

# SIRT1 overexpression attenuates offspring metabolic and liver disorders as a result of maternal high-fat feeding

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## Key points

- Maternal high-fat diet (MHF) consumption led to metabolic and liver disorders in male offspring, which are associated with reduced sirtuin (SIRT)1 expression and activity in the offspring liver
- SIRT1 overexpression in MHF offspring reduced their body weight and adiposity and normalized 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 liver of offspring.

**Abstract** Maternal obesity can increase the risk of metabolic disorders in the offspring. However, the underlying mechanism responsible for this 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. In the present study, 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) $\gamma$  ( $P < 0.01$ ), PPAR $\gamma$ -coactivator 1-alpha ( $P < 0.05$ ) and sterol regulatory element-binding protein-1c in EpiWAT ( $P < 0.01$ ), whereas there was increased expression of PPAR $\gamma$  in RpWAT ( $P < 0.05$ ). In the liver, PPAR $\gamma$  mRNA expression,

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as well as Akt protein expression and activity, were increased ( $P < 0.05$ ), whereas fatty acid synthase and carbohydrate response element binding protein were downregulated ( $P < 0.05$ ), supporting increased insulin sensitivity and reduced lipogenesis in the liver. In addition, hepatic expression of endogenous anti-oxidants, 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 present study provides direct evidence of the mechanistic significance of SIRT1 in maternal HFD-induced metabolic dysfunction in offspring and suggests that SIRT1 is a promising target for fetal reprogramming.

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## 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 as a result of poor compliance and late engagement in these programmes (Catalano, 2015). Clearly, understanding all of the contributing factors and transmission pathways in maternal obesity-related DOHaD is crucial to the development of 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 playing an important role in DNA repair and replication, as well as genetic and epigenetic regulation. SIRT1 activity strictly depends on the cellular availability of  $NAD^+$ , which fluctuates in accordance with the 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 ageing and obesity negatively affect SIRT1 expression and activity (Pedersen *et al.* 2008; dos Santos Costa *et al.* 2010; Mariani *et al.* 2015). Conversely, over-expression of SIRT1 mimics caloric restriction (Wood *et al.* 2004), leading to a 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 studies have reported reduced SIRT1 expression/activity in the fetus and neonatal tissues as a result of maternal HFD feeding (MHF) (Suter *et al.* 2012; Nguyen *et al.* 2017).

In the present study, we induced a systemic over-expression of SIRT1 in the offspring to confirm the causative role of SIRT1 regulation in maternal obesity-related DOHaD. The results of the present study suggest that SIRT1 is a promising target for fetal reprogramming of metabolic disorders as a result of maternal obesity.

## Methods

### Animals

The present 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<sup>-1</sup>, 43.5% calorie as fat; Specialty Feed, Glen Forest, WA, Australia) (MHF,  $n = 21$ ) or standard rodent chow (11 kJ g<sup>-1</sup>, 14% calorie as fat; Gordon's Speciality Stockfeeds, Yanderra, 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 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-Coll1a1<sup>tm1(CAG-Sirt1)Dsin</sup>/Mmjax, derived from C57BL/6. The original Tg colony was a generous gift from Dr Lindsay Wu (University of New South Wales, Sydney, NSW, Australia).

As we have previously shown sex-specific down-regulation of SIRT1 by MHF in male offspring (Nguyen *et al.* 2017), only male offspring were examined in the present study. To limit the difference in milk competition between the litters (Chen *et al.* 2008), the newborn litters were adjusted to four to six pups per 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 ~30% of the litter size and there was typically one Tg male mouse per litter. The offspring were genotyped at postnatal (P)14 in accordance to the Jackson Laboratory Genotyping Protocol for B6.Cg-Coll1a1<sup>tm1(CAG-Sirt1)Dsin</sup>/Mmjax strain using crude tail DNA extracted with DirectPCR Lysis Reagent (Mouse Tail) (Viagen Biotech, Los Angeles, CA, USA).

At weaning (P20), all pups were deeply anaesthetized with 3% isoflurane and killed upon cardiac puncture for blood collection after 5 h of fasting. PBS (1%) was used for whole body perfusion. Tissues were snap frozen or embedded in optimal cutting temperature (OCT) compound and stored at  $-80^{\circ}\text{C}$  or fixed in neutral buffered formalin (10%) for ~36 h 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 Bregma +0.5 mm. A second coronal cut was made 3.2 mm caudal to the second cut. This section (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 5 h prior to IPGTT (Chen *et al.* 2008) and then D-glucose was injected ( $2\text{ g kg}^{-1}\text{ i.p.}$ ). Tail blood glucose level was recorded prior to glucose injection, and then at 15, 30, 60 and 90 min after injection using a glucometer (Accu-Chek<sup>®</sup> glucose meter; Roche Diagnostics, Basel, Switzerland). The area under the curve was calculated for each animal. The Quantitative Insulin Sensitivity Check Index (QUICKI) was calculated as reported previously (Chen *et al.* 2005; Cacho *et al.* 2008).

### Milk intake estimation

At P19, offspring were separated from their dams and fasted for 5 h. They were weighed before returning to their dams. After 2 h of feeding, the offspring were weighed again. The difference in the body weight during the 2 h

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 in accordance with previously reported protocols (Nguyen *et al.* 2017) using Roche triglyceride reagent GPO-PAP (Roche Life Science, North Ryde, NSW, Australia) and the Pierce BCA Protein Assay Kit (Thermo Scientific, Scoresby, VIC, Australia) in accordance with the manufacturer's instructions. Lipid concentrations were normalized to the weight of tissue homogenized. Protein concentrations were standardized to  $5\text{ }\mu\text{g }\mu\text{L}^{-1}$ .

### Quantitative RT-PCR

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were followed (Bustin *et al.* 2009). Total RNA was extracted from liver tissues using an RNeasy Plus Mini Kit (Qiagen Pty Ltd, Valancia, CA, USA) in accordance with the manufacturer's instructions, whereas RNA in fat tissues and the hypothalamus was extracted using Trizol Reagent (Sigma-Aldrich, St Louis, MO, USA). The purified total RNA was used as a template to generate cDNA using the First Strand cDNA Synthesis Kit (Roche Life Science). The amplicons of target genes were amplified with SYBR Green probes. Primer sequences have been reported in our previous study (Nguyen *et al.* 2018) and are summarized in Table 1. Before acquiring the actual data, all of 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 quantitative PCR reaction was 200 nM. Several commonly used housekeeping genes in the literature, including 18s,  $\alpha$ -tubulin and  $\beta$ -actin, were tested. B-actin showed the least variation in mRNA expression among the groups. Therefore, gene expression was standardized to  $\beta$ -actin mRNA and log-transformed.

### Immunoblotting

The same amount of protein (20  $\mu\text{g}$ ) was electrophoresed and electroblotted onto the Hybond nitrocellulose membrane (Amersham Pharmacia Biotech, Amersham, UK), which was then incubated with a primary antibody at  $4^{\circ}\text{C}$  overnight. Antibody information and the applied

**Table 1. RT-PCR primers sequences**

Number	Gene	Forward primer sequence	Reverse primer sequence
1	IL1B	GGATGATGATGATAACCTGC	CATGGAGAATATCACTTGTGG
2	Leptin	GAGACCCCTGTGTCGGTTC	GACTGCGTGTGTGAAATGTC
3	MC4R	GACGGAGGATGCTATGAG	GCAGGTTCTTGTCTTGGC
4	MCP-1	GCCTGCTGTCACAGTTGC	CAGGTGAGTGGGGCGTTA
5	NPY	GGCTGTGTGGACTGACCCT	GATGTAGTGTGCGAGAGCGG
6	NPY1R	TGATCTCCACCTGCGTCAAC	ATGGCTATGGTCTCGTAGTC
7	Ob-Rb	CCAGGTGAGGAGCAAGAG	CTGCACAGTGCTTCCCAC
8	POMC	GAGATTCTGCTACAGTCGCTC	TTGATGATGGCGTTCTTGAA
9	TGFBR1	CAGCTCCTCATCGTGTGG	CAGAGGTGGCAGAAACTG
10	TGFBR2	TCCATCTGTGAGAAGCCACA	GGGTCATGGCAAAGTCTC
11	GLUT1	AGCCTGCAAACCTCACTGCTC	CCTACCCTCAATCCCACAAGC
12	GLUT2	TCGCCCTGCTTCCAGTAC	GAACACGTAAGGCCAAGGA

**Table 2. Antibody information**

Target	Catalogue number	Size (kDa)	Dilution (WB)	Dilution (IHC)	Company	Address
GAPDH	sc-47724	36	1:2000		Santa Cruz Biotechnology	Santa Cruz, TX, USA
SIRT1	07-131	110	1:2000		EMD Millipore	North Ryde, NSW, Australia
PGC-1 $\alpha$	NBP1-04676	91	1:2000		Novus Biologicals	Littleton, 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 Signaling	Danvers, MA, USA
Akt	9272	60	1:1000		Cell Signaling	MA, USA
p-AMPK $\alpha$ (T172)	2535	62	1:1000		Cell Signaling	MA, USA
AMPK $\alpha$	5831	62	1:1000		Cell Signaling	MA, USA
Collagen I	ab34710			1:750	Abcam	Cambridge, UK
Collagen III	ab7778			1:750	Abcam	Cambridge, UK

WB, western blot.

concentrations are shown in Table 2. Two commonly used housekeeping genes in the literature including  $\beta$ -actin and GAPDH were tested for loading normalization in a western blot. GAPDH showed less variation in expression among groups compared to  $\beta$ -actin and hence was selected for the present 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, Burlington, MA, USA) to the membrane and imaged using ImageQuant LAS 4000 (Fujifilm, Tokyo, Japan). ImageJ (National Institutes of Health, Bethesda, MD, USA) was used for densitometric analyses.

### SIRT1 activity assay

Nuclear protein was extracted in accordance with 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 MgCl<sub>2</sub> and 10 mM KCl, 1 mM dithiothreitol), followed by centrifugation to separate nuclear and cytoplasmic fractions. The nuclear proteins were extracted using extraction buffer (20 mM HEPES, pH 7.9, with 1.5 mM MgCl<sub>2</sub>, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM dithiothreitol). 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) in accordance with the manufacturer's instructions.

### Tissue morphology

Tissues were fixed in 10% formalin for 36 h and embedded in paraffin or frozen-embedded in OCT solution (Tissue-Tek; Sakura Finetek USA, Inc., Torrance, CA, USA). Paraffin sections and frozen sections were prepared at 4 and 12  $\mu$ m thickness, respectively, and mounted on microscope slides (Trajan Scientific and Medical, Ringwood, VIC, Australia).

The sections were stained with haematoxylin and eosin (H&E) for general morphology. For adipocyte frequency analysis, H&E-stained sections were analysed using a bright-field microscope (Leica Microsystems, Wetzlar, Germany) and six random non-overlapping fields were captured at 200 $\times$  magnification. All slides were randomly coded by an independent person before analysis. Adipocyte size analysis was conducted using Adiposoft software (<https://imagej.net/Adiposoft>) (Galarraga *et al.* 2012). For collagen staining, paraffin sections were stained with Fast Green for 30 min followed by incubation in Picro-Sirius Red (PSR) for another 30 min. Cytoplasm was stained in orange or light red while collagen was stained in dark red. For lipid droplet visualization, both H&E and Oil Red O (ORO) staining was used. In ORO staining, frozen tissues were sectioned at a thickness of 12  $\mu$ m and stained with ORO (Sigma-Aldrich, St Louis, MO, USA) for 15 min.

### Immunohistochemistry (IHC)

IHC staining was performed as described previously (Nguyen *et al.* 2017). Antigen-retrieval was performed at 99°C for 20 min in 0.01 M (pH 6.0) citric buffer. Endogenous peroxidase was deactivated with 3% H<sub>2</sub>O<sub>2</sub> (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-conjugated streptavidin (Dako). All slides were randomly coded and then assessed by two independent investigators. Using bright field microscopy, six consecutive non-overlapping fields from each liver section were photographed under high magnification (200 $\times$ ). ImageJ (National Institutes of Health) 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 the Macro function in ImageJ. 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 the results of the ANOVA. 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 the mean  $\pm$  SD unless otherwise specified.  $P < 0.05$  was considered statistically 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 the MHF-WT group ( $P < 0.001$ ) (Table 3). Net liver weight was also increased ( $P < 0.001$ ) proportionally to body weight. Plasma levels of non-esterified fatty acid (NEFA) were elevated to a small extent as a result of maternal HFD consumption (two-way ANOVA,  $P_{\text{MHF}} < 0.05$ ). Similarly, plasma triglyceride levels were increased in MHF-WT offspring compared to the MC-WT group ( $P < 0.01$ ).

MHF-Tg offspring had a significantly lower body weight compared to MHF-WT offspring ( $P < 0.001$ ) (Table 3). The net weight and percentage of body weight of EpiWAT and RpWAT in these offspring was 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 as a result of SIRT1 overexpression in the offspring (Table 3).

### SIRT1 overexpression does not change milk intake in MHF offspring

As shown in Fig. 1A and B, MHF-WT offspring had a higher milk intake ( $P < 0.001$  *vs.* MC-WT) and plasma leptin level ( $P < 0.01$  *vs.* MC-WT) compared to the control group. Concomitantly, there were significant reductions of hypothalamic mRNA expression of SIRT1 ( $P < 0.001$ ) (Fig. 1C) and leptin receptor (Ob-Rb) ( $P < 0.05$  *vs.* MC-WT) (Fig. 1D). In addition, mRNA expression of the orexigenic neuropeptide Y (NPY) was significantly decreased ( $P < 0.05$ ) in MHF-WT animals, probably as result of 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 Y1 receptor (NPY-1R), pro-opiomelanocortin (POMC) and melanocortin 4 receptor (MC4R).

Plasma levels of leptin were significantly reduced in MHF-Tg offspring ( $P < 0.05$ ) (Fig. 1B). However, despite



**Table 3. Anthropometric results**

	MC-WT ( <i>n</i> = 21) (mean ± SD)	MC-Tg ( <i>n</i> = 8) (mean ± SD)	MHF-WT ( <i>n</i> = 26) (mean ± SD)	MHF-Tg ( <i>n</i> = 11) (mean ± SD)	Main effect	MC-WT vs. MC-Tg	MC-WT vs. MHF-WT	MHF-WT vs. MHF-Tg
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. Compared to 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. <sup>a</sup>*P*<sub>interaction</sub> < 0.05. <sup>b</sup>*P*<sub>MHF</sub> < 0.05. <sup>c</sup>*P*<sub>SIRT1-tg</sub> < 0.05.

the trend to normalized mRNA expression of leptin receptor (*P* = 0.06) and MC4R (two-way ANOVA, *P*<sub>SIRT1-Tg</sub> < 0.05), no reduction in milk intake was detected (Fig. 1A). STAT3 was elevated by SIRT1 overexpression in MC offspring only (MC-WT vs. MC-Tg, *P* < 0.05) (Fig. 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) (Fig. 2A), suggesting glucose intolerance. This was associated with hyperinsulinaemia (*P* < 0.05) and significant reduction of QUICKI (*P* < 0.05) (Fig. 2B), indicating impaired insulin sensitivity in the offspring as a result of maternal HFD consumption. SIRT1 overexpression prevented glucose intolerance (*P* < 0.01 vs. MC-WT) (Fig. 1A) and normalized 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 transporters (GLUT)1 and 2 in the liver was also downregulated (two-way ANOVA, *P*<sub>MHF</sub> < 0.05) (Fig. 2C), suggesting impaired insulin signalling and glucose transport in offspring liver as a result of maternal HFD consumption. In line with the quantitative RT-PCR result, the immunoblotting result also indicated reduced GLUT2 protein expression in MHF-WT offspring (two-way ANOVA, *P*<sub>MHF</sub> < 0.05) (Fig. 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) (Fig. 2D). AMPK expression was unchanged and the pAMPK/AMPK ratio was significantly reduced (*P* < 0.05), suggesting reduced activity rather than expression. Because both pAkt and Akt levels were reduced, the relative ratio of pAkt/Akt was unchanged. Consistent with previous findings (Borengasser *et al.* 2014), liver expression of SIRT1 was reduced as a result of 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 (Fig. 2C and D).

### SIRT1 overexpression attenuates adipocyte hypertrophy in MHF offspring

H&E staining of EpiWAT revealed enlarged adipocytes in MHF offspring compared to the control (two-way ANOVA, *P*<sub>MHF</sub> < 0.05) (Fig. 3A), which was associated with a significant reduction in SIRT1 mRNA expression (two-way ANOVA, *P*<sub>MHF</sub> < 0.05) (Fig. 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) (Fig. 3B). No change was found in PPARγ mRNA levels. Similar to EpiWAT, RpWAT showed a significant reduction in SIRT1 mRNA levels in MHF offspring (two-way ANOVA, *P*<sub>MHF</sub> < 0.05) (Fig. 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) (Fig. 3B), whereas SREBP1c levels were unchanged.

SIRT1 overexpression significantly attenuated adipocyte hypertrophy in MHF-Tg offspring EpiWAT (*P* < 0.01) (Fig. 3A). The effect was associated with

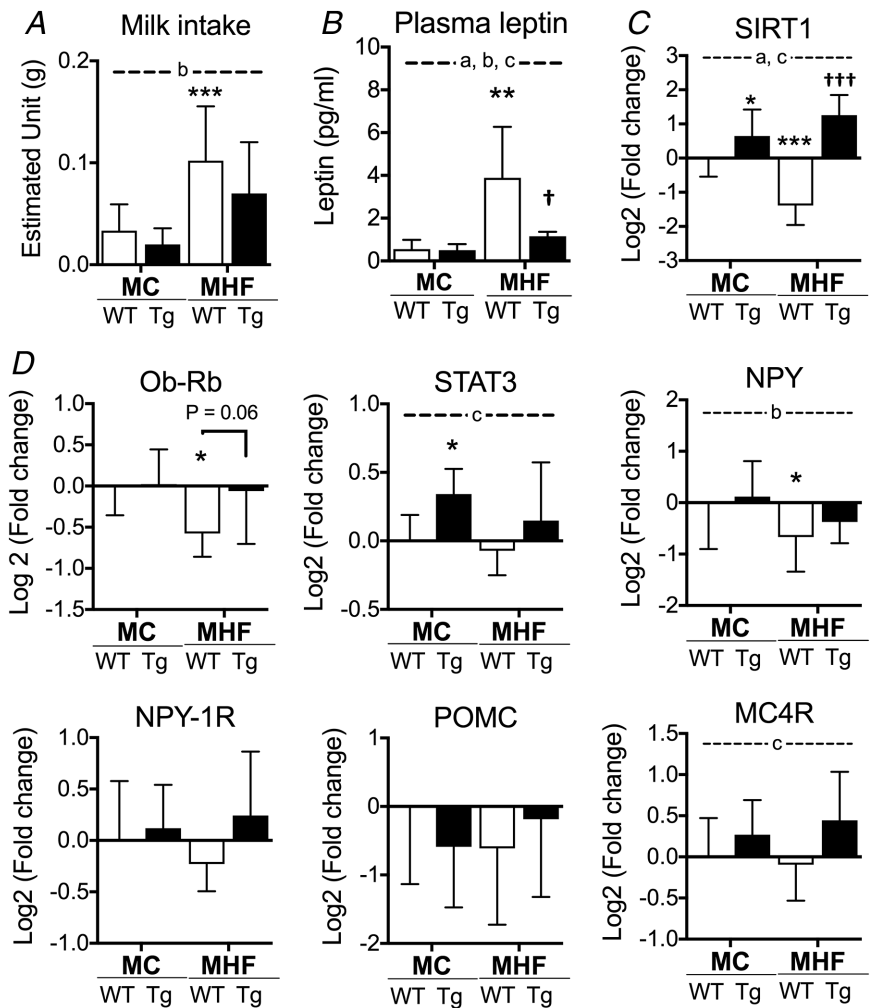
marked reductions in the mRNA expression of PGC-1 $\alpha$ , SREBP-1c and PPAR $\gamma$  ( $P < 0.01$ ,  $P < 0.05$  and  $P < 0.01$ , respectively) (Fig. 3B). There was a significant interaction between maternal diet and SIRT1 overexpression on mRNA expression of PGC-1 $\alpha$  and SREBP-1c in EpiWAT (two-way ANOVA,  $P_{\text{interaction}} < 0.05$ ). 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-1 $\alpha$  and SREBP-1c mRNA expression as a result of SIRT1 overexpression. Conversely, PPAR $\gamma$  expression was significantly increased ( $P < 0.01$ ) (Fig. 3B).

**SIRT1 overexpression attenuates liver lipogenesis and lipotoxicity in MHF offspring**

In the present study, MHF offspring showed increased lipid accumulation as reflected by liver TG levels, ORO staining (both  $P < 0.001$ ) (Fig. 4A), as well as H&E staining (Fig. 4B), in association with significantly reduced SIRT1 protein expression (Fig. 4B). The mRNA expression

of SREBP-1c, fatty acid synthase (FASN) and fatty acid uptake fatty acid binding protein (FABP)1 was significantly increased in MHF offspring ( $P < 0.05$ ,  $P < 0.05$  and  $P < 0.01$ , respectively) (Fig. 4D), suggesting lipotoxicity. Carbohydrate-responsive element-binding protein (ChREBP), a hepatic lipogenesis marker that acts in a glucose concentration-dependent manner, was also slightly upregulated as a result of MHF (two-way ANOVA,  $P_{\text{MHF}} < 0.05$ ). PPAR $\alpha$  mRNA expression was also increased (two-way ANOVA,  $P_{\text{MHF}} < 0.05$ ). By contrast, PGC-1 $\alpha$  and PPAR $\gamma$  mRNA expression were significantly downregulated (MHF-WT vs. MC-WT,  $P < 0.05$ ) (Fig. 4C). The results of SREBP1 and PGC-1 $\alpha$  were validated by protein expression analysis (MHF-WT vs. MC-WT,  $P < 0.05$ ) (Fig. 4D). mRNA expression of liver X receptor (LXR) $\beta$ , a marker of cholesterol efflux, was unchanged as a result of maternal HFD consumption.

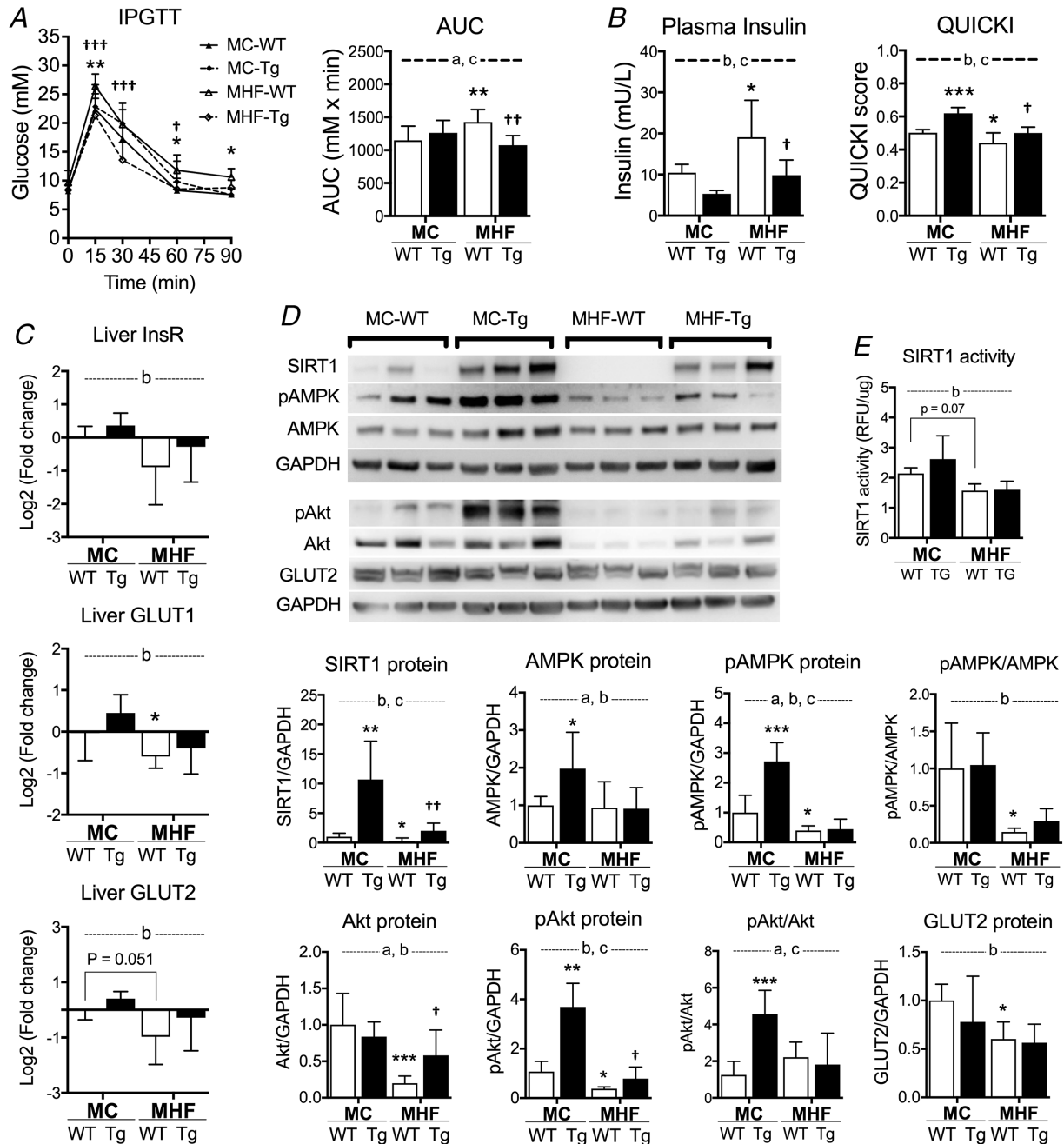
SIRT1 overexpression significantly decreased TG and ORO staining levels in the liver of MHF offspring ( $P < 0.05$  and  $P < 0.01$ ) (Fig. 4A and B). mRNA but not protein levels of PGC-1 $\alpha$  were upregulated in Tg offspring (two-way



**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 ( $n = 8$ ). Compared to MC-WT: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; vs. MHF-WT: † $P < 0.05$ , ††† $P < 0.001$ .  
<sup>a</sup>Two-way ANOVA,  $P_{\text{interaction}} < 0.05$ .  
<sup>b</sup>Two-way ANOVA,  $P_{\text{MHF}} < 0.05$ . <sup>c</sup>Two-way ANOVA,  $P_{\text{SIRT1-tg}} < 0.05$ .

ANOVA,  $P_{\text{SIRT1-tg}} < 0.05$ ). By contrast, the mRNA level of SREBP-1c was not significantly changed ( $P = 0.06$ ) (Fig. 4C), although its protein level was significantly suppressed ( $P < 0.05$ ) (Fig. 4D). Similar to the RpWAT, the PPAR $\gamma$  mRNA level in the liver was normalized by

SIRT1 overexpression in MHF-Tg offspring ( $P < 0.05$ ) (Fig. 4C). On the other hand, FASN and ChREBP were significantly suppressed (all  $P < 0.05$ ) (Fig. 4C). There was an interactive effect between maternal diet and SIRT1 overexpression on LXR $\beta$  expression (two-way ANOVA,



**Figure 2. Perinatal SIRT1 overexpression increases glucose tolerance and insulin sensitivity in MHF offspring**

A, IPGTT and area under the curve ( $n = 11, 8, 13$  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 ( $n = 6$ ). Compared to MC-WT: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; vs. MHF-WT: † $P < 0.05$ , †† $P < 0.01$ , ††† $P < 0.001$ . <sup>a</sup>Two-way ANOVA,  $P_{\text{interaction}} < 0.05$ . <sup>b</sup>Two-way ANOVA,  $P_{\text{MHF}} < 0.05$ . <sup>c</sup>Two-way ANOVA,  $P_{\text{SIRT1-tg}} < 0.05$ .



$P_{\text{interaction}} < 0.05$ ), with a significant decrease in MHF-Tg compared to MHF-WT offspring ( $P < 0.05$ ).

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

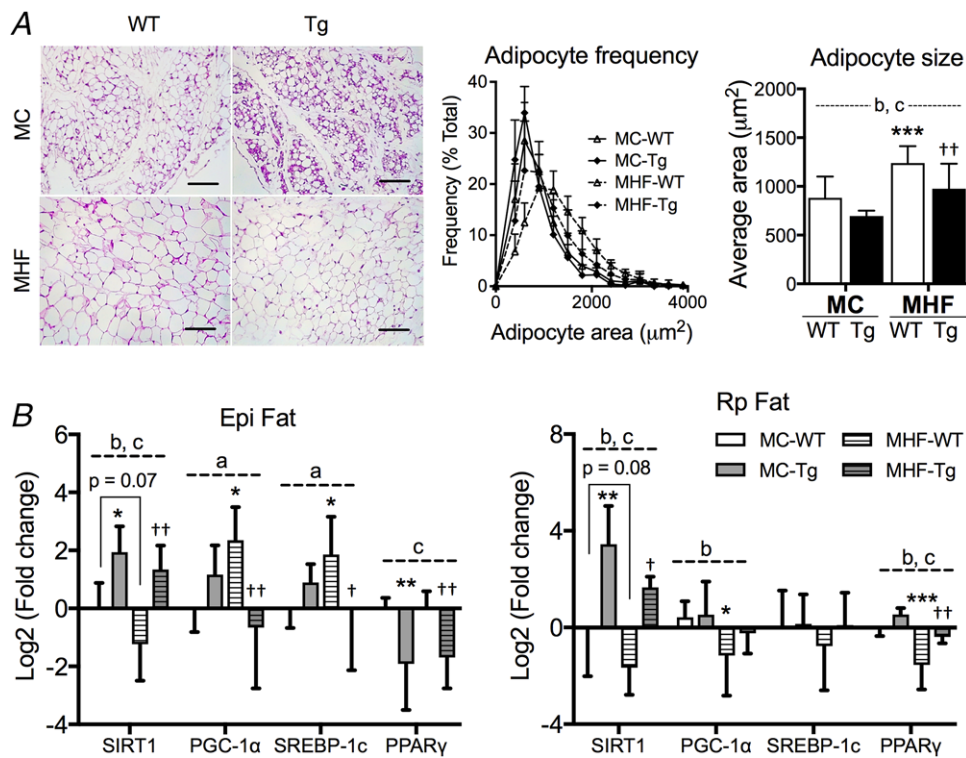
In association with increased lipid accumulation and altered levels of lipotoxicity markers, MHF-WT offspring also demonstrated increased mRNA expression of monocyte chemotactic protein (MCP)1, an inflammatory marker, and reduced expression of TGFβ receptor (TGFβR) types 1 and 2 in the liver (all  $P < 0.05$ ) (Fig. 5A). mRNA expression of anti-oxidant enzymes, including superoxide dismutase (SOD)2, glutathione peroxidase (GPx)-1 and catalase, in MHF-WT offspring liver was significantly suppressed by maternal HFD consumption (all  $P < 0.01$ ) (Fig. 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 liver, as reflected by IHC staining (all  $P < 0.05$ ) (Fig. 5C). The results were confirmed by PSR staining for collagens ( $P < 0.001$ ) (Fig. 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 normalized hepatic levels of TGFβR2 ( $P < 0.01$ ) but had little effect on the other inflammatory and macrophage markers in offspring liver (Fig. 5A). It also increased mRNA expression of NAPDH oxidase 2 (two-way ANOVA,  $P_{\text{SIRT1-tg}} < 0.05$ ) but concomitantly improved the levels of GPx-1 ( $P < 0.01$ ) and catalase ( $P < 0.05$ ) (Fig. 5B), which may reflect increased reactive oxygen species production and appropriate anti-oxidant 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 ( $P < 0.01$ ) (Fig. 5C).

**Discussion**

In the present 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 offspring at weaning. Importantly, we demonstrate that SIRT1 is a key player in this process, the systemic overexpression of which resulted in the

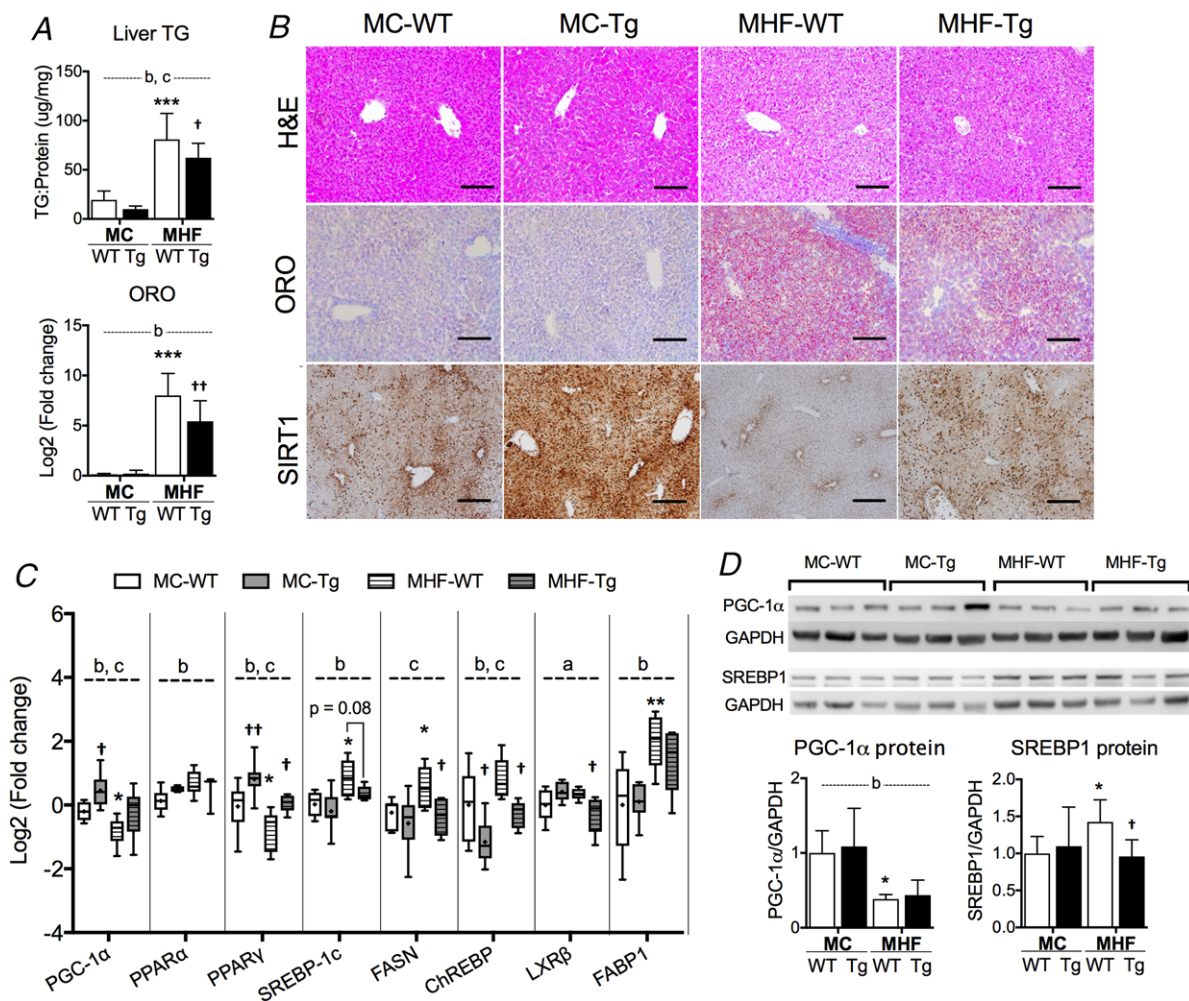


**Figure 3. Perinatal SIRT1 overexpression reversed hypertrophy in white adipose tissue in MHF offspring**  
 A, H&E staining and adipocyte frequency analysis. 200× magnification ( $n = 6$ ). B, quantitative RT-PCR analysis of offspring EpiWAT and RpWAT ( $n = 6$ ). Compared to MC-WT: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; vs. MHF-WT: †† $P < 0.01$ . <sup>a</sup>Two-way ANOVA,  $P_{\text{interaction}} < 0.05$ . <sup>b</sup>Two-way ANOVA,  $P_{\text{MHF}} < 0.05$ . <sup>c</sup>Two-way ANOVA,  $P_{\text{SIRT1-tg}} < 0.05$ . Scale bar = 200 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

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 the present study. Because both WT and Tg offspring were born to the same WT dams, offspring of both genotypes probably received similar nutrients during gestation and lactation. As such, the key difference that underlines the reduced body weight and adiposity in Tg offspring probably 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-1 $\alpha$  and increase fatty acid oxidation in muscles (Gerhart-Hines *et al.* 2007), with concordant suppression of PPAR $\gamma$  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 (i.e. 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-1 $\alpha$  was also increased, probably suggesting an adaptive mechanism. SIRT1 overexpression suppressed SREBP1c and PPAR $\gamma$ , which is in line with reduced EpiWAT mass and fat cell size in MHF-Tg offspring.



**Figure 4. Perinatal SIRT1 overexpression reduced liver weight and lipotoxicity in MHF offspring**

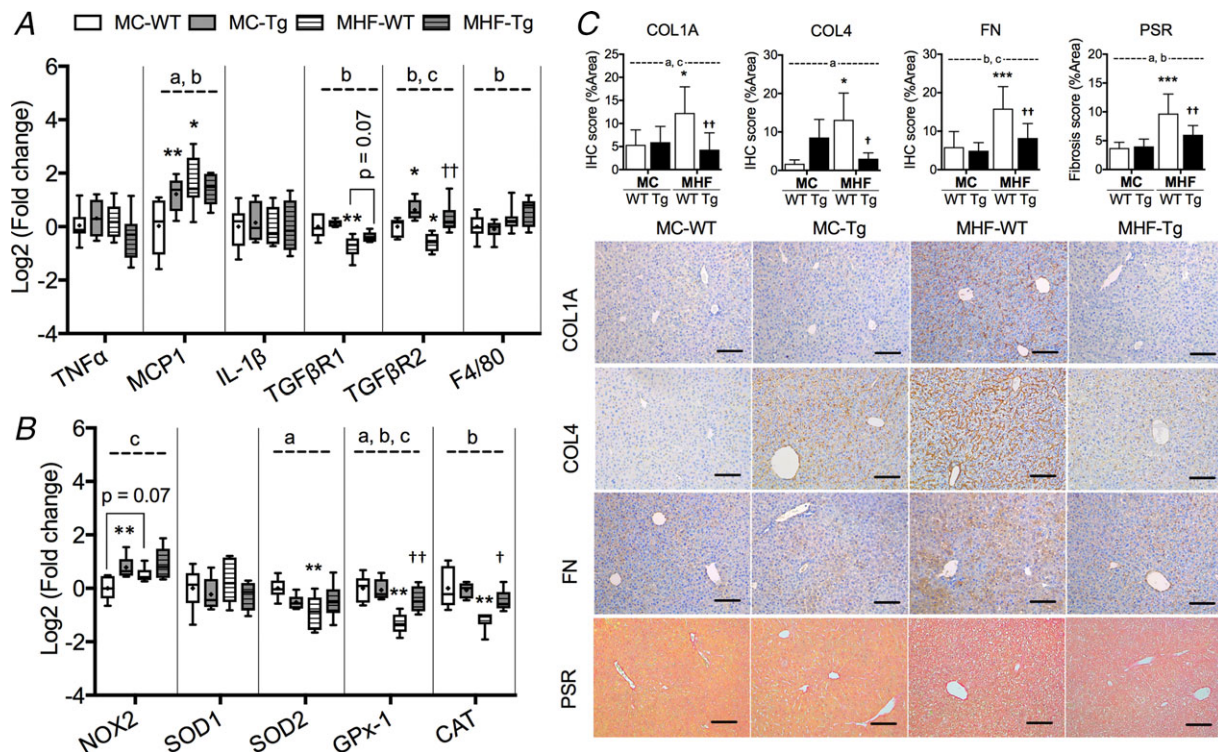
**A**, liver triglyceride (TG) and Oil Red O (ORO) staining quantitation ( $n = 6$ ). **B**, representative images of H&E, ORO and IHC staining of SIRT1. **C**, box plots of RT-PCR analysis of offspring liver. The boxes extend from the 25th to 75th percentiles, the central lines indicate the median. Whiskers extend from minimum to maximum values ( $n = 6$ ). **D**, protein expression of genes in SIRT1 signalling network ( $n = 6$ ). Compared to MC-WT: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; vs. MHF-WT: † $P < 0.05$ , †† $P < 0.01$ . <sup>a</sup>Two-way ANOVA,  $P_{\text{interaction}} < 0.05$ . <sup>b</sup>Two-way ANOVA,  $P_{\text{MHF}} < 0.05$ . <sup>c</sup>Two-way ANOVA,  $P_{\text{SIRT1-tg}} < 0.05$ . Scale bar = 100 µm. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

By contrast to EpiWAT, the RpWAT in MHF offspring showed a significant reduction of PGC-1 $\alpha$  and PPAR $\gamma$ , which is associated with smaller RpWAT mass and increased systemic insulin sensitivity. It has been shown that the expression of PPAR $\gamma$  and SREBP-1c in s.c. WAT is suppressed in obese patients with insulin resistance (Kolehmainen *et al.* 2001; Dubois *et al.* 2006). Thus, PPAR $\gamma$  agonists are used clinically as insulin sensitizers (Lebovitz *et al.* 2001; Ahmadian *et al.* 2013) and MHF mouse offspring treated with pioglitazone, a PPAR $\gamma$  agonist, have reduced body weight, reduced visceral WAT gain, increased s.c. WAT mass in association with lower levels of fasting glucose and insulin resistance (Kalanderian *et al.* 2013). In the present 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 PPAR $\gamma$  in the offspring RpWAT.

The reduced expression of GLUTs, InsR, pAMPK and pAkt indicates impaired glucose transport and insulin signalling in the offspring liver as a result of 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 that SIRT1 overexpression regulates liver insulin sensitivity via an Akt-dependent mechanism in our model. To support this hypothesis, PGC-1 $\alpha$ , 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<sup>+</sup> and AMP as a result of a 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. By 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, PPAR $\gamma$  expression was also upregulated in Tg offspring, which suggests another pathway for improved insulin sensitivity in the liver (Tiikkainen *et al.* 2004). Importantly, PPAR $\gamma$  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



**Figure 5. Perinatal SIRT1 overexpression attenuates inflammatory dysregulation and oxidative stress as a result of 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 minimum to maximum values ( $n = 6$ ). C, IHC and PSR staining quantification ( $n = 6$ ). Compared to MC-WT: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; vs. MHF-WT: † $P < 0.05$ , †† $P < 0.01$ . <sup>a</sup>Two-way ANOVA,  $P_{\text{interaction}} < 0.05$ . <sup>b</sup>Two-way ANOVA,  $P_{\text{MHF}} < 0.05$ . <sup>c</sup>Two-way ANOVA,  $P_{\text{SIRT1-Tg}} < 0.05$ . Scale bar = 100  $\mu\text{m}$ . [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



SREBP-1c, ChREBP and FASN, probably also contributes to such an effect. LXR $\beta$  not only is the master regulator of cholesterol, but also is involved in lipogenesis by stimulating the expression of SREBP-1c and FASN (Grefhorst *et al.* 2002). The downregulation of LXR $\beta$  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 liver was unchanged. As noted, AMPK/PGC-1 $\alpha$  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 the present study, SIRT1-mediated suppression of *de novo* lipogenesis by itself can not fully normalize lipid accumulation in the liver as a result of maternal HFD consumption.

Maternal HFD consumption was associated with inflammatory dysregulation, as reflected by increased expression of MCP-1 and reduced expression of TGF $\beta$  receptors 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 TGF $\beta$  signalling pathways is required to determine whether these abnormalities reflect inflammatory disorders or compensatory responses. In the present study, maternal HFD also led to significant suppression of endogenous anti-oxidants, including SOD2, GPx-1 and CAT, suggesting increased oxidative damage in offspring 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 anti-oxidant capacity and attenuate fibrogenesis in the liver as a result of 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 the present 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 probably minimal.

Collectively, the present study provides direct evidence of the importance of SIRT1 in linking maternal HFD consumption to metabolic dysfunction in the offspring, and suggests that targeting SIRT1 in the offspring in early developmental periods may reprogram metabolic disorders as a result of maternal HFD feeding. Further studies are required to examine the long-term effects of these approaches in adulthood and across generations. In addition, because 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.

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## Additional information

### Competing interests

The authors declare that they have no competing interests.

### Author contributions

LN designed and conducted all of the main experiments, performed the data analysis, and prepared the figures and the manuscript. AZ assisted with the tissue processing for histology. HC, CP and SS co-ordinated the execution of the project and were involved in the experimental design. HC, CP and SS reviewed the data analysis and the manuscript. All authors have critically revised and approved the final copy of the manuscript submitted for publication.

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