Non-alcoholic fatty liver disease
Sheedfar, Fareeba

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Sheedfar, F. (2015). Non-alcoholic fatty liver disease: understanding the role of aging, fatty acid transport and epigenetics. [S.l.]: [S.n].

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 6

Genetic ablation of macrohistone H2A1 leads to increased leanness, glucose tolerance and energy expenditure in mice fed a high fat diet

Fareeba Sheedfar¹, Mathilde Vermeer¹, Valerio Pazienza², Joan Villarroya³, Francesca Rappa⁴, Francesco Cappello⁴,⁵, Gianluigi Mazzoccoli⁶, Francesc Villarroya³, Henk van der Molen¹, Marten H. Hofker¹, Debby P. Koonen¹, Manlio Vinciguerra²,⁵,⁷

¹University of Groningen, University Medical Center Groningen (UMCG), Department of Pediatrics, Section Molecular Genetics, Groningen, The Netherlands; ²Department of Medical Sciences, Gastroenterology Unit, IRCCS “Casa Sollievo della Sofferenza” Hospital, San Giovanni Rotondo (FG) Italy; ³Department of Biochemistry and Molecular Biology, and Institute of Biomedicine (IBUB), University of Barcelona, Barcelona, Spain; ⁴CIBER Fisiopatologia de la Obesidad y Nutricion, Instituto de Salud Carlos III, Spain; ⁵Department of Experimental Biomedicine and Clinical Neurosciences, Section of Human Anatomy, University of Palermo, Palermo, Italy; ⁶Euro-Mediterranean Institute of Science and Technology (IEMEST), Palermo, Italy; ⁷Department of Medical Sciences, Division of Internal Medicine and Chronobiology Unit, IRCCS “Casa Sollievo della Sofferenza” Hospital, San Giovanni Rotondo (FG) Italy; ⁸University College London (UCL) – Institute for Liver and Digestive Health, Division of Medicine, Royal Free Hospital, London, UK.

* = contributed equally

Abstract

In the context of obesity, epigenetic mechanisms regulate cell specific chromatin plasticity, perpetuating gene expression responses to nutrient excess. MacroH2A1, a variant of histone H2A, emerged as key chromatin regulator sensing small nutrients during cell proliferation and differentiation. Mice genetically ablated for macroH2A1 (knock-out; KO) do not show overt phenotypes under a standard diet. Our objective was to analyse the in vivo role of macroH2A1 in response to nutritional excess. 12 weeks old whole-body macroH2A1 KO male mice were given a high-fat diet (60% energy from lard) for 12 weeks until sacrifice, and examined for glucose and insulin tolerance, and for body fat composition. Energy expenditure was assessed using metabolic cages and by measuring the expression levels of genes involved in thermogenesis in the brown adipose tissue (BAT) or in adipogenesis in the visceral adipose tissue (VAT). Under a chow diet, macroH2A1 KO mice did not differ from their wild type (WT) littermates for body weight, and sensitivity to glucose or insulin. However, KO mice displayed decreased heat production (p<0.05), and enhanced total activity during the night (p<0.01). These activities related to protection against diet-induced obesity in KO mice, which displayed decreased body weight due to a specific decrease in fat mass (p<0.05), increased tolerance to glucose (p<0.05), and enhanced total activity during the day (p<0.05), compared to WT mice. KO mice displayed increased expression of thermogenic genes (Ucp1, p<0.05; Glut4 p<0.05; Cox4 p<0.01) in BAT and a decreased expression of adipogenic genes (Pparγ, p<0.05; Fabp4, p<0.05; Glut4, p<0.05) in VAT compared to WT, indicative of augmented energy expenditure. Genetic eviction of macroH2A1 confers protection against diet-induced obesity and metabolic derangements in mice. Inhibition of macroH2A1 might be a helpful strategy for epigenetic therapy of obesity.

Introduction

The current pandemic in obesity/metabolic syndrome is a risk factor for many types of diseases, including cardiovascular diseases and cancer (Louie et al., 2013; Pedersen, 2013). Epigenetic mechanisms of nuclear chromatin remodelling, such as DNA methylation, post-translational modifications of histones and incorporation of histone variants into the chromatin are increasingly recognized as crucial factors in the pathophysiology of obesity and related complications (Podrini et al., 2013). In fact, metabolic alterations in peripheral tissues are triggered at the cellular level by changes in gene transcriptional patterns dependent on the degree of nuclear chromatin compaction. The latter is regulated at several levels, allowing transcriptional plasticity (Goldberg,
Chapter 6

122

2007): one way is the replacement of canonical histones around which DNA is wrapped (H2A, H2B, H3 and H4) with the incorporation of histone variants. The histone variant of H2A, known as macroH2A1, is believed to act as a strong transcriptional modulator that can either repress transcription (Doye, 2006; Ladurner, 2003), or activate it in response to as yet undefined nutrients or growth signals (Gamble, 2010). The transcriptional activity of macroH2A1 has come to take a center stage in the plasticity of stem cell differentiation and in the pathogenesis of many cancer types (Barrero et al., 2013; Cantarino et al., 2013; Creppe et al., 2012; Gaspar-Maia et al., 2013). MacroH2A1 is composed of a domain displaying 66% homology with histone H2A, and a domain called macro that is conserved in multiple functionally unrelated proteins throughout the animal kingdom and that can bind in vitro with tight affinity ADP-ribose-like metabolites produced by NAD+-dependent histone deacetylase Sirtuins or by polyADP-ribose polymerase enzymes (Pehrson and Fried, 1992; Posavec et al., 2013). This binding represents a direct molecular interaction between intermediate metabolism and the chromatin, whereby a metabolite can impinge on and tweak gene expression in vitro (Kustatscher et al., 2005; Ladurner, 2006; Pazienza et al., 2014; Timinszky et al., 2009). Interestingly, in mice models of non-alcoholic fatty liver disease (NAFLD), a disorder that is present in 90% of obese subjects, hepatic content of macroH2A1 is augmented (Pogribny et al., 2009) (Rappa et al., 2013). Two mice models knock-out (KO) for macroH2A1 have been reported under a standard diet feeding. In the first model, generated in the pure C57Bl/6J background, developmental changes in macroH2A1-mediated gene regulation were observed (Changolkar et al., 2007; Changolkar et al., 2010). In the second model, KO for macroH2A1 in a mixed background led to a variable hepatic lipid accumulation in 50% of the females (Boulard et al., 2010). Therefore, despite compelling in vitro evidence that macroH2A1 modulates gene expression programs involved in cell metabolism, proliferation and differentiation (Barrero et al., 2013; Cantarino et al., 2013), its role at the organism level under nutritional stress conditions, especially during obesity, is not understood. In this study we challenged macroH2A1KO mice (Changolkar et al., 2007; Changolkar et al., 2010) with an obesogenic diet for 12 weeks: we found that genetic ablation of this histone increased leanness, glucose tolerance and energy expenditure, protecting the body against high fat diet (HFD)-induced obesity.

Materials and Methods

Animals and diet

Viable and fertile macroH2A1 knockout (KO) mice were kindly provided by Prof. John Pehrson (UPenn) (Changolkar et al., 2007). MacroH2A1
KO and their littermates (WT) mice were bred and all procedures were approved by the University of Groningen Ethics Committee for Animal Experiments, which adheres to the principles and guidelines established by the European Convention for the Protection of Laboratory Animals. Mice were housed in a temperature-controlled room under a 12 h light-dark cycle with ad libitum access to water and food (2181, RMH-B Arie Blok, Woerden, the Netherlands). At the age of 12-14 weeks mice were individually housed and fed a HFD composed of 60% Kcal fat from lard (D12492, Research Diets, New Brunswick, USA) for 12 weeks until sacrifice.

Oral Glucose Tolerance Test and Insulin Tolerance Test

Mice were fasted for 6 hours day time, and given a glucose bolus (2 g/kg of 20% glucose solution) by oral gavage (OGTT) or intraperitoneally (IP) injected with human recombinant insulin (0.5 U/kg bodyweight, Actrapid, Novo Nordisk Canada inc., Ontario, Canada) (ITT). Blood was collected from the tail vein and glucose levels were measured with an OneTouch Ultra glucometer (Lifescan Benelux, Beerse, Belgium) before and 15, 30, 60, 90, 120 and 150 minutes after the gavage/injection.

Dual-Energy X-ray Absorptiometry (DEXA) scan analysis

Fat and lean mass was determined in HF-fed mice following 11 weeks of dietary intervention using Dual-Energy X-ray Absorptiometry (p-DEXA, Norland Stratec Medizintechnik GmbH, Birkenfeld, Germany). Mice were scanned under fed conditions while anesthetized using isoflurane and data were analyzed according to the manufacturer’s instructions.

In vivo metabolic analyses

Groups of 7-8 mice per genotype on high-fat diet were placed individually in indirect calorimetric cages (LabMaster TSE systems, Bad Homburg, Germany) to enable real time and continuous monitoring of metabolic gas exchange. Following an initial 24-h acclimatization period, mice were monitored every 13 min for 24 h for 4 consecutive days. Heat production, food intake and total activity were analyzed. The respiratory exchange ratio RER = VCO2(volume of CO2 produced)/VO2(volume of O2 consumed) was used to estimate the percent of carbohydrates and fat contribution to whole-body energy metabolism of mice in vivo. Carbohydrate and fat oxidation rates were calculated from VO2 and VCO2 according to Perronnet and Massicotte (Peronnet and Massicotte, 1991). Faeces were collected and measured by scale.

Analysis of plasma parameters

Insulin was determined in plasma from 6 h fasted mice using an enzyme-linked immunosorbent assay kit (Mercodia ultrasensitive mouse insulin-linked immunosorbent assay, Orange Medica, Tilburg, the Netherlands). Triglycerides (TG) and total cholesterol
(TC) were determined by commercially available kits, according to the manufacturer’s instructions (TG: Hitachi, Roche, Woerden, the Netherlands; TC: cholesterol CHOD-PAP, Roche, Woerden, the Netherlands;). Roche Percimat Glycerol standard (16658800) and Cholesterol Standard FS (DiaSys Diagnostic Systems Gmbh, Holzheim, Germany) was used as a reference.

Biochemical analyses of Lipids
Total lipids were extracted from the liver according to Bligh and Dyer (Bligh and Dyer, 1959). Liver and plasma lipid analysis were performed using the available reagent kits (Tri/GB kit Roche®, Mannheim, Germany) based on the manufactures protocol.

Immunoblot analyses in mice
The mice were fasted for 6 h and subjected to an IP injection with saline or human recombinant insulin (0.75 U/kg body weight, Actrapid, Novo Nordisk Canada inc., Ontario, Canada) 15 minutes before sacrifice. Liver, adipose tissue (interscapular or visceral) and skeletal muscle tissues were rapidly removed and frozen in liquid nitrogen. Powdered tissue was homogenized as described previously (Veyrat-Durebex et al., 2009). Equal amounts of protein were separated by SDS-PAGE, transferred to PVDF membrane (Amersham, Buckinghamshire, UK) and the resulting immune-complex was visualized using the molecular imager ChemiDoc XRS + system (Bio-Rad, Veenendaal, The Netherlands). Antibodies against phospho- and total AKT were purchased from Cell Signaling (Leiden, the Netherlands). β-Actin (Sigma, Zwijndrecht, the Netherlands) was used as loading control. Densitometry was performed using Image Lab Software (Bio-Rad, Veenendaal, The Netherlands).

Histology
Paraffin embedded sections of the liver or adipose tissue (4 μm) were processed by haematoxylin and eosin (H&E) for histological evaluation, as previously described (Kleiner et al., 2005; Salamone et al., 2012). Diagnostic classification of NAFLD was performed by applying a semiquantitative scoring system that grouped histological features into broad categories (steatosis, hepatocellular injury, portal inflammation, fibrosis, and miscellaneous features) (Kleiner et al., 2005). Adipocyte area and perimeter were evaluated using the ImagePro Plus software (Media Cybernetics, Inc. Bethesda, MD, USA).

Gene expression analyses
Quantitative reverse transcriptase PCR experiments were performed as previously described (Planavila et al., 2012). After homogenization of tissue samples in RLT buffer (Qiagen, Hilden, Germany), RNA was isolated using a column-affinity based methodology that included on-column DNA digestion (RNaseasy; Qiagen). One microgram of RNA was transcribed into cDNA using MultiScribe reverse transcriptase and
random-hexamer primers (TaqMan Reverse Transcription Reagents; Applied Biosystems, Foster City, California, CA, USA). For quantitative mRNA expression analysis, TaqMan reverse transcriptase (RT)-polymerase chain reaction (PCR) was performed on the ABI PRISM 7700HT sequence detection system (Applied Biosystems). The TaqMan RT-PCR reactions were performed in a final volume of 25 µl using TaqMan Universal PCR Master Mix, No AmpErase UNG reagent and primer pair probes specific for uncoupling protein-1 (Ucp1, Mm00494069), fibroblast growth factor-21 (Fgf21, Mm00840165), peroxisome proliferator-activated receptor-γ (Pparγ, Mm00440945), glucose transporter-4 (Glut4, Slc2a4, Mm00436615), cytochrome c oxidase-4 (Cox4, Mm00438289), fatty acid binding protein-4 (Fabp4, Mm00445880) and interleukin-6 (Il-6, Mm0044619). Controls with no RNA, primers, or RT were included in each set of experiments. Each sample was run in duplicate, and the mean value of the duplicate was used to calculate the mRNA levels for the genes of interest. Values were normalized to that of the reference control (Ppia, Mm02342430) using the comparative 2-deltaCT method, following the manufacturer’s instructions and represented as fold induction in comparison to WT group.

Rhythmicity of expression of considered genes was verified using CircaDB, a data set of time course expression experiments from mice and humans deposited as publicly available microarray studies and highlighting circadian gene expression cycles (https://www.bioinf.itmat.upenn.edu/circa).

Statistical analysis

Results are expressed as means ± SEM. Comparisons between groups were performed with the parametric Student’s t-test or the non-parametric Mann-Whitney U test, as appropriate, using GraphPad Prism Software (version 5.00 for Windows, San Diego, CA, USA); a p-value ≤ 0.05 was considered significant.

Results

MacroH2A1 KO mice are leaner and more glucose tolerant under a HFD

To determine the impact of histone variant macroH2A1 on obesity-associated increase in body weight gain, wild type and macroH2A1 KO mice were fed either a chow diet or a HFD. Body weight at sacrifice in HFD fed mice was ~10% lower in macroH2A1 KO mice compared to WT littermates (p<0.001) (Figure 1A). Next, we employed a DEXA (Dual Energy X-ray Absorptiometry) analyzer, an effective method in characterizing fat content and body composition, to examine the content of lean and fat masses: macroH2A1 KO displayed a significant lower amount of fat content compared to WT mice (15.58±0.48 gr versus
17.82±1.23 gr, p<0.05), whereas there was no statistical significant difference between the lean mass content in the groups (Figures 1B, 1C). In 90% of cases, human obesity is accompanied by an accumulation of lipid droplets in the hepatic parenchyma, termed NAFLD. NAFLD affects about one third of the overall population, and it is a main risk factor for the development of non-alcoholic steatohepatitis (NASH), characterized not only by increased lipid content, but also by oxidative stress, inflammation and deposition of extracellular matrix (Pinzani and Macias-Barragan, 2010). We thus sought to analyze if the lipid content in the liver of macroH2A1 KO was lower than in WT mice: surprisingly, histological analysis did not reveal evident differences between KO and WT mice, with a comparable mixed

![Figure 1](image-url)

Figure 1. MacroH2A1 KO mice are protected against body weight gain and obesity after HFD. (A) Total body weights were measured. (B) Representative pictures of mice under DEXA scan. (C) Quantification of lean and fat mass as determined by DEXA scan analysis. (D) Plasma Triglyceride (TG) and (E) plasma total cholesterol (TC) concentrations. (F) Fasted glucose was measured before sacrificing. Values shown are means ± SEM (n = 7-8; except body weight, n = 14-15). *, p<0.05, ***, p<0.001, macroH2A1 KO vs. WT.
micro/macro centrolobular steatosis (Figure S1A). Consistently, steatosis grade and NAFLD Activity Score (NAS) score in the centrolobular areas (zone 3 of the Rappaport’s acinus) were similar in macroH2A1 KO and WT mice (Figures S1B, S1C), liver TG and TC content were unaltered (Figures S1D, S1E) as well as the liver weight/body weight ratios (Figure S1F), between the two mice cohorts. Plasma levels of TG and TC were similar in the KO and WT mice under HFD (Figures 1D, 1E). However, livers of 6 out of 15 WT mice showed increased accumulation of lymphocytes in the periportal areas (zone 1 of the Rappaport’s acinus), while this was absent in macroH2A1 KO mice (Figure S2A-B), indicating the existence of periportal inflammation in WT but not in macroH2A1 KO. Circulatory glucose is kept within a tightly regulated range to provide a constant fuel supply for cell metabolism, and obesity notoriously interferes with it, often triggering the development of type 2 diabetes (T2D). Fasting plasma glucose levels were markedly reduced in KO mice upon HFD compared to WT (p<0.05) (Figure 1F). A previous report described slight glucose intolerance in macroH2A1 KO male mice under a chow diet, with higher concentrations of blood glucose in intra-peritoneal glucose tolerance tests (GTT) at all times except time point zero (Changolkar et al., 2007); we were not able to reproduce these findings using an oral GTT (OGTT). To the contrary, upon HFD macroH2A1 KO were largely more glucose tolerant at all times tested, with larger differences observed at 90, 120 and 150 minutes (Figure 2A). This was reflected by a ~20% lower area under the curve (AUC: 0-150 minutes time points) (Figure 2B). Insulin tolerance tests (ITT) showed insulin-mediated time-

![Figure 2. MacroH2A1 KO mice display increased sensitivity to glucose. (A) Oral glucose tolerance test (OGTT) was performed in WT and macroH2A1 KO mice fed a HFD following a 6-h fast. (B) Area under the curve (AUC) for the OGTT. (C) Insulin tolerance test (ITT) performed in WT and macroH2A1 KO mice fed a HFD following a 6-h fast. Values shown are means ± SEM (n = 7-8). *, p<0.05, macroH2A1 KO vs. WT.](image-url)
dependent decreases in glycemia to a similar extent in macroH2A1 KO mice versus WT littermates (Figure 2C). To gain insight into the mechanism by which systemic glucose tolerance is maintained in HFD–fed macroH2A1 KO mice, we characterized insulin-induced AKT signaling in the skeletal muscle, liver and adipose tissues under insulin-stimulated conditions (0.75 U/kg body weight, injected 15 minutes before sacrifice) (Figure 3A–C). Interestingly, AKT phosphorylation (T308/S473) tended to be increased in the skeletal muscle tissues of macroH2A1 KO mice fed a HFD compared to WT controls (Figure 3A). This trend was absent in the liver (Figure 3B) and in the adipose tissues (Figure 3C). Altogether these findings show that the systemic absence of macroH2A1 confers mild protection from HFD-

![Image](Figure 3. Increased glucose clearance is not due to enhanced insulin sensitivity in muscle, liver and adipose tissue. Mice were injected with insulin (0.75 U/kg) 15 minutes prior to sacrifice, after which phosphorylation status of AKT (T308/S473) was determined by western blot. Representative immunoblots are shown in (A) skeletal muscle and (B) liver and (C) adipose tissue. Immunoblots were quantified by densitometry and normalized against total protein levels of AKT. Actin was used as loading control. Data are expressed as means ± SEM, N=6-7 per group.)
induced obesity as it protects from diet-induced weight gain. Although macroH2A1 KO are not protected against HFD-induced NAFLD, the total absence of inflammatory infiltrates in the periportal areas suggests that the presence of macroH2A1 gene could have a pro-inflammatory effect in the progression of NAFLD. Regarding glucose metabolism, macroH2A1 KO mice exhibited decreased fasted glucose plasma levels and mice remained sensitive to a glucose challenge when fed an obesogenic HFD.

Food intake, heat production and respiratory exchange ratio (RER) in macroH2A1 KO mice

Using metabolic cages we ascertained if the observed difference in body weight in macroH2A1 KO mice versus WT littermates under a HFD could be due to decreased food intake and/or increased heat production. As shown in Figure 4A and 4C, under a standard, chow diet, macroH2A1 KO mice show a tendency towards a lower food intake and faeces production. When fed a HFD for 12 weeks, this trend was no more evident (Figure 4B and 4D). Heat production was found slightly lower in macroH2A1 KO only during the day (p<0.05) and under a chow diet (Figure 5A). These differences were not anymore evident upon feeding with a HFD (Figure 5B). The respiratory exchange ratio (RER) is the ratio between the amount of CO2 produced and O2 consumed by breathing. Measuring this ratio can be used for estimating which fuel (carbohydrate or fat) is being metabolized to supply the body with energy. RER was unaltered in macroH2A1 KO compared to WT mice regardless the time of the day or the diet administered (chow or HFD) (data not shown). These negative data suggest that, despite some minor differences in food intake and heat production when fed a chow diet, macroH2A1 KO mice fed an obesogenic HFD diet display similar basal metabolic parameters compared to their WT littermates.

MacroH2A1 KO mice have enhanced total activity during night time under a chow diet, and during day time under HFD

Recent studies have pointed out the strong relationship between energy homeostasis and circadian activities at the molecular, behavioural and physiological level (Bass and Takahashi, 2010). Experiments on rodents have shown that HFD affects total circadian activities as well as energy metabolism (Froy, 2012) and alters eating behaviour. These patterns were observed because calorie intake during inactive period (day time) increased when eating a HFD more than when eating a chow diet. Here, we measured total activity of macroH2A1 KO and WT mice under a chow diet or HFD during day and night time (12-h light/12-h dark). As shown in Figure 5C, total activity was significantly increased (p<0.001) in macroH2A1 KO mice under chow diet compared to WT littermates only during
night time, which is the active period. Under HFD, macroH2A1 KO similarly displayed a slightly higher level of total activity, this time observable only during day time, which is the inactive period. These data suggest that i) the systemic absence of macroH2A1 might trigger epigenetically an increase in total activity and ii) a shift in nocturnal mouse activity into day time occurs upon a HFD.

Control of genes involved in energy expenditure in BAT and VAT by macroH2A1 under HFD

An increase in total activity is usually mirrored in an increase in energy expenditure, which might underlie the reason of the increased leanness of macroH2A1 KO mice under HFD. Obesity is a state of excessive accumulation of TG in visceral adipose tissues because of a prolonged positive energy balance. Adipocytes are the major storage site for fat in the form of TG. Histological analyses of visceral white adipose tissues (VAT) from macroH2A1 and WT mice fed a HFD revealed normal size and perimeter of the adipocytes on average upon quantification (Figures 6A-C), despite a decreased amount of total fat mass (Figure 1). The two types of adipose tissue, VAT and brown adipose tissue (BAT) are quite different in their location and physiological functions. VAT is the primary site of energy storage, while BAT is specialized for energy expenditure, and it is important for regulating body temperature: thermogenesis in BAT.

Figure 4. Food intake and faeces production are unaltered in macroH2A1 KO mice. Food intake was measured during in vivo metabolic analyses in mice fed a (A) chow diet or (B) high fat diet. Faeces production were measured in mice fed a (C) chow diet or (D) high fat diet. Values shown are means ± SEM (n = 7-8).
Histone macroH2A1 and diet-induced obesity

is fundamental to energy balance in mice and humans (Whittle et al., 2012). We sought to perform qRT-PCR gene expression studies to obtain a snapshot of the metabolic state of BAT and VAT in macroH2A1 KO mice fed an obesogenic diet. We analyzed a panel of genes involved in BAT-related energy expenditure (Ucp1 and Fgf21), local inflammation (interleukin-6, Il-6), mitochondrial oxidative capacity (Cox4), overall adipogenesis (Pparγ and Fabp4), and insulin-dependent glucose uptake capacity (Glut4). In BAT from macroH2A1 mice under a HFD, we detected a significant increase in Ucp1, Glut4 and Cox4 mRNA levels compared to WT mice (Figure 6D). These changes could be indicative of enhanced thermogenesis in BAT. In VAT the mRNA levels of the markers of adipogenesis Pparγ, Glut4 and Fabp4 were lower in the KO animals (Figure 6E), indicative of lowered adipogenic process. In conclusion, these data suggest that when fed an obesogenic HFD diet, macroH2A1 KO mice develop enhanced thermogenic gene expression in BAT and impaired pro-adipogenic gene expression in VAT.

Discussion

MacroH2A1 is a variant of histone H2A whose transcriptional activity is implicated mechanistically in vitro in hepatocyte lipid accumulation,
which accompanies 90% of the cases of obesity, and in vivo in cell stemness and tumorigenesis (Barrero et al., 2013; Boulard et al., 2010; Cantarino et al., 2013; Changolkar et al., 2007; Changolkar et al., 2010; Creppe et al., 2012; Gaspar-Maia et al., 2013; Pogribny et al., 2009; Rappa et al., 2013). MacroH2A1 KO mice have been generated by two independent groups and they both reported mild metabolic effects (Changolkar et al., 2007; Changolkar et al., 2010). To understand if macroH2A1 could have systemic effects on fat accumulation and obesity, we challenged macroH2A1 KO mice with a HFD. We surprisingly found that whole body macroH2A1 KO mice take on less fat mass than their WT littermates, without significant variations in the lean mass. Although the ablation of macroH2A1 did not alter HFD-induced lipid accumulation in comparison to WT mice, histological analysis showed perportal inflammation in 6 out of 15 of the WT mice analyzed, while this was completely absent in macroH2A1 KO mice. Migration of lymphocytes towards the perportal area is very common in NAFLD spectrum (Bigorgne...
et al., 2008). During NASH, periportal infiltration can then extend to the all the lobule (Desmet, 2004; Tilg and Moschen, 2010). Livers from HF-fed WT mice showed increased accumulation of lymphocytes around the periportal areas but not the centrolobular areas, while all HF-fed macroH2A1 KO mice were protected (Figure S1 and S2). MacroH2A1 has been reported to modulate NF-kB activity in reconstituted nucleosomes (Angelov et al., 2003), and to regulate IL-8 production in B cells (Agelopoulos and Thanos, 2006). The mechanism by which the absence of macroH2A1 blocks the formation of inflammatory infiltrates warrants further investigation. The resistance of macroH2A1 KO mice to HFD was accompanied by decreased fasting glucose levels, an increased tolerance for glucose by OGTT, and a tendency for increased insulin sensitivity in the skeletal muscle, without enhanced insulin sensitivity in liver or skeletal muscle. Increased glucose tolerance occurred despite the development of NAFLD in the KO mice. Although NAFLD is a strong and independent predictor for the development of type 2 diabetes, the link between insulin resistance and NAFLD has not always been demonstrated and there are a number of studies reporting dissociation of NAFLD from insulin resistance in genetic mouse models and in patients (Aparicio-Vergara et al., 2013; Sheedfar et al., 2013; Sun and Lazar, 2013). Our results diverge from those of Changolkar et al. (Changolkar et al., 2007; Changolkar et al., 2010), reporting glucose intolerance in male mice fed a chow diet. It must be noted that we used OGTT, while this previous report used intra-peritoneal GTT. OGTT represents the most physiological route of entry of glucose, and it has been shown to be more sensitive than intra-peritoneal GTT to detect glucose tolerance (Andrikopoulos et al., 2008); in addition, our mice were fed an obesogenic HFD diet. Moreover, a functional link between glucose intolerance and increased expression of lipogenic genes (Lpl, Serpina7, CD36), despite the absence of steatosis, was proposed in the liver of macroH2A1 KO mice (Changolkar et al., 2007; Changolkar et al., 2010). This is surprising, since 80–90% of the infused glucose is uptaken by the skeletal muscle (Ferrannini et al., 1988), rather than the liver, consistent with the phenotype we observed in our fat model. Food intake and heat production tended to be lower in macroH2A1 KO fed a chow diet, differences that were mitigated in presence of a HFD. Interestingly, macroH2A1 KO mice have increased total activity and display a shift in nocturnal activity into day time upon a HFD. HFD is known to affect total circadian activities as well as energy expenditure (Froy, 2012), because calorie intake during inactive period increased when eating HFD. This phenotype is reminiscent of the recently proposed “work for food” paradigm that occurs in mice when reduced food intake shifts the activity phase from night time into day time and eventually causes nocturnal hypothermia (Hut
et al., 2011), i.e. decreased heat production during night time. Whereas food restriction and HFD are extreme nutritional scenarios, we hypothesize that systemic genetic depletion of macroH2A1 might lead to adaptive flexibility in circadian organization of the behavioural timing, allowing mice to exploit the diurnal temporal niche. The functional or evolutionary advantages of these adaptive phenomena require further study, but establish a new unappreciated epigenetic link between metabolism and circadian rhythms (cycles iterating with a period of 24±4 hours). The biological clocks are hardwired at the cellular level by molecular oscillators working through transcriptional/translational feedback loops operated by genes and proteins fluctuating rhythmically with circadian pattern. These clock drive the temporal variations of expression of genes encoding proteins involved in lipid and glucose metabolism (biosynthesis, transport, binding, and lysis) (Mazzoccoli et al., 2012; Mazzoccoli et al., 2014). The expression of a huge number of these genes is influenced by the presence of macroH2A1 variants (Changolkar et al., 2007; Pazienza et al., 2014) and shows an evident circadian rhythmicity of variation, with the exception of Apoa1, which is characterized by ultradian rhythmicity (period < 20 hours) (Supplementary Table 1). Despite the gross analysis of energy expenditure did not indicate major alterations in macroH2A1 KO mice, the assessment of gene expression in BAT revealed coordinated induction of marker genes of enhanced mitochondrial thermogenesis and glucose consumption, consistently with enhanced BAT-mediated energy expenditure in these mice. The parallel reduction in the expression of genes related to adipose accretion in VAT mirrors a scenario in which metabolic fuel is preferentially driven to energy consuming processes (BAT) at the expense of fat deposition (VAT). The reduced fat content in KO mice would be the result of such alterations. Possibly, the mild magnitude of the process (differential accumulation of % fat in macroH2A1 KOs versus WT mice across weeks of HF diet) explains why gross measurements of energy expenditure did not allow detecting some minor, but persistent, enhancement in energy expenditure as pointed out by molecular markers in BAT. Considering that promotion of BAT-mediated energy expenditure is emerging as a potential strategy for protection against obesity and metabolic alterations (hyperglycemia, hyperlipidemia), current data indicates macroH2A1 inhibition as a strategy worth to explore. In this respect, functional partners of the macroH2A1 transcriptional complexes in the adipose tissue are unknown. In cancer cells, macroH2A1 recruits to the promoters of its target genes PELP1, a strong potentiator of retinoid X receptor (RXR) activation (Hussey et al., 2014; Singh et al., 2006). In the adipose tissue, RXR activation is crucial for reprogramming towards energy storage or thermogenesis (Imai et al.,
Histone macroH2A1 and diet-induced obesity

Moreover, adipose tissue is at the nexus of mechanisms involved in life span and age-related metabolic dysfunction (Tchkonia et al., 2010). Obesity is associated with accelerated onset of diseases common in old age, and macroH2A1 is also a major driver in the formation of senescent-associated heterochromatin foci, one of the most prominent features of cellular senescence (Tchkonia et al., 2010; Zhang et al., 2005). It is tempting to speculate that this epigenetic player could be at the crossroad of metabolism, energy expenditure and cellular aging.

Acknowledgments

We appreciate Dr. Bastiaan Moesker, Paulina Bartuzi and Dr. Marcela Aparicio-Vergara for their technical helps. FS is supported by a PhD scholarship from the Graduate School for Drug Exploration (GUIDE), University of Groningen. DK and MH are supported by the Center for Translational Molecular Medicine (http://www.ctmm.nl), project PREDICCT (grant 01C-104), and by the Dutch Heart Foundation, Dutch Diabetes Research Foundation and Dutch Kidney Foundation. MV is a recipient of a My First AIRC Grant (MFAG) from Associazione Italiana per la Ricerca sul Cancro, Italy. FC is funded by Euro-Mediterranean Institute of Science and Technology, Palermo, Italy.

Declaration of interest

The authors declare that they have no competing financial interests or other conflicts of interest.
References


Supplemental figures

Figure S1. Genetic ablation of macroH2A1 does not affect hepatic steatosis. (A) Representative H&E pictures of liver sections from HFD fed mice. (B) Pathological score for steatosis (microvesicular and macrovesicular) grade 0 < 5%; 1=5-33%; 2=33-66%; 3= > 66% grade around the peri-centrolobular vein. (C) NAS = NAFLD activity Score (sum of steatosis + lobular inflammation + ballooning); score >=5 SH; Score <3 NSH. Quantitative measurement of (D) hepatic TG levels and (E) TC levels. (F) fasted liver ratio over % body weight were measured. Data are expressed as means ± SEM, (n = 14-15, except liver TG and TC, n = 7-8).

Figure S2. Genetic ablation of macroH2A1 protects against portal inflammation. (A) Representative pictures of H&E stainings from periportal area of HF-fed mice and (B) pathological portal inflammation score. Stainings were categorized as 0 (no lymphocyte) and 1 (lymphocyte). The arrow shows accumulation of lymphocytes around the branch of the portal vein present in the periportal area. Data are expressed as means ± SEM, (n = 14-15). *p<0.05, macroH2A1 KO vs. WT.
### Supplemental Tables

**Supplementary Table 1. Genes whose expression is influenced by manipulation of macroH2A1 expression in vitro and/or in vivo**

<table>
<thead>
<tr>
<th>Official Symbol</th>
<th>Official Full Name</th>
<th>Rhythmicity</th>
<th>Period (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette, sub-family A (ABC1), member 1</td>
<td>Circadian</td>
<td>20</td>
</tr>
<tr>
<td>ACACA</td>
<td>acetyl-CoA carboxylase alpha</td>
<td>Circadian</td>
<td>28</td>
</tr>
<tr>
<td>ACADL</td>
<td>acyl-CoA dehydrogenase, long chain</td>
<td>Circadian</td>
<td>21</td>
</tr>
<tr>
<td>ACLY</td>
<td>ATP citrate lyase</td>
<td>Circadian</td>
<td>28</td>
</tr>
<tr>
<td>ACOX1</td>
<td>acyl-CoA oxidase 1, palmitoyl</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>ACSL5</td>
<td>acyl-CoA synthetase long-chain family member 5</td>
<td>Circadian</td>
<td>25</td>
</tr>
<tr>
<td>ACSM3</td>
<td>acyl-CoA synthetase medium-chain family member 3</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>ADIPOR1</td>
<td>adiponectin receptor 1</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>ADIPOR2</td>
<td>adiponectin receptor 1</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>AKT1</td>
<td>v-akt murine thymoma viral oncogene homolog 1</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>APOA1</td>
<td>apolipoprotein A-I</td>
<td>Ultradian</td>
<td>8</td>
</tr>
<tr>
<td>APOB</td>
<td>apolipoprotein B</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>APOC3</td>
<td>apolipoprotein C-III</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
<td>Circadian</td>
<td>28</td>
</tr>
<tr>
<td>APOE</td>
<td>apolipoprotein E</td>
<td>Circadian</td>
<td>28</td>
</tr>
<tr>
<td>ATP5C1</td>
<td>ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>ATP11a</td>
<td>ATPase, class VI, type 11A</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>CASP3</td>
<td>caspase 3, apoptosis-related cysteine peptidase</td>
<td>Circadian</td>
<td>28</td>
</tr>
<tr>
<td>CD36</td>
<td>CD36 molecule (thrombospondin receptor)</td>
<td>Circadian</td>
<td>23</td>
</tr>
<tr>
<td>CEBPB</td>
<td>CCAAT/enhancer binding protein (C/EBP), beta</td>
<td>Circadian</td>
<td>28</td>
</tr>
<tr>
<td>CNBP</td>
<td>CCHC-type zinc finger, nucleic acid binding protein</td>
<td>Circadian</td>
<td>25</td>
</tr>
<tr>
<td>COX4</td>
<td>cytochrome c oxidase subunit IV</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>CPT1A</td>
<td>carnitine palmitoyltransferase 1A (liver)</td>
<td>Circadian</td>
<td>21</td>
</tr>
<tr>
<td>CPT2</td>
<td>carnitine palmitoyltransferase 2</td>
<td>Circadian</td>
<td>28</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>cytochrome P450, family 2, subfamily E, polypeptide 1</td>
<td>Circadian</td>
<td>22</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>cytochrome P450, family 7, subfamily A, polypeptide 1</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>DGAT2</td>
<td>diacylglycerol O-acyltransferase 2</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>FABP1</td>
<td>fatty acid binding protein 1, liver</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>FABP3</td>
<td>fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)</td>
<td>Circadian</td>
<td>28</td>
</tr>
<tr>
<td>FABP4</td>
<td>fatty acid binding protein 4, adipocyte</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>FABP5</td>
<td>fatty acid binding protein 5 (psoriasis-associated)</td>
<td>Circadian</td>
<td>28</td>
</tr>
<tr>
<td>Gene/Protein Name</td>
<td>Description</td>
<td>Circadian/ Ultradian</td>
<td>Value</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>--------------------------------------------------</td>
<td>----------------------</td>
<td>-------</td>
</tr>
<tr>
<td>FAS (TNFRSF6)</td>
<td>Fas (TNF receptor superfamily member 6)</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>FASN</td>
<td>fatty acid synthase</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>FGF21</td>
<td>fibroblast growth factor 21</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>FOXA2 (HNF3B)</td>
<td>forkhead box A2</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>FOXO1</td>
<td>forkhead box O1</td>
<td>Circadian</td>
<td>20</td>
</tr>
<tr>
<td>G6PC</td>
<td>glucose-6-phosphatase, catalytic subunit</td>
<td>Circadian</td>
<td>27</td>
</tr>
<tr>
<td>G6PD</td>
<td>glucose-6-phosphate dehydrogenase</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>GCK</td>
<td>glucokinase (hexokinase 4)</td>
<td>Circadian</td>
<td>23</td>
</tr>
<tr>
<td>GSK3B</td>
<td>glycogen synthase kinase 3 beta</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>GTPBP4</td>
<td>GTP binding protein 4</td>
<td>Ultradian</td>
<td>13</td>
</tr>
<tr>
<td>HMGCR</td>
<td>3-hydroxy-3-methylglutaryl-CoA reductase</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>HNF4A</td>
<td>hepatocyte nuclear factor 4, alpha</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>IFNG</td>
<td>interferon, gamma</td>
<td>Circadian</td>
<td>22</td>
</tr>
<tr>
<td>IGF1</td>
<td>insulin-like growth factor 1 (somatomedin C)</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>IGFBP1</td>
<td>insulin-like growth factor binding protein 1</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>IL1β</td>
<td>interleukin 1, beta</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>IL6</td>
<td>interleukin 6</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>IL10</td>
<td>interleukin 10</td>
<td>Circadian</td>
<td>28</td>
</tr>
<tr>
<td>INSR</td>
<td>insulin receptor</td>
<td>Circadian</td>
<td>25</td>
</tr>
<tr>
<td>IRS1</td>
<td>insulin receptor substrate 1</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>KRT1-23</td>
<td>keratin complex 1, acidic, gene 23</td>
<td>Ultradian</td>
<td>16</td>
</tr>
<tr>
<td>LDLR</td>
<td>low density lipoprotein receptor</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>LEPR</td>
<td>leptin receptor</td>
<td>Circadian</td>
<td>27</td>
</tr>
<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>MAPK1 (ERK2)</td>
<td>mitogen-activated protein kinase 1</td>
<td>Circadian</td>
<td>18</td>
</tr>
<tr>
<td>MLXIPL</td>
<td>MLX interacting protein-like</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>MTOR</td>
<td>mechanistic target of rapamycin (serine/threonine kinase)</td>
<td>Circadian</td>
<td>23</td>
</tr>
<tr>
<td>NDUFB6</td>
<td>NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6, 17kDa</td>
<td>Circadian</td>
<td>23</td>
</tr>
<tr>
<td>NFKB1</td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B-cells 1</td>
<td>Circadian</td>
<td>22</td>
</tr>
<tr>
<td>NR1H2</td>
<td>nuclear receptor subfamily 1, group H, member 2</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>NR1H3</td>
<td>nuclear receptor subfamily 1, group H, member 3</td>
<td>Circadian</td>
<td>26</td>
</tr>
<tr>
<td>NR1H4</td>
<td>nuclear receptor subfamily 1, group H, member 4</td>
<td>Circadian</td>
<td>25</td>
</tr>
<tr>
<td>PCK2</td>
<td>phosphoenolpyruvate carboxykinase 2 (mitochondrial)</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>PDK4</td>
<td>pyruvate dehydrogenase kinase, isozyme 4</td>
<td>Circadian</td>
<td>24</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Circadian</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------------------------------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>PIK3CA</td>
<td>phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>PIK3R1</td>
<td>phosphoinositide-3-kinase, regulatory subunit 1 (alpha)</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>PKLR</td>
<td>pyruvate kinase, liver and RBC</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>PNPLA3</td>
<td>patatin-like phospholipase domain containing 3</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>PPA1</td>
<td>pyrophosphatase (inorganic) 1</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>PPARA</td>
<td>peroxisome proliferator-activated receptor alpha</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>PPARG</td>
<td>peroxisome proliferator activated receptor gamma</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>PPARGC1A</td>
<td>peroxisome proliferative activated receptor, gamma, coactivator 1 alpha</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>PRKAA1</td>
<td>protein kinase, AMP-activated, alpha 1 catalytic subunit</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>PTPN1</td>
<td>protein tyrosine phosphatase, non-receptor type 12</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>RBP4</td>
<td>retinol binding protein 4, plasma</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>RXRA</td>
<td>retinoid X receptor alpha</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>SCD2</td>
<td>stearoyl-CoA desaturase (delta-9-desaturase)</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>SERPINE1</td>
<td>serine (or cysteine) peptidase inhibitor, clade E, member 1</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>SERPINA7</td>
<td>serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>SLC27A5</td>
<td>solute carrier family 27 (fatty acid transporter), member 5</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>SLC2A1</td>
<td>solute carrier family 2 (facilitated glucose transporter), member 1</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>SLC2A2</td>
<td>solute carrier family 2 (facilitated glucose transporter), member 2</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>SLC2A4</td>
<td>solute carrier family 2 (facilitated glucose transporter), member 4</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>SOCS3</td>
<td>suppressor of cytokine signaling 3</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>SREBF1</td>
<td>sterol regulatory element binding transcription factor 1</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>SREBF2</td>
<td>sterol regulatory element binding transcription factor 2</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>SUCNR1</td>
<td>succinate receptor 1</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>THRSP</td>
<td>thyroid hormone responsive</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>UCP1</td>
<td>uncoupling protein 1 (mitochondrial, proton carrier)</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>XBP1</td>
<td>X-box binding protein 1</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

Source articles: PMID: 24473773, PMID: 17242180.

CIRCA-DB: https://www.bioinf.itmat.upenn.edu/circa