*) mRNA expression in Hepa1-6 cells following treatment with lipopolysaccharides (LPS). Cells were collected after 30, 120 and 180 min of treatment with 10 μg/ml LPS and total RNA was extracted. One microgram of RNA was reverse-transcribed with random hexamers to generate cDNA, and qPCR was performed with specific primers (Table I) using SYBR-Green I chemistry. Geometric mean expression of Mrpl36, Hmbs and Mcoln1 was used for normalization. Data are presented as the means ± SEM, n=3. Statistical significance was assessed by the Mann-Whitney U test. *P<0.05, **P<0.001 indicate statistical significance; ns, not significant.

expression (19). Unexpectedly, neither the Pol2 nor the NF-κB levels corresponded to the increased mRNA levels of *Tnfa* and *Ccl2* observed in the livers of the obese mice. Additionally, Pol2 abundance at *Ccl2* was decreased in these mice.

Subsequently, in order to elucidate the contribution of the state of the chromatin at the *Tnfa* and *Ccl2* loci to their transcription, histone modification markers associated with active transcription were examined (Fig. 2). The acetylation of lysines eliminates their positive charge, which has been shown to have a negative effect on the higher order structure of chromatin, making it less compact (11). The acetylation of histone H3 lysine 9 and 18 (H3K9K18Ac) is associated with actively transcribed genes (12). The levels of H3K9K18Ac at the *Tnfa* gene were significantly higher at the first and last exon in the obese mice. The H3K9K18Ac levels at the *Ccl2* gene were also increased, although this did not reach the significance threshold. These results are consistent with those of several studies on histone acetylation patterns of actively transcribed genes, as reviewed by Medzhitov and Horng (20).

The trimethylation of histone H3 lysine 4 (H3K4me3) is regarded as an indicator of active chromatin at the 5' ends of actively transcribed genes (12). The H3K4me3 levels at *Tnfa* were not changed in obese animals. In addition, the abundance of this mark at the first exon of *Ccl2* was significantly decreased in the obese animals, contrary to the expectation that H3K4me3 correlates with an elevated mRNA expression. The trimethylation of histone H3 lysine 36 (H3K36me3) is associated with transcription elongation and is expected to gradually increase toward the 3' end of a transcribed gene (12). The level of H3K36me3 was unaffected at the *Tnfa* gene, while its level at the first exon of *Ccl2* mirrored the changes observed for H3K4me3. Taken together, these results demonstrate that the increased acetylation of K9/K18 residues at histone H3 along inflammatory genes in murine fatty liver is an epigenetic mark that correlates with their expression.

Cell culture studies. Unexpectedly, only the acetylation of histone H3 differed in the livers of the obese mice compared to the control mice. To confirm that our methodology reliably determined the epigenetic make-up at these inflammatory genes, we used LPS to induce an inflammatory response in Hepa1-6 cells, a cell line derived from murine hepatocytes. LPS mediates an inflammatory response through TLRs and differentially regulates the expression of numerous genes involved in cell migration, tissue repair and remodeling, antimicrobial defense, phagocytosis, metabolic reprogramming and the regulation of adaptive immune responses (20).

The Hepa1-6 cells were treated with LPS at a concentration of 10 μg/ml. This induced a substantial increase in the mRNA expression of *Tnfa* and *Ccl2*, with a peak expression at 120 min (Fig. 3). Subsequently, we stimulated the Hepa1-6 cells with LPS for 30, 60 and 90 min to capture the epigenetic changes at the *Tnfa* and *Ccl2* loci that preceded the maximum mRNA expression of these genes. ChIP analyses were performed for the same factors and histone marks as analyzed in the experiments on chromatin isolated from liver samples.

In response to LPS treatment, Pol2 levels at *Tnfa* and *Ccl2* progressively increased, with a peak at 90 min (Fig. 4). This LPS-mediated increase in Pol2 density was associated with higher levels of NF-κB and H3K9/18Ac at these loci. We also examined NF-κB binding at the ends of the genes since the presence of transcription factors in the body of an activated gene, rather than simply at the promoter region, has been observed, and this binding followed the Pol2 distribution pattern (21). Furthermore, NF-κB distribution along chromosome 22 has been reported, suggesting that it binds to sites other than the promoter (22). In concordance with these observations, NF-κB binding at *Tnfa* and *Ccl2* following LPS stimulation resembled the pattern of Pol2 recruitment (Fig. 4).

Notably, H3K9/18Ac abundance also mirrored Pol2 and NF-κB density at these genes; however, it was more robust and, although not significant, already pronounced after 30 min of LPS stimulation (Fig. 4). Surprisingly, no significant differences were observed in the H3K4me3 and H3K36me3 marks in this experimental setup, with the exception of an increase in the H3K4me3 levels at the end of C*cl2* after 30 min of LPS stimulation. At the same time, LPS had no effect on the expression of the Gapdh housekeeping gene, which was characterized by high basal levels of H3K9/18Ac, H3K4me3 and Pol2 at its promoter (data not shown). In conclusion, our survey of epigenetic changes at *Tnfa* and *Ccl2* in cells treated with

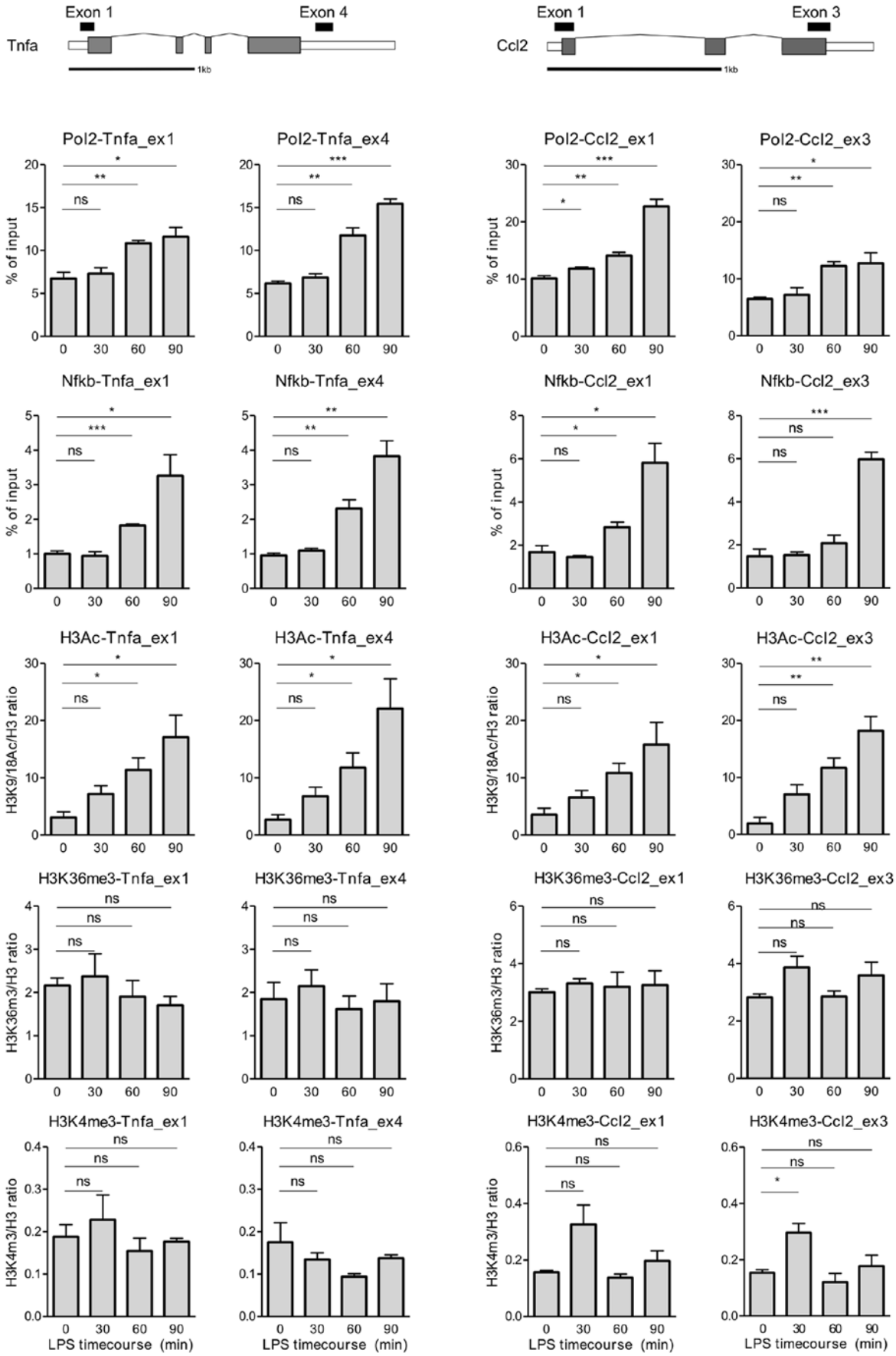

Figure 4. ChIP assay of RNA polymerase II (Pol2), nuclear factor (NF)-κB and histone modifications at tumor necrosis factor (*Tnfa*) and monocyte chemotactic protein 1 (*Ccl2*) in Hepa1-6 cells following treatment with lipopolysaccharides (LPS). Cells were formaldehyde-fixed after 30, 60 and 90 min of treatment with 10 μg/ml LPS and cross-linked chromatin was sheared and used in ChIP analysis on a Matrix-ChIP platform. ChIP DNA was analyzed at the first and last exons by qPCR. Data are presented as the means ± SEM (n=3), expressed as either the percentage (%) of input DNA or as the ratio of modified histone to total histone H3. Statistical significance was assessed by the Mann-Whitney test. *P<0.05, **P<0.001, ***P<0.0001 indicate statistical significance; ns, not significant.

LPS confirmed that the acetylation of histone H3 is strongly associated with the increased transcription of these genes.

Discussion

The interplay between chromatin states and gene expression represent key mechanisms underlying the development of numerous disease phenotypes, including the modulation of the expression of pro-inflammatory factors produced by a wide variety of cells (23). Non-alcoholic fatty liver disease (NAFLD)-associated phenotypes, both in humans and animal models of obesity, are associated with the altered expression of genes involved in a number of processes, including hepatic glucose and lipid metabolism, insulin signaling, inflammation, coagulation, cell adhesion, oxygen stress and chaperone activity (24). Some of these processes are modulated by Tnfa, Il-6 and interferon-γ and their increased expression reflects both global chronic inflammation and local liver injury (25-27).

Given the link between obesity and low-grade inflammation in the liver, we set out to survey epigenetic changes at two of the inflammatory genes that accompany this process, i.e., *Tnfa* and *Ccl2*. As expected, both *Tnfa* and *Ccl2* mRNA levels were significantly increased in ob/ob and old HFD mice (Fig. 1) and corresponded with liver steatosis, as determined by histopathological evaluation (9). These expression data are in accordance with a previous study by Stanton *et al* (28), in which fatty liver was induced by a high cholesterol diet.

To the best of our knowledge, no previous studies have investigated epigenetic changes at inflammatory genes in liver tissue upon steatosis. As *Tnfa* and *Ccl2* mRNA levels were elevated in our model of obesity, we surveyed epigenetic events at the chromatin of these two loci. However, out of several chromatin features tested, only the acetylation of histone H3 was associated with an increase in the mRNA levels of these two inflammatory genes in the livers of obese mice (Figs. 1 and 2). Although post-transcriptional mechanisms may account for the observed changes in the mRNA levels of these inflammatory genes (29-31), the survey of Pol2 complexes along the locus provided a means to assess the rates of transcription. Although conditions of acute endotoxemia accompany elevated plasma levels of inflammatory mediators and potentially contribute to multi-organ failure, including acute renal failure (15,32), in our study, we did not observe an increase in Pol2 transcriptional complex density at these genes (Fig. 2). On the other hand, the observed increase in histone H3 acetylation may result in the higher accessibility of the Pol2 complex to chromatin, thereby allowing more robust Pol2 traveling along the gene. In contrast to *in vivo* studies of the LPS-induced inflammatory response (Fig. 3), LPS-induced acute inflammation in Hepa1-6 cells was accompanied by an increase in the binding of Pol2 transcriptional complexes (Fig. 4).

Liver steatosis is a chronic state, in contrast to the acute inflammatory response induced in cultured cells challenged with LPS (15). It is likely that this difference is the reason we did not observe the same results *in vitro* and *in vivo*. Chronic liver steatosis in which acetylation marks are already established can render inflammatory loci more transcription-permissive without inducing measurable changes in Pol2 levels. Another possible reason for the differences in the results obtained from analyses of liver samples and cells in culture may stem from the general cellular heterogeneity of organs and tissues. When certain epigenetic changes are present only in a minority cell type, the analysis of samples taken from whole tissues has an obvious limitation, as cells that compose the majority of the tissue can mask the probed epigenetic marks. These limitations may be addressed with a combination approach utilizing laser capture microdissection and more sensitive ChIP methods requiring the input of fewer cells.

Histone acetylation is a well-established PTM associated with transcriptional activity in eukaryotic cells (33), and increased histone acetylation at inflammatory gene loci has been shown in both *in vivo* and *in vitro* studies (20). For example, increased histone H3 and H4 acetylation at the *Tnfa* promoter is associated with the induction of transcription by LPS, high glucose concentrations, a diabetic state and systemic lupus erythematosus. However, it must be stressed that the majority of these studies were performed on monocytes and macrophages *in vivo* or in cell culture (34-37). Consequently, *Tnfa* transcription has been shown to be regulated by a number of HATs, including CBP/p300, p/CAF and GCN5 (38). Histone acetyl-lysine modifications also serve as docking sites for factors that promote transcription through interactions with BRD-containing proteins. Recently developed small molecule inhibitors targeting BRD-containing proteins have been shown to selectively interfere with inflammatory gene expression (39). In line with the action of these drugs are the effects of knockdown of BRD-containing proteins, particularly Brd2. *Brd2* disruption in mice has been shown to ameliorate obesity-induced inflammatory responses, protect animals from insulin resistance, glucose intolerance, and pancreatic beta cell dysfunction and uncoupled obesity from the onset of diabetes (40,41). The role of chronic inflammation and metabolic dysfunction in NAFLD is a considerable public health issue, and new epigenetic drugs, such as those targeting BRD-containing proteins, may provide a novel means of treating or preventing obesity-related diseases (42).

While the level of H3K4m3 marks has generally been shown to correlate with gene expression and histone H3 acetylation (43), our measurements displayed no such dependency at the *Tnfa* and *Ccl2* genes in either liver or cell culture (Figs. 2 and 4). Furthermore, together with upregulated gene expression, an increase in H3K36me3 histone marks is expected at the 3' end of actively transcribed genes (44). However, the present study did not demonstrate such a pattern at *Tnfa* and *Ccl2*. In line with our observation regarding the lack of changes in H3K4me3 and H3K36me3 upon *Tnfa* and *Ccl2* activation are recent findings in yeast by Zhang *et al* (45), who provided evidence that a marked increase in transcription can occur in the context of little, if any, covalent histone modification including the two aforementioned marks. Our data therefore suggest that these two permissive epigenetic modifications may not act in concert with histone H3 acetylation to generate a transcriptional response at *Tnfa* and *Ccl2* in the liver and liver-derived cell lines.

In conlcusion, the use of a multiplex Matrix-ChIP platform allowed us to assess obesity-mediated epigenetic changes in the liver at two pro-inflammatory genes, *Tnfa* and *Ccl2*, suggesting that histone H3 acetylation is associated with obesity.

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