SIRT1 overexpression attenuates offspring metabolic and liver disorders due to maternal high-fat feeding

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Running title: SIRT1 attenuates maternal obesity-induced fetal metabolic programming

Key words: obesity, sirtuin, developmental programming, metabolism, liver

Key points

- *Maternal high-fat diet (MHF) consumption led to metabolic and liver disorders in male offspring, which are associated with reduced SIRT1 expression and activity in the offspring liver*
- *SIRT1 overexpression in MHF offspring reduced their body weight and adiposity and normalised lipid metabolic markers in epididymal and retroperitoneal adipose tissues*
- *SIRT1 overexpression in MHF offspring improved glucose tolerance as well as systemic and hepatic insulin sensitivity*
- *SIRT1 overexpression ameliorated MHF-induced lipogenesis, oxidative stress and fibrogenesis in the offspring's liver.*

ABSTRACT

It is well-established that maternal obesity can increase the risk of metabolic disorders in offspring. However, the underlying mechanism is not clearly understood. Previous evidence implied that sirtuin (SIRT)1, a potent regulator of energy metabolism and stress responses, may play an important role. Here we have shown in C57BL/6 mice that maternal high-fat diet (HFD) consumption can induce a pre-diabetic and non-alcoholic fatty liver disease phenotype in the offspring, associated with reduced SIRT1 expression in the hypothalamus, white adipose tissues (WAT) and liver. Importantly, the overexpression of SIRT1 in these offspring significantly attenuated the excessive accumulation of epididymal (Epi) white adipose tissue (WAT) and retroperitoneal (Rp) WAT ($P<0.001$), glucose intolerance and insulin resistance (both $P<0.05$) at weaning age. These changes were associated with the suppression of peroxisome proliferator-activated receptor gamma (PPAR)γ ($P<0.01$), PPARγ-coactivator (PGC-1α, $P<0.05$) and sterol regulatory element-binding protein (SREBP)-1c in EpiWAT ($P<0.01$), but increased expression of PPARγ in RpWAT ($P<0.05$). In the liver, PPARγ mRNA expression, as well as Akt protein expression and activity were increased ($P<0.05$), whereas fatty acid synthase and carbohydrate response element binding protein (ChREBP) were downregulated ($P<0.05$), supporting increased insulin sensitivity and reduced lipogenesis in the liver. In addition, hepatic expression of endogenous antioxidants including glutathione peroxidase 1 and catalase was increased ($P<0.01$ and $P<0.05$ respectively), whereas collagen and fibronectin deposition was suppressed ($P<0.01$). Collectively, the study provides direct evidence of the mechanistic significance of SIRT1 in maternal HFD-induced metabolic dysfunction in offspring and suggest that SIRT1 is a promising target for fetal reprogramming.

INTRODUCTION

The term of developmental origin of health and diseases (DOHaD) or fetal programming refers to the effects of parental lifestyle and health conditions on subsequent generations (Armitage *et al.*, 2008). Maternal obesity and high-fat diet (HFD) consumption in particular have been shown to predispose offspring to obesity and related metabolic disorders (Howie *et al.*, 2009; Oben *et al.*, 2010; Glastras *et al.*, 2015; Saben *et al.*, 2016). Importantly, clinical trials of lifestyle intervention during pregnancy only marginally improve postnatal outcomes, partially due to poor compliance and late engagement in these programs (Catalano, 2015). Clearly understanding all contributing factors and transmission pathways in maternal obesity-related DOHaD is crucial to developing therapeutic strategies. Recent studies suggest that maternal obesity during pregnancy can cause the transmission of excessive nutrients to the fetus, which further induces oxidative stress and inflammatory responses, leading to epigenetic modifications in fetal tissues to reprogram energy metabolism (Heerwagen *et al.*, 2010).

A particular protein has been suggested to play a central role in this multidimensional mechanism, namely Sirtuin (SIRT)1 (Nguyen *et al.*, 2016). As a histone deacetylase, SIRT1 has a direct regulatory effect on chromatin tertiary structure, thus plays an important role in DNA repair, replication, as well as genetic and epigenetic regulation. SIRT1 activity strictly depends on the cellular availability of NAD^+, which fluctuates according to circadian rhythm and energy status (Houtkooper *et al.*, 2010). These characteristics make SIRT1 a potent regulator of lifespan, metabolism and stress responses (Haigis & Sinclair, 2010; Houtkooper *et al.*, 2012). Both aging and obesity negatively affect SIRT1 expression and activity (Pedersen *et al.*, 2008; dos Santos Costa *et al.*, 2010; Mariani *et al.*, 2015). Conversely, overexpression of SIRT1 mimics caloric restriction (Wood *et al.*, 2004), leading to longer lifespan and resistance to obesity-related disorders in various animal models (Wood *et al.*, 2004; Milne *et al.*, 2007; Pfluger *et al.*, 2008). With respect to DOHaD, several reports have described reduced SIRT1 expression/activity in the fetus and neonatal tissues due to maternal HFD feeding (MHF) (Suter *et al.*, 2012; Nguyen *et al.*, 2017).

In this study, we induced a systemic overexpression of SIRT1 in the offspring to confirm the causative role of SIRT1 regulation in maternal obesity-related DOHaD. The study suggests that SIRT1 is a promising target for fetal reprogramming of metabolic disorders due to maternal obesity.

METHODS

Animals

The study was approved by the Animal Care and Ethics Committee of the University of Sydney (RESP/15/22). All methods were performed in accordance with the relevant guidelines and regulations

in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Eight-week old female wild-type C57BL/6 mice (WT) were fed a HFD (20 kJ/g, 43.5% calorie as fat, Specialty Feed, WA, Australia) (MHF, n = 21) or standard rodent chow (11 kJ/g, 14% calorie as fat, Gordon's Speciality Stockfeeds, NSW, Australia) (MC, n = 13) for 6 weeks before mating, throughout gestation and lactation (Glastras *et al.*, 2015). To test the hypothesis that SIRT1 expression in the offspring per se can attenuate maternal obesity-induced metabolic programming, these female mice were mated with hemizygous transgenic sires (Tg) to produce both WT and Tg offspring without maternal genotypic modification (resulting in the following offspring groups: MC-WT n=21, MC-Tg n=8, MHF-WT, n=26, MHF-Tg n=11). The strain of male breeders was B6.Cg-Col1a1$^{tm1(CAG-Sirt1)Dsin}$/Mmjax, derived from C57BL/6. The original Tg colony was a generous gift from Dr. Lindsay Wu (University of New South Wales, Australia).

As we have previously shown sex-specific downregulation of SIRT1 by MHF in male offspring (Nguyen *et al.*, 2017), only male offspring were examined in the current study. To limit the difference in milk competition between the litters (Chen *et al.*, 2008), the newborn litters were adjusted to 4-6 pups/litter. Because HFD-fed dams tend to cannibalize their pups, the number of pups per litter was subject to how many male mice survived until weaning and how many of these were genotyped to be wild-type and transgenic. The proportion of Tg offspring was roughly 30% of the litter size and there was typically 1 Tg male mouse per litter. The offspring were genotyped at postnatal (P)14 in accordance to the Jackson Laboratory Genotyping Protocol for B6.Cg-Col1a1$^{tm1(CAG-Sirt1)Dsin}$/Mmjax strain using crude tail DNA extracted with DirectPCR Lysis Reagent (Mouse Tail) (Viagen Biotech, California, USA).

At weaning (P20), all pups were deeply anaesthetised with 3% isoflurane and euthanised upon cardiac puncture for blood collection after 5h fasting. Phosphate-buffered saline (PBS, 1%) was used for whole body perfusion. Tissues were snap frozen or embedded in Optimal Cutting Temperature (OCT) compound and stored at −80 °C or fixed in Neutral buffered formalin (10%) for approximately 36h for later analyses.

Hypothalamus dissection

The first coronal cut was made at the rostral edge of the hypothalamus, using the sulcus as a guide, at approximately Bregma +0.5 mm. A second coronal cut was made ~3.2 mm caudal to the second cut. This section (approximately Bregma +0.5 mm to -2.7 mm), contains the hypothalamus. It was then placed on its rostral surface and the hypothalamic area dissected by cutting at the level of the

hypothalamic sulcus. The hypothalamus was then removed by cutting just above the top of the third ventricle

Intraperitoneal glucose tolerance test (IPGTT)

At P18, the animals were weighed and fasted for 5h prior to IPGTT (Chen *et al*. 2009), then D-glucose was injected (2g/kg, i.p.). Tail blood glucose level was recorded prior to glucose injection, then at 15, 30, 60 and 90min post injection using a glucometer (Accu-Chek® glucose meter; Roche Diagnostics). The area under the curve (AUC) was calculated for each animal. Quantitative Insulin Sensitivity Check Index (QUICKI) was calculated as previously published (Chen *et al.*, 2005; Cacho *et al.*, 2008)

Milk intake estimation

At P19, offspring were separated from their dams and fasted for 5h. They were weighed before returning to their dams. After 2h of feeding, the offspring were weighed again. The difference in the body weight during the 2h period was used for milk intake estimation. No access to solid food was provided during the lactation phase to ensure no Chow/HFD consumption by the pups. The same method has been described previously (Jara-Almonte & White, 1972; Del Prado *et al.*, 1997).

Protein and lipid extraction from tissues

The tissues were homogenized in Triton X-100 lysis buffer (pH 7.4, 150 mM NaOH, 50 mM Tris-HCl, 1% Triton X-100, Roche protease inhibitor) using TissueRuptor (Qiagen, Hilden, Germany). Lipid and protein were extracted and quantified according to our previously published protocols (Nguyen *et al.*, 2017) using Roche triglyceride reagent GPO-PAP (Roche Life Science, NSW, Australia) and Pierce BCA Protein Assay Kit (Thermo Scientific, VIC, Australia) according to the manufacturer's instructions. Lipid concentrations were normalised to the weight of tissue homogenized. Protein concentrations were standardised to 5 μg/μl.

Quantitative RT-PCR

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines was followed. Total RNA was extracted from liver tissues using RNeasy Plus Mini Kit (Qiagen Pty Ltd, CA, USA) according to the manufacturer's instructions, while RNA in the fat tissues and hypothalamus was extracted using Trizol Reagent (Sigma-Aldrich). The purified total RNA was used as a template to generate cDNA using the First Strand cDNA Synthesis Kit (Roche Life Science, NSW, Australia). The amplicons of target genes were amplified with SYBR Green probes. Primer sequences have been published in our previous study (Nguyen *et al.*, 2018), and summarised in Table 1. Before acquiring the actual data, all the new primers were tested for amplification efficiency (90 –

110%) and specificity (single peak in dissociation curve analysis). The final concentration for all primers in a qPCR reaction was 200nM. Several commonly used housekeeping genes in the literature including 18s, α-tubulin, and β-actin were tested. β–actin showed the least variation in mRNA expression among the groups. Therefore, gene expression was standardized to β-actin mRNA and log-transformed.

Immunoblotting

The same amount of protein (20 μg) was electrophoresed and electroblotted onto the Hybond nitrocellulose membrane (Amersham Pharmacia Biotech, Amersham, UK), which was then incubated with a primary antibody at 4°C overnight. Antibodies information and applied concentrations were described in Table 2. Two commonly used housekeeping genes in the literature including β-actin and GAPDH were tested for loading normalisation in western blot. GAPDH showed less variation in expression among groups compared to β-actin, hence was selected in this study. Subsequently, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibodies. The immunoblots were developed by adding the Luminata Western HRP Substrates (Millipore, MA, USA) to the membrane and imaged using ImageQuant LAS 4000 (Fujifilm, Tokyo, Japan). ImageJ (National Institutes of Health, USA) was used for densitometric analyses.

SIRT1 activity assay

Nuclear protein was extracted according to the protocol 'Nuclear protein extraction without the use of detergent' (Sigma-Aldrich, Dublin, Ireland) using a hypotonic lysis buffer (10 mM HEPES, pH 7.9, with 1.5 mM MgCl2 and 10 mM KCl, 1 mM DTT), followed by centrifuge to separate nuclear and cytoplasmic fractions. The nuclear proteins were extracted using extraction buffer (20 mM HEPES, pH 7.9, with 1.5 mM MgCl2, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) Glycerol, 1 mM DTT). No protease inhibitor was used in the extraction to avoid interference with SIRT1 activity measurement. SIRT1 activity was then measured using the SIRT1 activity assay kit (Abcam, Cambridge, UK) as per manufacturer's instruction.

Tissue morphology

Tissues were fixed in 10% formalin for 36-h and embedded in paraffin or frozen-embedded in OCT solution (Tissue-Tek). Paraffin sections and frozen sections were prepared at 4 μm and 12 μm thickness respectively and mounted on microscope slides (Trajan Scientific and Medical, VIC, Australia).

The sections were stained with hematoxylin and eosin (H&E) for general morphology. For adipocyte frequency analysis, H&E-stained sections were analysed using a bright-field microscope (Leica Microsystems, Germany) and 6 random non-overlapping fields were captured at 200X magnification. All slides were randomly coded by an independent person before analysis. Adipocyte size analysis was done by Adiposoft software (Galarraga *et al.*, 2012). For collagen staining, paraffin sections were stained with Fast Green for 30 mins followed by incubation in Picro-Sirius Red (PSR) for another 30 mins. Cytoplasm was stained in orange or light red while collagen was stained in dark red. For lipid droplet visualisation, both H&E and Oil Red O (ORO) staining was used. In ORO staining, frozen tissues were sectioned at 12μm thickness and stained with ORO (Sigma-Aldrich, MO, USA) for 15 mins.

Immunohistochemistry (IHC)

IHC staining was performed as previously described (Nguyen *et al.*, 2017). Antigen-retrieval was performed at 99 °C for 20 min in 0.01M, pH 6.0 citric buffer. Endogenous peroxidase was deactivated with 3% H_2O_2 (Sigma-Aldrich, Dublin, Ireland). The liver slices were then blocked (Protein Block Serum-Free, Dako, Glostrup, Denmark), and incubated with primary antibodies overnight at 4°C, and then biotinylated with secondary anti-rabbit IgG antibodies (Dako) and horseradish peroxidase (HRP)-conjugated streptavidin (Dako). All slides were randomly coded and then assessed by two independent investigators. Using bright field microscopy, 6 consecutive non-overlapping fields from each liver section were photographed under high magnification (x200). Image J (National Institutes of Health, USA) was used for estimation of the specific stained area. Briefly, colour deconvolution was performed on the image and the DAB channel was selected for analysis. The same threshold was applied to all images, above which positive staining area was measured. The whole process was automated using Image J's Macro function. IHC score was determined by the stained area.

Statistical analysis

The data were analysed using two-way ANOVA (two factors: maternal diet and offspring genotype) and planned pairwise comparisons (MC-WT vs MC-Tg, MC-WT vs MHF-WT, and MHF-WT vs MHF-Tg), which were performed independently of ANOVA results. Because the former two comparisons were to confirm previous findings in the literature, and only the comparison between MHF-WT and MHF-Tg addresses the hypothesis, no correction for multiple comparison was performed. Data are presented as mean ± standard deviation (SD) unless otherwise specified. $P < 0.05$ was considered as significant.

RESULTS

SIRT1 overexpression reduces body weight, fat and liver mass but not hyperlipidaemia in MHF offspring

Consistent with our previous studies, body weight was significantly increased in the MHF-WT offspring compared to MC-WT offspring ($P < 0.001$, Table 3). The net weight and percentage of body weight of epididymal (Epi) and retroperitoneal (Rp) white adipose tissue (WAT) were also significantly higher in MHF-WT group ($P < 0.001$, Table 3). Net liver weight was also increased ($P < 0.001$) proportionally to the body weight. Plasma levels of non-esterified fatty acid (NEFA) were elevated to a small extent due to maternal HFD consumption (2-way ANOVA, $P_{MHF} < 0.05$). Similarly, plasma triglyceride levels were increased in MHF-WT offspring compared to MC-WT group ($P < 0.01$).

MHF-Tg offspring had a significantly lower body weight in comparison to MHF-WT offspring ($P < 0.001$, Table 3). The net weight and percentage of body weight of EpiWAT and RpWAT in these offspring were also significantly lower than in MHF-WT offspring ($P < 0.001$, $P < 0.05$, $P < 0.01$ and $P < 0.01$ respectively). Liver weight (net and %) was also significantly lower in Tg offspring born to either chow- or HFD-fed dams (all $P < 0.05$). Despite reduced adiposity, no changes were found in NEFA and triglyceride levels due to SIRT1 overexpression in the offspring (Table 3).

SIRT1 overexpression does not change milk intake in MHF offspring

As shown in Figure 1A and 1B, MHF-WT offspring had a higher milk intake ($P < 0.001$ vs MC-WT) and plasma leptin level ($P < 0.01$ vs MC-WT) in comparison to the control group. Concomitantly, there were significant reductions of hypothalamic mRNA expression of SIRT1 ($P < 0.001$, figure 1C) and leptin receptor (Ob-Rb) ($P < 0.05$ vs MC-WT, Figure 1D). In addition, mRNA expression of the orexigenic neuropeptide Y (NPY) was significantly decreased ($P < 0.05$) in MHF-WT animals, likely due to the appetite suppressing effect of leptin. Maternal HFD consumption did not affect mRNA expression of signal transducer and activator of transcription (STAT)3, a downstream marker in the leptin signalling pathway, as well as neuropeptide Y 1 receptor (NPY-1R), pro-opiomelanocortin (POMC) and melanocortin 4 receptor (MC4R).

Plasma levels of leptin were significantly reduced in MHF-Tg offspring ($P < 0.05$, Figure 1B). However, despite the trend to normalised mRNA expression of leptin receptor ($P = 0.06$) and MC4R (2-way ANOVA, $P_{SIRT1\text{-}Tg} < 0.05$), no reduction in milk intake was detected (Figure 1A). STAT3 was elevated by SIRT1 overexpression in MC offspring only (MC-WT vs MC-Tg, $P < 0.05$, Figure 1D). Similarly, there were no differences between groups in the mRNA expression of NPY1, NPY1R, and POMC.

SIRT1 overexpression increases glucose tolerance and insulin sensitivity in MHF offspring

As expected, MHF-WT offspring showed significantly higher levels of blood glucose during IPGTT ($P < 0.01$ vs MC-WT, Figure 2A), suggesting glucose intolerance. This was associated with hyperinsulinaemia ($P < 0.05$) and significant reduction of QUICKI ($P < 0.05$, Figure 2B), indicating impaired insulin sensitivity in the offspring due to maternal HFD consumption. SIRT1 overexpression prevented glucose intolerance ($P < 0.01$ vs MC-WT, Figure 1A), and normalised plasma levels of insulin as well as QUICKI.

In association with the reduction of systemic insulin sensitivity in MHF-WT offspring, mRNA expression of insulin receptor and glucose transporter (GLUT) 1 and 2 in the liver was also downregulated (2-way ANOVA, $P_{MHF} < 0.05$, Figure 2C), suggesting impaired insulin signaling and glucose transport in the offspring liver due to maternal HFD consumption. In line with the RT-qPCR result, immunoblot result also indicated reduced GLUT2 protein expression in MHF-WT offspring (2-way ANOVA, $P_{MHF} < 0.05$, Figure 2D). AMPK and Akt are both important regulators of insulin sensitivity, which are phosphorylated upon activation (pAMPK and pAkt) (Khamzina *et al.*, 2005). Our results indicate that the protein levels of pAMPK, Akt and pAkt were significantly suppressed in MHF-WT offspring ($P < 0.05$, $P < 0.001$, and $P < 0.05$ respectively, Figure 2D). AMPK expression was unchanged and the pAMPK/AMPK ratio was significantly reduced ($P < 0.05$), suggesting reduced activity rather than expression. Since both pAkt and Akt levels were reduced, the relative ratio of pAkt/Akt was unchanged. Consistently with previous findings (Borengasser *et al.*, 2014), liver expression of SIRT1 was reduced due to maternal HFD consumption ($P < 0.05$, MHF-WT vs MC-WT). SIRT1 overexpression significantly reversed the expression of Akt and pAkt ($P < 0.05$), but not that of InsR, GLUT, AMPK or pAMPK (Figure 2C and 2D).

SIRT1 overexpression attenuates adipocyte hypertrophy in MHF offspring

H&E staining of EpiWAT revealed enlarged adipocytes in MHF offspring compared to the control (2-way ANOVA, $P_{MHF} < 0.05$, Figure 3A), which was associated with significant reduction in SIRT1 mRNA expression (2-way ANOVA, $P_{MHF} < 0.05$, Figure 3B). Concomitantly, mRNA expression of peroxisome proliferator-activated receptor gamma (PPAR) coactivator 1-alpha (PGC-1α) and sterol regulatory element-binding protein (SREBP)1c in MHF-WT offspring were both significantly upregulated ($P < 0.05$, Figure 3B). No change was found in PPARγ mRNA levels. Similar to EpiWAT, RpWAT showed a significant reduction in SIRT1 mRNA levels in MHF offspring (2-way ANOVA, $P_{MHF} < 0.05$, Figure 3B). However, in contrast to EpiWAT, PGC-1α and PPARγ mRNA

expression in RpWAT were significantly decreased in MHF-WT offspring ($P < 0.05$ and $P < 0.01$ respectively, Figure 3B), whereas SREBP1c levels were unchanged.

SIRT1 overexpression significantly attenuated adipocyte hypertrophy in MHF-Tg offspring's EpiWAT ($P < 0.01$, Figure 3A). The effect was associated with marked reductions in the mRNA expression of PGC-1α, SREBP-1c and PPARγ ($P < 0.01$, $P < 0.05$, and $P < 0.01$ respectively, Figure 3B). There was a significant interaction between maternal diet and SIRT1 overexpression on mRNA expression of PGC-1α and SREBP-1c in EpiWAT (2-way ANOVA, $P_{interaction} < 0.05$). These two markers appear to be upregulated in MC-Tg offspring but downregulated in MHF-Tg offspring. In RpWAT, no significant change was found in PGC-1α and SREBP-1c mRNA expression due to SIRT1 overexpression. Conversely, PPARγ expression was significantly increased ($P < 0.01$, Figure 3B).

SIRT1 overexpression attenuates liver lipogenesis and lipotoxicity in MHF offspring

In this study, MHF offspring showed increased lipid accumulation as reflected by liver TG levels, ORO staining (both $P < 0.001$, Figure 4A) as well as H&E staining (Figure 4B) in association with significantly reduced SIRT1 protein expression (Figure 4B). The mRNA expression of SREBP-1c, fatty acid synthase (FASN), and fatty acid uptake fatty acid binding protein (FABP)1 were significantly increased in MHF offspring ($P < 0.05$, $P < 0.05$, and $P < 0.01$ respectively, Figure 4D), suggesting lipotoxicity. Carbohydrate-responsive element-binding protein (ChREBP), a hepatic lipogenesis marker which acts in a glucose concentration-dependent manner, was also slightly upregulated due to MHF (2-way ANOVA, $P_{MHF} < 0.05$). PPARα mRNA expression was also increased (2-way ANOVA, $P_{MHF} < 0.05$). In contrast, PGC-1α and PPARγ mRNA expression was significantly downregulated (MHF-WT vs MC-WT, $P < 0.05$, Figure 4C). The results of SREBP1 and PGC-1α were validated by protein expression analysis (MHF-WT vs MC-WT, $P < 0.05$, Figure 4D). mRNA expression of liver X receptor (LXR)β, a marker of cholesterol efflux, was unchanged due to maternal HFD consumption.

SIRT1 overexpression significantly decreased TG and ORO staining levels in the liver of MHF offspring ($P < 0.05$ and $P < 0.01$, Figure 4A and 4B). mRNA but not protein levels of PGC-1α were upregulated in Tg offspring (2-way ANOVA, $P_{SIRT1\text{-}tg} < 0.05$). In contrast, the mRNA level of SREBP-1c was not significantly changed ($P = 0.06$, Figure 4C), but its protein level was significantly suppressed ($P < 0.05$, Figure 4D). Similar to the RpWAT, PPARγ mRNA level in the liver was normalised by SIRT1 overexpression in MHF-Tg offspring ($P < 0.05$, Figure 4C). On the other hand, FASN and ChREBP and were significantly suppressed (all $P < 0.05$, Figure 4C). There was an interactive effect between maternal diet and SIRT1 overexpression on LXRβ expression (2-way

ANOVA, $P_{interaction} < 0.05$), with a trend to increase in MC-Tg compared to MC-WT offspring, and a significant decrease in MHF-Tg compared to MHF-WT offspring.

SIRT1 overexpression attenuates inflammatory disorder, oxidative stress and fibrogenesis in the liver of MHF offspring

In association with increased lipid accumulation and altered levels of lipotoxicity markers, MHF-WT offspring also demonstrated increased mRNA expression of monocyte chemotactic protein (MCP)1, an inflammatory marker, and reduced expression of TGFβ receptor (TGFβR) type 1 and 2 in the liver (all $P < 0.05$, Figure 5A). mRNA expression of antioxidant enzymes including superoxide dismutase (SOD)2, glutathione peroxidase (GPx)-1 and catalase in MHF-WT offspring's liver were all significantly suppressed by maternal HFD consumption (all $P < 0.01$, Figure 5B). Maternal HFD consumption also significantly increased protein expression of liver extracellular matrix (ECM) markers including collagen (COL)1A, COL4 and fibronectin (FN) in offspring's liver, as reflected by IHC staining (all $P < 0.05$, Figure 5C). The results were confirmed by Picro-Sirius Red (PSR) staining for collagens ($P < 0.001$, Figure 5C). No significant change in plasma alanine aminotransferase (ALT) was detected among these groups (data not shown), suggesting these early changes have not induced measurable liver cell injury.

SIRT1 overexpression normalised hepatic levels of TGFβR2 ($P < 0.01$), but had little effects on the other inflammatory and macrophage markers in the offspring liver (Figure 5A). It also increased mRNA expression of NAPDH oxidase (NOX)2 (2-way ANOVA, $P_{SIRT1\text{-}tg} < 0.05$), but concomitantly improved the levels of GPx-1 ($P < 0.01$) and catalase ($P < 0.05$) Figure 5B), which may reflect increased reactive oxygen species (ROS) production and appropriate antioxidant defence. The Tg offspring also showed significantly attenuated levels of COL1A, COL4 and FN, suggesting reduced fibrogenesis (all $P < 0.05$), which is supported by the PSR staining ($P < 0.01$, Figure 5C).

DISCUSSION

In this study, we confirm the negative effects of maternal HFD consumption on increased body weight and adiposity, reduced glucose tolerance and insulin sensitivity, as well as induced hepatic steatosis, inflammatory and oxidative stress responses and fibrogenesis in the offspring at weaning. Importantly, we demonstrate that SIRT1 is a key player in this process, systemic overexpression of which resulted in the attenuation in all the above-mentioned disorders in the offspring of HFD-fed dams, suggesting improved metabolic homeostasis.

Although hypothalamic SIRT1 has been shown to play an important role in appetite regulation (Çakir *et al.*, 2009; Sasaki *et al.*, 2010), no difference in milk intake was found between WT and Tg offspring in this study. As both WT and Tg offspring were born to the same WT dams, it is likely that offspring of both genotypes received similar nutrients during gestation and lactation. As such, the key difference that underlines the reduced body weight and adiposity in Tg offspring likely lies in the increased metabolic rate, which is a well-established characteristic of SIRT1-overexpressed mice (Pfluger *et al.*, 2008).

SIRT1 has been shown to upregulate PGC-1α to increase fatty acid oxidation in muscles (Gerhart-Hines *et al.*, 2007), with concordant suppression of PPARγ and SREBP1c expression in adipose tissue, leading to reduced adipogenesis (Picard *et al.*, 2004; Ponugoti *et al.*, 2010). We demonstrate that these markers are differentially regulated in two types of adipose tissues, EpiWAT and RpWAT, in the offspring by maternal HFD and SIRT1 overexpression respectively. In EpiWAT, SREBP1c was increased in MHF offspring, which is consistent with the adipocyte hypertrophy phenotype. Interestingly, PGC-1α was also increased, likely suggesting an adaptive mechanism. SIRT1 overexpression suppressed SREBP1c and PPARγ, which is in line with reduced EpiWAT mass and fat cell size in MHF-Tg offspring. In contrast to EpiWAT, the RpWAT in MHF offspring showed a significant reduction of PGC-1α and PPARγ, which is associated with smaller RpWAT mass and increased systemic insulin sensitivity. It has been shown that the expression of PPARγ and SREBP-1c in subcutaneous WAT is suppressed in obese patients with insulin resistance (Kolehmainen *et al.*, 2001; Dubois *et al.*, 2006). Thus, PPARγ agonists are used clinically as insulin sensitizers (Lebovitz *et al.*, 2001; Ahmadian *et al.*, 2013), and MHF mouse offspring treated with pioglitazone, a PPARγ agonist, have reduced body weight, reduced visceral WAT gain, increased subcutaneous WAT mass in association with lower levels of fasting glucose and insulin resistance (Kalanderian *et al.*, 2013). In this study, IPGTT was reduced and QUICKI was improved in MHF-Tg offspring, suggesting the positive effect of SIRT1 overexpression on systemic insulin sensitivity and glucose homeostasis. This may be partially attributed to the regulation of PPARγ in the offspring RpWAT.

The reduced expression of GLUTs, InsR, pAMPK and pAkt indicates impaired glucose transport and insulin signaling in the offspring liver due to maternal HFD consumption (Nawano *et al.*, 1999; Iglesias *et al.*, 2002), which may also underpin the dysregulation of systemic glucose. SIRT1 overexpression only reversed the expression and activity of Akt and not the other markers, suggesting SIRT1 overexpression regulates liver insulin sensitivity via an Akt-dependent mechanism in our model. To support this hypothesis, PGC-1α, the common downstream marker of both SIRT1 and AMPK, was also not upregulated in MHF-Tg offspring. An explanation for this is the lack of

important cofactors NAD^+ and AMP due to positive energy balance that is required for SIRT1 and AMPK to become active. The unchanged activity of SIRT1 in the MHF-Tg offspring liver is also in line with this hypothesis. In contrast, SIRT1 has been shown to promote phosphorylation of Akt via deacetylation-independent pathways (Wang *et al.*, 2011; Ramakrishnan *et al.*, 2014).

Apart from Akt, PPARγ expression was also upregulated in Tg offspring, which suggests another pathway for improved insulin sensitivity in the liver (Tiikkainen *et al.*, 2004). Importantly, PPARγ is also involved in liver lipid metabolism, of which activation has been shown to reduce liver lipid contents (Tiikkainen *et al.*, 2004). The reduced expression of *de novo* lipogenesis markers including SREBP-1c, ChREBP and FASN is also likely to contribute to such effect. LXRβ is the master regulator of cholesterol but also involved in lipogenesis by stimulating the expression of SREBP-1c and FASN (Grefhorst *et al.*, 2002). The downregulation of LXRβ in MHF-Tg offspring supports a reduction of hepatic steatosis (Patel *et al.*, 2011). Conversely, no change in FABP1 expression was found, suggesting that lipid uptake in MHF-Tg offspring's liver was unchanged. As stated, AMPK/PGC-1α signalling was still impaired despite SIRT1 overexpression, suggesting no improvement in lipolysis (Viollet *et al.*, 2006). Considering that liver lipid content is the consequence of the balance between *de novo* lipogenesis, lipid uptake, and lipolysis, it is understandable why in our study, SIRT1-mediated suppression of *de novo* lipogenesis by itself can not fully normalise lipid accumulation in the liver due to maternal HFD consumption.

Maternal HFD consumption was associated with inflammatory dysregulation, reflected by increased expression of MCP-1 and reduced expression of TGFβ receptor 1 and 2. The result is consistent with our previous findings in the kidneys of MHF offspring in rats (Nguyen *et al.*, 2017). Further investigation regarding TGFβ signalling pathways is required to determine whether these abnormalities reflect inflammatory disorders or compensatory responses. In this study, maternal HFD also led to significant suppression of endogenous antioxidants including SOD2, GPx-1 and CAT, suggesting increased oxidative damage in the offspring's liver. As such, the expression of fibrogenic markers COL1A, COL4 and FN was significantly elevated, which reflects increased susceptibility for liver fibrosis. Despite significant liver remodelling, there was no change in the plasma levels of ALT in MHF offspring, which suggests that no major liver damages had occurred. However, additional exposure to postnatal HFD can cause significant lipotoxicity, oxidative stress and inflammation later in life (McCurdy *et al.*, 2009) SIRT1 overexpression in the offspring was able to enhance antioxidant capacity and attenuate fibrogenesis in the liver due to maternal HFD consumption. On one hand, these positive effects can be partially attributed to the effects of SIRT1 to suppress glucotoxicity and lipotoxicity in Tg offspring. On the other hand, SIRT1 has been shown to have direct regulatory

effects on inflammation, oxidative stress and fibrosis in models of acute tissue injuries (He *et al.*, 2010; Wu *et al.*, 2015).

In this study, we did not control for single pup per litter, which can be a limitation in DOHaD research (Dickinson *et al.*, 2016). However, given that there was typically one transgenic male mouse per litter, the possible bias in the analysis of the effects of SIRT1 overexpression is likely to be minimal.

Collectively, the study provides direct evidence of the importance of SIRT1 in linking maternal HFD consumption to metabolic dysfunction in the offspring, and suggest that targeting SIRT1 in the offspring in early developmental periods may reprogram metabolic disorders due to maternal HFD feeding. Further studies are required to examine the long-term effects of these approaches in adulthood and across generations. In addition, as SIRT1 has been shown to modulate zygotic histone code (Adamkova *et al.*, 2017), examination of epigenetic modifications including DNA methylation and histone acetylation can provide additional understanding of the fetal reprogramming effects of SIRT1 in the setting of maternal obesity.

ADDITIONAL INFORMATION

Competing interests

No potential conflicts of interest relevant to this article were reported.

Author contributions

L.N. designed and conducted all main experiments, performed data analysis, prepared figures and the manuscript. A.Z. assisted with tissue processing for histology. H.C, C.P., and S.S. coordinated the execution of the project and involved in experiment design. H.C, C.P., and S.S reviewed data analysis and the manuscript.

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Table 1. RT-PCR primers sequences

No.	Gene	Forward primer sequence	Reverse primer sequence
1	IL1B	GGATGATGATGATAACCTGC	CATGGAGAATATCACTTGTTGG
2	leptin	GAGACCCCTGTGTCGGTTC	GACTGCGTGTGTGAAATGTC
3	MC4R	GACGGAGGATGCTATGAG	GCAGGTTCTTGTTCTTGGC
4	MCP-1	GCCTGCTGTTCACAGTTGC	CAGGTGAGTGGGGCGTTA
5	NPY	GGCTGTGTGGACTGACCCT	GATGTAGTGTCGCAGAGCGG
6	NPY1R	TGATCTCCACCTGCGTCAAC	ATGGCTATGGTCTCGTAGTC
7	Ob-Rb	CCAGGTGAGGAGCAAGAG	CTGCACAGTGCTTCCCAC
8	POMC	GAGATTCTGCTACAGTCGCTC	TTGATGATGGCGTTCTTGAA
9	TGFBR1	CAGCTCCTCATCGTGTTGG	CAGAGGTGGCAGAAACACTG
10	TGFBR2	TCCATCTGTGAGAAGCCACA	GGGTCATGGCAAACTGTCTC
11	GLUT1	AGCCTGCAAACTCACTGCTC	CCTACCCTCAATCCCACAAGC
12	GLUT2	TCGCCCTCTGCTTCCAGTAC	GAACACGTAAGGCCAAGGA

Table 2. Antibody information

Target	Cat#	Size (kDa)	Dilution (WB)	Dilution (IHC)	Company	Address
GAPDH	sc-47724	36	1:2000		Santa Cruz	TX, USA
SIRT1	07-131	110	1:2000		EMD Millipore	NSW, Australia
PGC-1α	NBP1-04676	91	1:2000		Novus Biologicals	CO, USA
SREBP1	04-469	62	1:500		EMD Millipore	NSW, Australia
GLUT2	07-1402	50	1:1000		EMD Millipore	NSW, Australia
p-Akt (S473)	13038	60	1:1000		Cell Signalling	MA, USA
Akt	9272	60	1:1000		Cell Signalling	MA, USA
p-AMPKα (T172)	2535	62	1:1000		Cell Signalling	MA, USA
AMPKα	5831	62	1:1000		Cell Signalling	MA, USA
Collagen I	ab34710			1:750	Abcam	Cambridge, UK
Collagen III	ab7778			1:750	Abcam	Cambridge, UK

Table 3. Anthropometric results

	MC-WT (n = 21)		MC-Tg (n = 8)		MHF-WT (n = 26)		MHF-Tg (n = 11)		Main effect	MC-WT vs MC-Tg	MC-WT vs MHF-WT	MHF-WT vs MHF-Tg
	Mean	SD	Mean	SD	Mean	SD	Mean	SD				
BW (g)	7.95	1.37	7.40	0.99	10.44	1.28	8.62	1.85	b, c	ns	***	†††
EpiWAT (mg)	30.9	9.1	15.8	8.9	113.7	31.8	76.8	35.4	b, c	ns	***	†††
EpiWAT (%BW)	0.39	0.07	0.21	0.11	1.01	0.23	0.83	0.31	b, c	ns	***	†
RpWAT (mg)	7.8	2.1	4.2	2.3	29.2	7.8	21.4	11.6	b, c	ns	***	††
RpWAT (%BW)	0.10	0.03	0.05	0.03	0.27	0.08	0.21	0.09	b, c	ns	***	††
Liver (mg)	425.4	91.5	305.5	70.1	597.9	103.8	376.2	113.4	b, c	*	***	†††
Liver (%)	4.74	0.79	4.04	0.75	5.16	0.61	4.38	0.70	c	*	ns	†
NEFA (mM)	0.36	0.06	0.35	0.08	0.44	0.09	0.41	0.09	b	ns	ns	ns
Triglyceride (g/L)	0.51	0.16	0.52	0.12	0.90	0.34	0.79	0.10	b	ns	**	ns

BW: Body weight. N = 6 for NEFA and triglyceride measurement. Vs MC-WT: *p < 0.05, **p < 0.01, ***p < 0.001; vs MHF-WT: †p < 0.05, ††p < 0.01, †††p < 0.001, ns: non-significant. a ($P_{interaction}$ < 0.05), b (P_{MHF} < 0.05), c ($P_{SIRT1\text{-}tg}$ < 0.05)

Figure legends

Figure 1. The effects of perinatal SIRT1 overexpression on estimated milk intake and appetite regulators in MHF offspring. (A) Estimated appetite (n = 10, 5, 13 and 5 respectively). (B) Plasma leptin levels. mRNA expression of (C) SIRT1 and (D) appetite regulators (n = 8). Vs MC-WT: *p < 0.05, **p < 0.01, ***p < 0.001; vs MHF-WT: †p < 0.05, †††p < 0.001. a (2-way ANOVA, $P_{interaction}$ < 0.05), b (2-way ANOVA, P_{MHF} < 0.05), c (2-way ANOVA, $P_{SIRT1\text{-}tg}$ < 0.05).

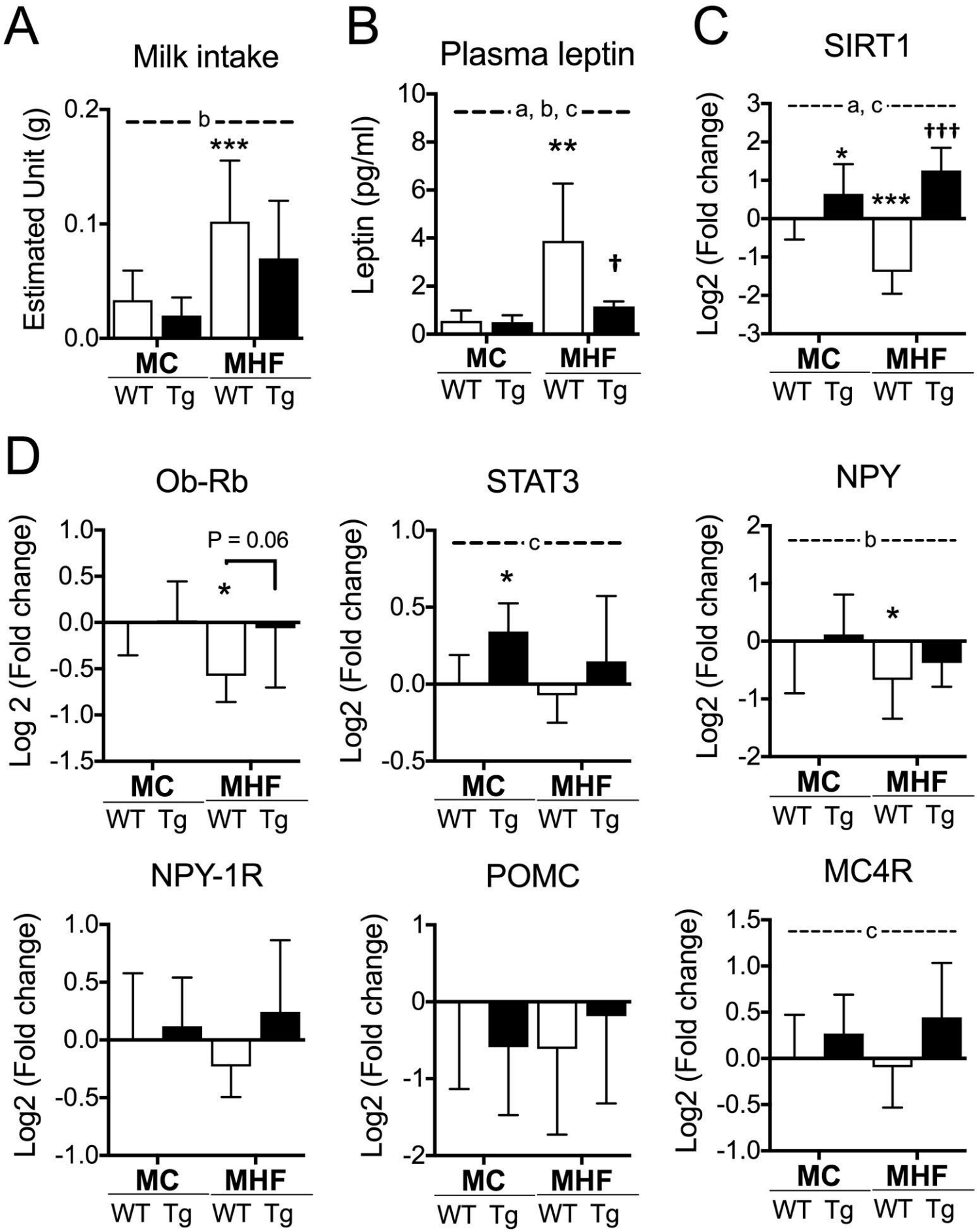
Figure 2. Perinatal SIRT1 overexpression increases glucose tolerance and insulin sensitivity in MHF offspring. (A) Intraperitoneal glucose tolerance test (IPGTT) and area under the curve (AUC) (n = 11, 8, 13 and 8 respectively). (B) Plasma insulin levels and quantitative insulin sensitivity check index (QUICKI) (n = 6)**.** (C) liver mRNA expression of insulin receptor and glucose transporter 1 and 2 (n = 6). (D) Protein expression of SIRT1 and insulin signalling regulators (n = 6). (E) SIRT1 activity (n = 6). Vs MC-WT: *p < 0.05, **p < 0.01, ***p < 0.001; vs MHF-WT: †p < 0.05, ††p < 0.01, †††p < 0.001). a (2-way ANOVA, $P_{interaction}$ < 0.05), b (2-way ANOVA, P_{MHF} < 0.05), c (2-way ANOVA, $P_{SIRT1\text{-}tg}$ < 0.05).

Figure 3. Perinatal SIRT1 overexpression reversed hypertrophy in white adipose tissue in MHF offspring. (A) H&E staining and adipocyte frequency analysis. 200X magnification (n = 6). (B) RT-qPCR analysis of offspring EpiWAT and RpWAT (n = 6). Vs MC-WT: *p < 0.05, **p < 0.01, ***p < 0.001; vs MHF-WT: ††p < 0.01). a (2-way ANOVA, $P_{interaction}$ < 0.05), b (2-way ANOVA, P_{MHF} < 0.05), c (2-way ANOVA, $P_{SIRT1\text{-}tg}$ < 0.05). Scale bar = 200 μm.

Figure 4. Perinatal SIRT1 overexpression reduced liver weight and lipotoxicity in MHF offspring. (A) Liver Triglyceride (TG) and Oil Red O (ORO) staining quantitation (n = 6). (B) Representative images of H&E, ORO and IHC staining of SIRT1. (C) Box plots of RT-PCR analysis of offspring liver. The boxes extend from the 25th to 75th percentiles, the central lines indicate the median. Whiskers extend from Min to Max values (n = 6). (D) Protein expression of genes in SIRT1 signalling network (n = 6). Vs MC-WT: *p < 0.05, **p < 0.01, ***p < 0.001; vs MHF-WT: †p < 0.05, ††p < 0.01. a (2-way ANOVA, $P_{interaction}$ < 0.05), b (2-way ANOVA, P_{MHF} < 0.05), c (2-way ANOVA, $P_{SIRT1\text{-}tg}$ < 0.05). Scale bar = 100 μm.

Figure 5. Perinatal SIRT1 overexpression attenuates inflammatory dysregulation and oxidative stress due to MHF in offspring liver. Box plots showing mRNA expression of (A) inflammatory markers, and (B) oxidative stress regulators. The boxes extend from the 25th to 75th percentiles, the

central lines indicate the median. Whiskers extend from Min to Max values (n = 6)**.** (C) IHC and Picro-Sirius Red (PSR) staining quantification (n = 6)**.** Vs MC-WT: *p < 0.05, **p < 0.01, ***p < 0.001; vs MHF-WT: †p < 0.05, ††p < 0.01). a (2-way ANOVA, $P_{interaction} < 0.05$), b (2-way ANOVA, $P_{MHF} < 0.05$), c (2-way ANOVA, $P_{SIRT1\text{-}tg} < 0.05$). Scale bar = 100 μm.


A
Milk intake
Estimated Unit (g)
0.2
0.1
0.0
b
***
MC
MHF
WT Tg WT Tg
B
Plasma leptin
Leptin (pg/ml)
10
8
6
4
2
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a, b, c
**
†
MC
MHF
WT Tg WT Tg
C
SIRT1
Log2 (Fold change)
3
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1
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-3
a, c
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MC
MHF
WT Tg WT Tg
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Ob-Rb
Log 2 (Fold change)
1.0
0.5
0.0
-0.5
-1.0
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P = 0.06
*
MC
MHF
WT Tg WT Tg
STAT3
Log2 (Fold change)
1.0
0.5
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MC
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WT Tg WT Tg
NPY
Log2 (Fold change)
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Log2 (Fold change)
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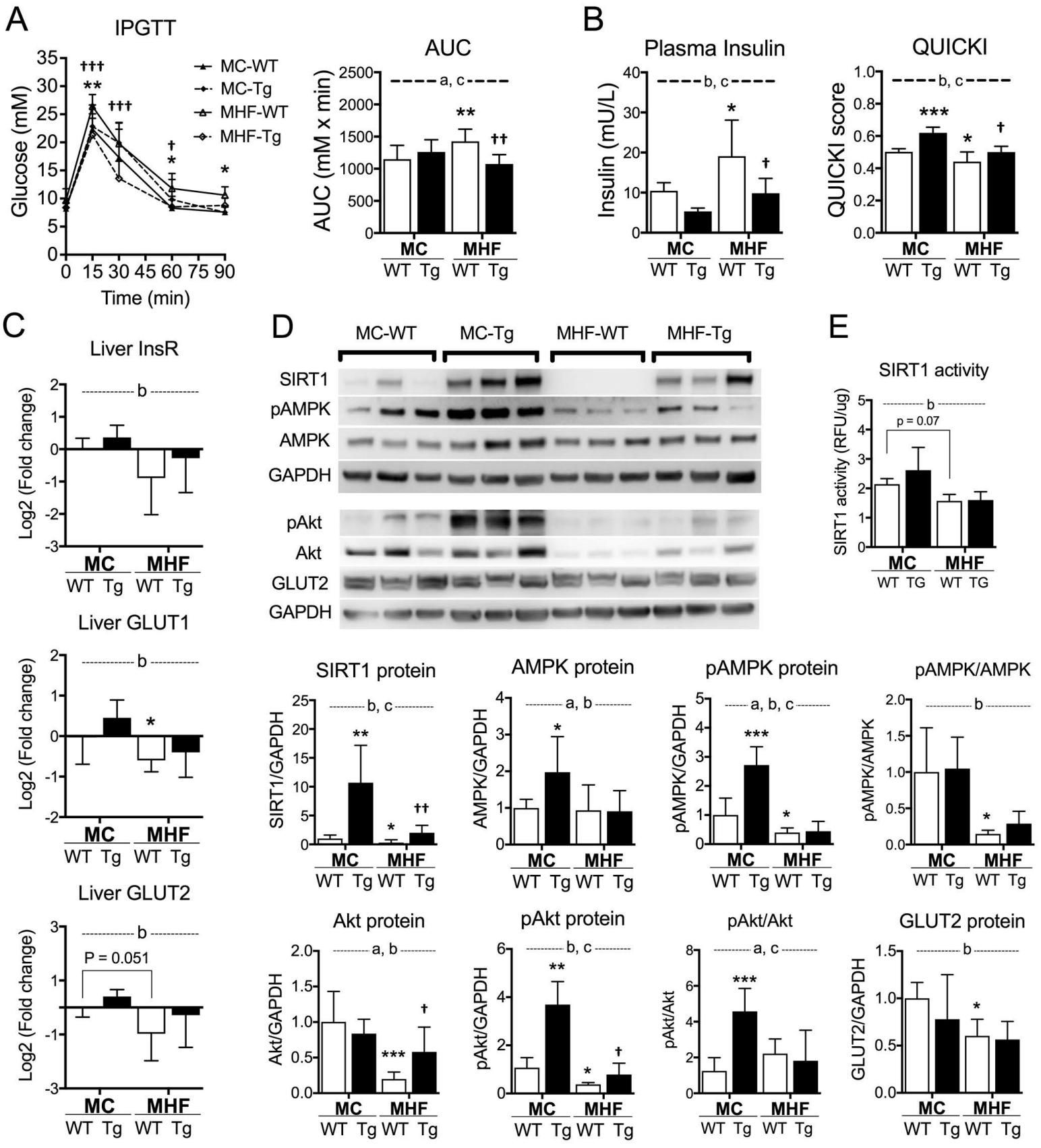
A
IPGTT
Glucose (mM)
Time (min)
MC-WT
MC-Tg
MHF-WT
MHF-Tg
AUC
AUC (mM x min)
a, c
MC
MHF
WT Tg WT Tg
B
Plasma Insulin
Insulin (mU/L)
b, c
QUICKI
QUICKI score
C
Liver InsR
Log2 (Fold change)
b
Liver GLUT1
Liver GLUT2
P = 0.051
D
MC-WT
MC-Tg
MHF-WT
MHF-Tg
SIRT1
pAMPK
AMPK
GAPDH
pAkt
Akt
GLUT2
E
SIRT1 activity
SIRT1 activity (RFU/ug)
p = 0.07
SIRT1 protein
SIRT1/GAPDH
AMPK protein
AMPK/GAPDH
pAMPK protein
pAMPK/GAPDH
pAMPK/AMPK
Akt protein
Akt/GAPDH
pAkt protein
pAkt/GAPDH
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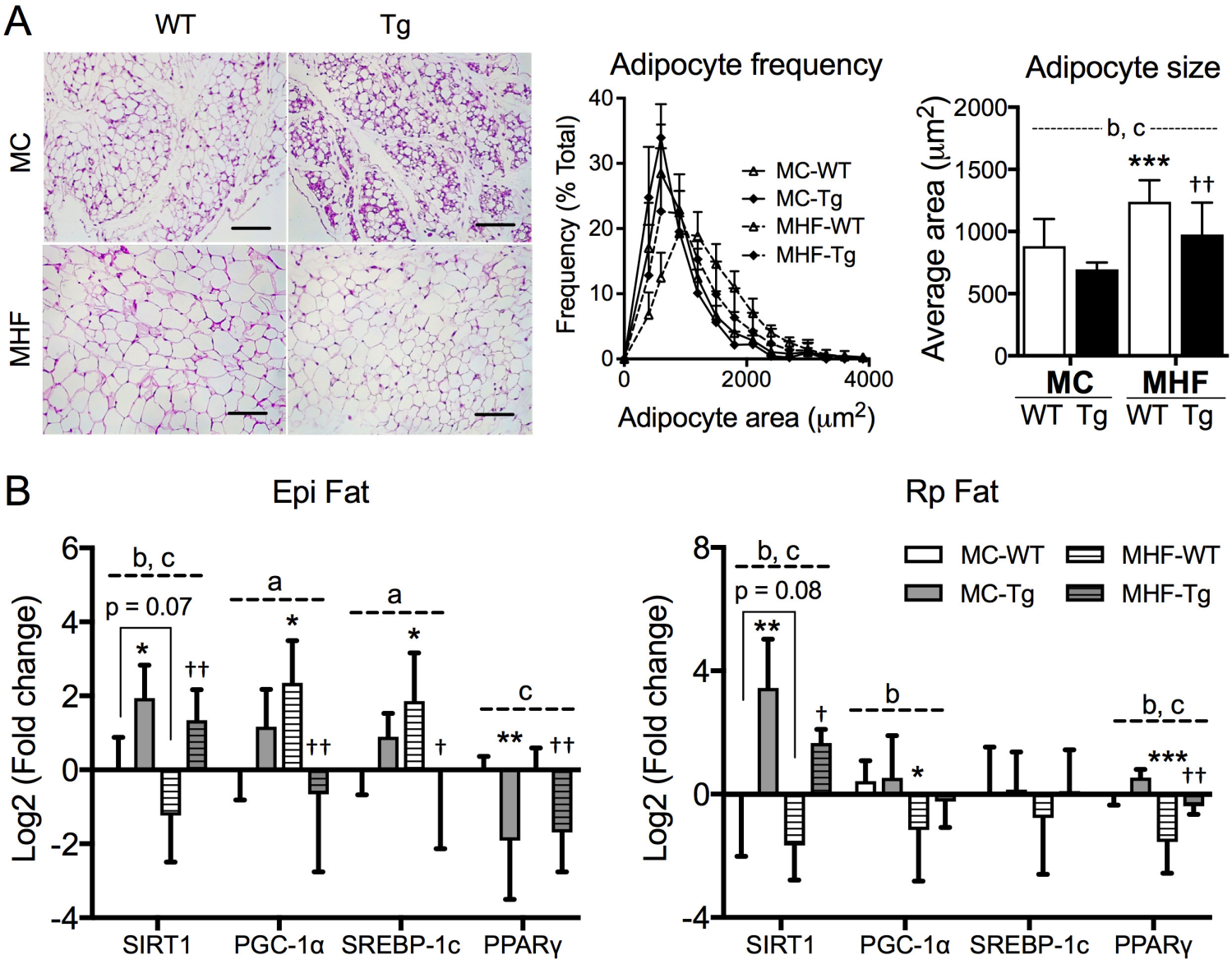
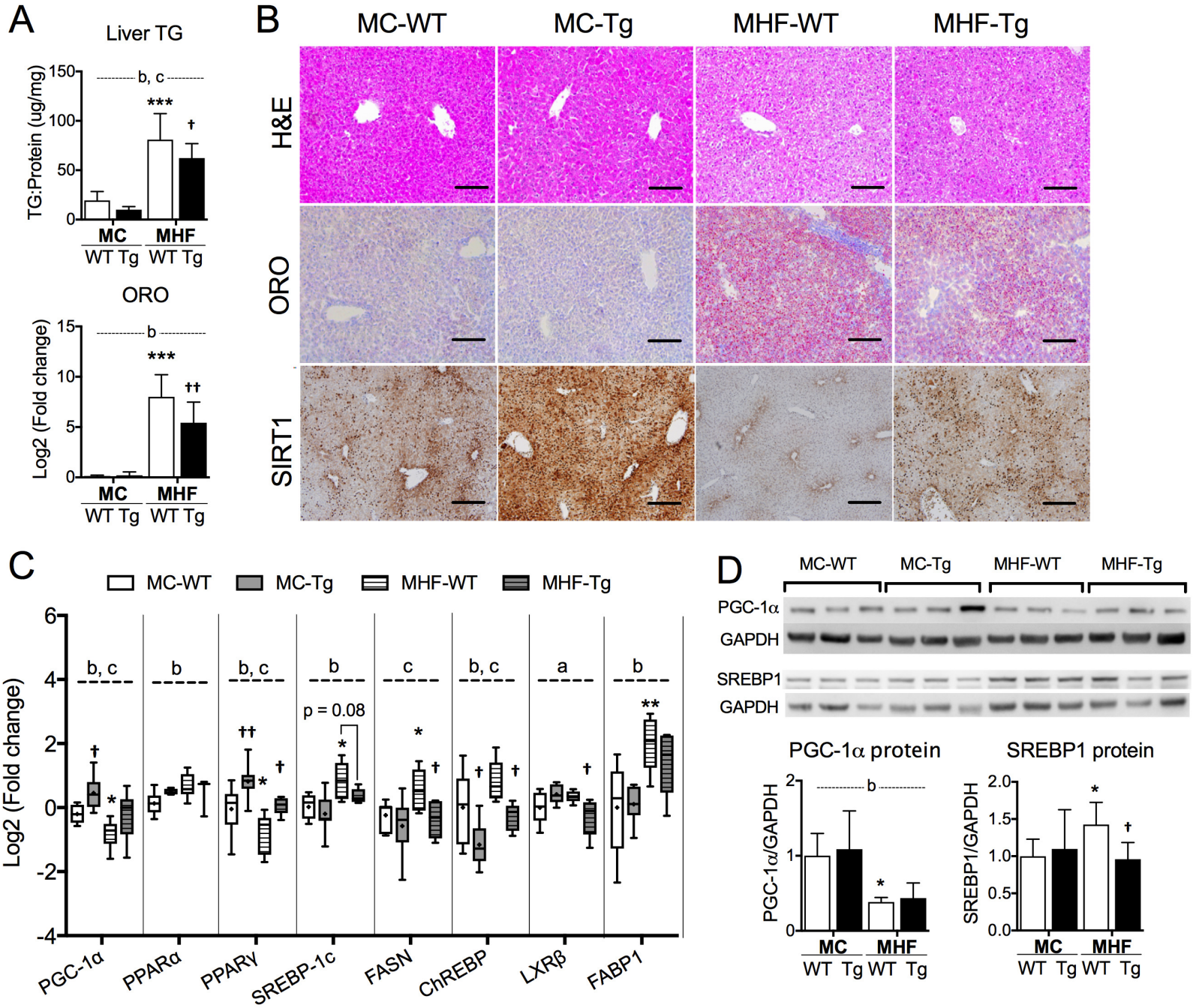
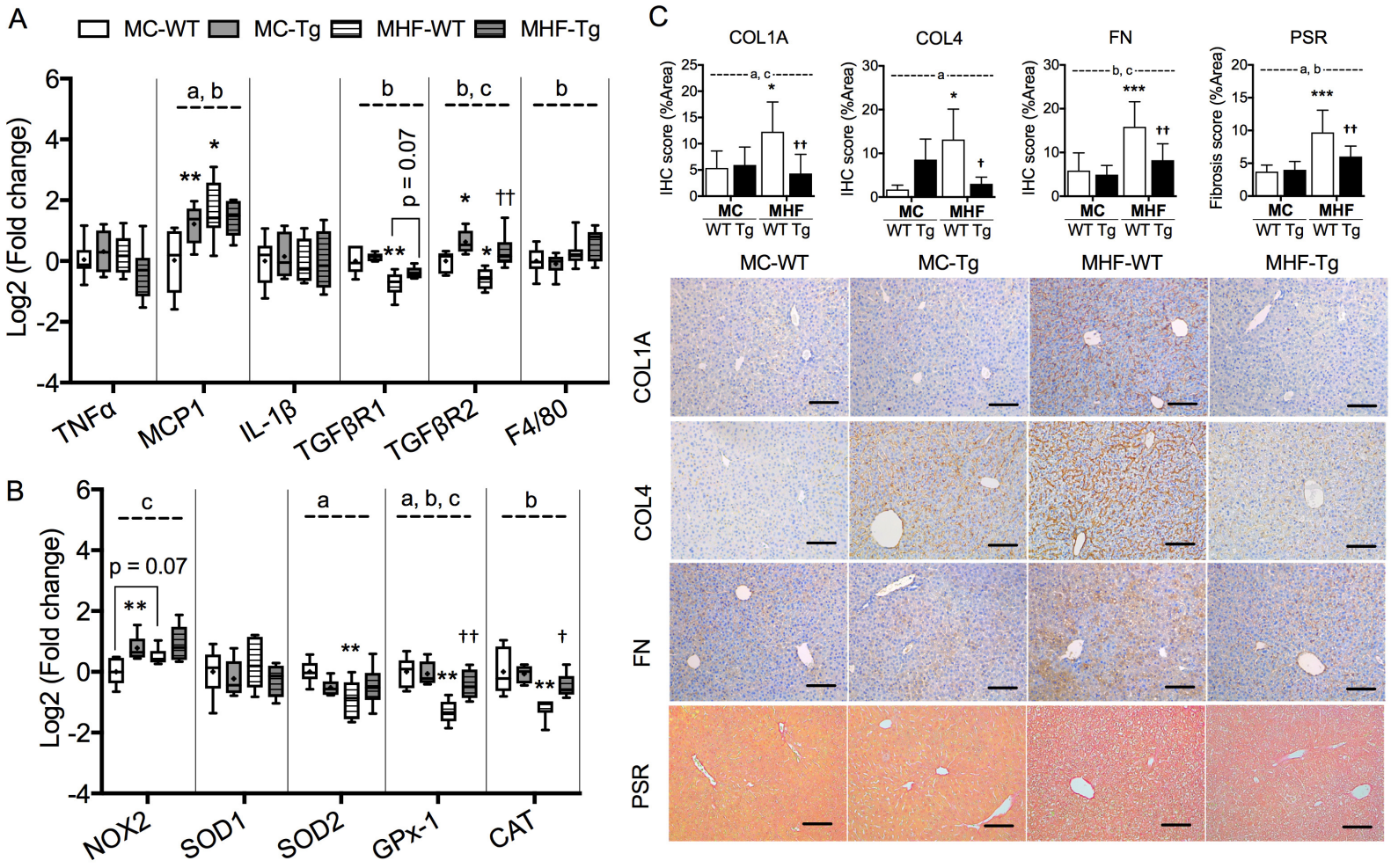
GLUT2 protein
GLUT2/GAPDH


A
WT
Tg
MC
MHF
Adipocyte frequency
Frequency (% Total)
Adipocyte area (μm2)
MC-WT
MC-Tg
MHF-WT
MHF-Tg
Adipocyte size
Average area (μm2)
b, c
***
††
MC
MHF
WT Tg WT Tg
B
Epi Fat
Rp Fat
Log2 (Fold change)
p = 0.07
p = 0.08
SIRT1
PGC-1α
SREBP-1c
PPARγ


A
Liver TG
TG:Protein (ug/mg)
b, c
***
†
MC
MHF
WT Tg WT Tg
ORO
Log2 (Fold change)
b
***
††
B
MC-WT
MC-Tg
MHF-WT
MHF-Tg
H&E
ORO
SIRT1
C
MC-WT
MC-Tg
MHF-WT
MHF-Tg
Log2 (Fold change)
b, c
b
b, c
b
p = 0.08
c
b, c
a
b
PGC-1α
PPARα
PPARγ
SREBP-1c
FASN
ChREBP
LXRβ
FABP1
D
MC-WT
MC-Tg
MHF-WT
MHF-Tg
PGC-1α
GAPDH
SREBP1
GAPDH
PGC-1α protein
PGC-1α/GAPDH
SREBP1 protein
SREBP1/GAPDH
MC
MHF
WT Tg WT Tg


A
MC-WT
MC-Tg
MHF-WT
MHF-Tg
Log2 (Fold change)
TNFα
MCP1
IL-1β
TGFβR1
TGFβR2
F4/80
a, b
b
b, c
p = 0.07
B
Log2 (Fold change)
NOX2
SOD1
SOD2
GPx-1
CAT
c
a
a, b, c
b
p = 0.07
C
COL1A
COL4
FN
PSR
IHC score (%Area)
Fibrosis score (%Area)
MC
MHF
WT Tg
MC-WT
MC-Tg
MHF-WT
MHF-Tg