

Original Article

Hepatic FoxO3 Supports Metabolic Resilience, Attenuates Age-Associated Obesity and Metabolic Dysfunction, and Mitigates Diet-Induced Steatohepatitis

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ABSTRACT: Forkhead box O3 (FoxO3) is implicated in aging and metabolic regulation; however, its roles in the local and systemic control of aging remain incompletely understood. In this study, we characterized the effects of hepatocyte-specific *Foxo3* deletion on hepatic metabolic and whole-body energy homeostasis during aging, and the susceptibility of mice to metabolic disease. Male hepatocyte-specific *Foxo3* knockout (*h-Foxo3*^{-/-}) mice developed moderate obesity and higher plasma insulin levels by middle age, despite no difference in food intake compared with wild-type mice. Indirect calorimetry revealed lower energy expenditure relative to body weight, a higher respiratory quotient, and less fatty acid oxidation, indicating a shift toward carbohydrate-dependent energy metabolism. Adipose tissue exhibited inflammatory cell infiltration and high expression of inflammatory and senescence markers, consistent with systemic insulin resistance. Although these findings suggested that hepatokine signaling regulates systemic metabolism, there was limited direct evidence for FoxO3-dependent hepatokine secretion. RNA-sequencing analysis suggested that fatty acid processing and xenobiotic and detoxification pathways were suppressed in *h-Foxo3*^{-/-} liver and that gluconeogenic and proinflammatory programs were activated. *h-Foxo3*^{-/-} mice exhibited hepatic steatosis and an increase in the oxidized glutathione concentration when consuming a regular diet, and those consuming a fat-, fructose-, and cholesterol-rich diet, which induces steatohepatitis, showed more severe fibrosis than controls. Collectively, these findings suggest that hepatic FoxO3 is a key regulator coordinating hepatic lipid processing, xenobiotic metabolism and detoxification, susceptibility to metabolic disorders, and systemic metabolic flexibility. Thus, hepatic FoxO3 contributes to the maintenance of metabolic resilience during aging.

Keywords: FoxO, Aging, Liver, Energy metabolism, Steatohepatitis

INTRODUCTION

Forkhead box O (FoxO) transcription factors are key regulators of nutrient sensing, insulin signaling, and stress response pathways, which collectively regulate metabolic homeostasis and longevity [1, 2]. Dietary restriction (DR), a robust intervention that extends lifespan [3], is associated with the activation of FoxOs, which coordinate adaptive metabolic and cytoprotective processes.

Insulin signaling suppresses FoxO activity during feeding and promotes glycolysis and lipid biosynthesis, whereas fasting-induced FoxO activation supports

glucose production, while limiting the use of excess nutrients by anabolic pathways [4, 5]. As in insulin resistance, the disruption of this regulatory axis leads to inappropriate activation of FoxOs, particularly FoxO1, which contributes to metabolic disorders such as nonalcoholic fatty liver disease and diabetes [6]. Previous studies have shown that the deficiency of FoxO1/3/4 or FoxO1/3 in the liver causes severe metabolic abnormalities, even in young mice [4, 7], whereas the deficiency of FoxO3 alone has only a minimal effect [4]. In other words, although previous research has suggested that there is functional redundancy among FoxO

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transcription factors [8], it has also demonstrated the importance of FoxO3 in the effects of DR on aging and in human longevity [9, 10]. These effects of FoxO3 are context-specific, but may be critical in the presence of physiologic or pathologic stress.

The liver functions as an endocrine organ that regulates whole-body energy homeostasis by secreting hepatokines. These liver-derived factors modulate metabolic processes in peripheral tissues, including skeletal muscle and adipose tissue, thereby influencing systemic insulin sensitivity, metabolic substrate use, and inflammatory responses [11]. The dysregulation of hepatokine signaling has been implicated in the development of obesity and insulin resistance, thereby linking the metabolic state in the liver to organismal energy balance. However, the transcriptional mechanisms that coordinate hepatokine production in response to nutritional and aging-related cues remain incompletely understood.

Aging is characterized by a progressive decline in metabolic flexibility and greater susceptibility to metabolic disease, which are often associated with impaired coordination between hepatic metabolism and peripheral energy use. Given the established roles of FoxO3 in stress resistance and longevity [2, 12], we hypothesized that hepatic FoxO3 contributes to systemic metabolic homeostasis by regulating intracellular metabolic pathways and modulating hepatokine-mediated endocrine signaling. To test this hypothesis, we characterized the phenotype of hepatocyte-specific Foxo3 knockout (*h-Foxo3*^{-/-}) mice during aging, focusing on the integration of hepatic metabolism and whole-body energy balance.

MATERIALS AND METHODS

Animal procedures

The present study was conducted in accordance with the provisions of, and approved by, the Ethics Review Committee for Animal Experimentation at Nagasaki University (approval numbers: 1911071575 and 2103111702). Hepatocyte-specific *Foxo3* knockout (*h-Foxo3*^{-/-}) mice were generated by crossing *albumin*-Cre mice (Alb-cre, B6.Cg-Speer6-ps1Tg(Alb-re)21^{Mgn}/J) with floxed-*Foxo3* (*Foxo3*^{fl/fl}, Stock *Foxo3*^{tml} Rdp/J, The Jackson Laboratory, Bar Harbor, MA, USA) mice, which were established on a C57BL6/J genetic background through more than 10 backcrosses at Nagasaki University School of Medicine. The Alb-cre mice were kindly provided by Professor Koichi Ikuta at the Institute for Frontier Life and Medical Sciences, Kyoto University, with permission for distribution from Professor Mark Magnuson at Vanderbilt University School of Medicine,

Nashville, TN, USA. A tail biopsy of weanling mice was performed for genotyping by PCR using specific primers (Supplementary text). The mRNA expression of exons 2–3 of the *Foxo3* gene was significantly lower in *h-Foxo3*^{-/-} livers than in the livers of control (Ctrl) mice (Supplementary Fig. 1A). In addition, the abundance of FoxO3 protein in the nuclear fraction of the livers of the *h-Foxo3*^{-/-} mice was approximately 25% of that of the Ctrl group (Supplementary Fig. 1B, C). By contrast, the abundance of nuclear FoxO1 did not differ significantly between Ctrl and *h-Foxo3*^{-/-} livers (Supplementary Fig. 1D, E).

The animal procedures have been reported elsewhere [13]. Briefly, male Ctrl flox-*Foxo3* and *h-Foxo3*^{-/-} mice were kept under specific pathogen-free conditions, at 22°C, under a 12-hour dark/light cycle, with free access to water and a regular CRF-1 diet (Oriental Yeast Co. Ltd., Tokyo, Japan) throughout the experiment. The body weights (BW) and food intakes of the mice were measured every 2 weeks from 10 to 24 weeks of age, and every 4 weeks thereafter. The mice were euthanized by exsanguination under isoflurane anesthesia during middle age (69–77 weeks of age, wk) or old age (96–110 wk) after overnight fasting or under *ad libitum*-fed conditions. Blood samples were obtained by cardiac puncture into tubes containing EDTA-2Na and aprotinin, and plasma was separated by centrifugation at 2,000 × *g* for 10 min at 4°C. In addition, the liver and the inguinal, perirenal, and peritesticular adipose depots were quickly removed from each mouse, diced, snap-frozen in liquid nitrogen. These samples were stored at -80°C until use, except for portions of the livers and peritesticular adipose depots, which were fixed in buffered 4% formaldehyde, routinely processed, and embedded in paraffin blocks for histologic and immunohistochemical examination.

Indirect calorimetry and micro-computed tomography

At 60–63 weeks of age, the mice underwent indirect calorimetry. The volumes of carbon dioxide they produced (VCO₂) and oxygen they consumed (VO₂) were measured in metabolic cages equipped with an indirect open-circuit calorimeter (MK-5000RQ, Muromachi Kikai Co., Tokyo, Japan). The gas contents were measured for 10 seconds every 3 minutes over 24 hours. Average values of these parameters were calculated every 30 minutes. The respiratory quotient (RQ), energy expenditure (EE), and fatty acid oxidation (FAO) of the mice were calculated using the following formulae [14, 15]:

$$RQ = VCO_2/VO_2$$

$$EE \text{ (kJ/h)} = 4.184 \times (3.815 + 1.232 \times RQ) \times VO_2 \text{ (L/kg BW/h)} \times BW \text{ (kg)}$$

$$FAO \text{ (kJ/h)} = EE \times (1 - RQ/0.3).$$

The visceral and subcutaneous fat contents of the mice were evaluated at 68 weeks of age using 3D micro-computed tomography (CT) (Rigaku Co., Tokyo, Japan). Abdominal CT images of the mice obtained under isoflurane anesthesia were analyzed using CT Axial Metabolic Analysis (v.4.05.06, Rigaku Co., Tokyo, Japan). Adipose tissue was identified using the settings specified by the manufacturer; adiposity is expressed as the percentage of the reference body area that was adipose tissue.

Blood chemistry

The glucose concentrations of blood samples collected by cardiac puncture were determined using a FreeStyle Precision Neo meter (Abbott Diabetes Care Inc., Alameda, CA, USA). Plasma insulin concentrations were measured using an Ultra-sensitive Mouse/Rat Insulin ELISA Kit (Morinaga Institute of Biological Science, Yokohama, Japan). The plasma triglyceride (TG) and non-esterified fatty acid (NEFA) concentrations were measured using LabAssay™ Triglyceride and LabAssay™ NEFA reagents (Fujifilm Wako Pure Chemical Corp., Osaka, Japan), according to the manufacturer's protocol. The plasma Mac-2 binding protein (Mac-2bp) concentration was measured using a Mouse Mac-2 Binding Protein Assay Kit (IBL Co., Ltd., Gunma, Japan). The plasma TG and total cholesterol concentrations and the aspartate aminotransferase (AST) activity were measured by Oriental Yeast Co., Ltd. (Tokyo, Japan).

The quantitative insulin sensitivity check index (QUICKI) was calculated using the following equation: $QUICKI = 1 / [\log(I_0) + \log(G_0)]$, where I_0 is the fasting insulin concentration (mIU/mL) and G_0 is the fasting glucose concentration (mg/dL) [16].

Quantitative PCR

RNA was isolated from frozen tissue using the ReliaPrep™ RNA tissue miniprep system (Promega, Madison, WI, USA), and then cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). The relative mRNA expression of target genes was measured by quantitative polymerase chain reaction (qPCR) using Thunderbird SYBR qPCR Mix (Toyobo) or Thunderbird Probe qPCR Mix (Toyobo), according to the manufacturer's protocol. qPCR was performed using a Light Cycler 480II (Roche Diagnostics, Mannheim, Germany), using the following program: 95°C for 1 min, then 45 cycles of amplification at 95°C for 15 s and 60°C for 1 min, followed by a dissociation curve analysis step. To quantify expression, standard curves were constructed by plotting the results of the

amplification of eight concentrations of liver cDNA from Ctrl-AL mice (arbitrary units). PCR assays were performed in duplicate. The expression of the target genes was normalized to that of ribosomal protein S18 (*Rps18*) or ATP synthase peripheral stalk-membrane subunit b (*Atp5f1*). The primer and probe sequences used are listed in Supplementary Table 1.

RNA-sequencing (seq) in the liver

RNA-seq analysis was performed on livers from middle-aged Ctrl and *h-Foxo3*^{-/-} mice, which were collected under overnight-fasted and non-fasted conditions (n = 3 each). RNA was isolated from frozen liver samples using the ReliaPrep™ RNA tissue miniprep system (Promega). The quality and quantity of the RNA samples obtained were evaluated using a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Rhexia, Inc. (Tokyo, Japan) prepared cDNA libraries and performed the sequencing. Strand-specific RNA-seq libraries were prepared using a NEBNext Ultra II Directional RNA Library Prep Kit (New England BioLabs, Ipswich, MA, USA) and a NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs), according to the manufacturer's instructions. Sequencing was performed on an Illumina NovaSeq X Plus (Illumina Inc., San Diego, CA, USA) platform to generate 150 bp paired-end reads. The quality of the raw paired-end sequence reads was evaluated using the quality control program FastQC (v.0.11.7; www.bioinformatics.babraham.ac.uk/projects/fastqc/), and adaptor sequence trimming was performed using Trimmomatic (v.0.38; www.usadellab.org/cms/?page=trimmomatic). Each mRNA species present was quantified using HISAT2 (v.2.1.0; <https://daehwankimlab.github.io/hisat2/>), featureCounts (v.1.6.3; <https://subread.sourceforge.net/featureCounts.html>), and DESeq2 (version 1.24.0; <https://bioconductor.org/packages/release/bioc/html/DESeq2.html>). The RNA-seq reads were then mapped to the mouse reference sequence genome (GRCmm10; www.ncbi.nlm.nih.gov/datasets/genome/GCF_000001635.20/). Normalization of the raw read counts was performed using the transcript per million (TPM) method and DESeq2. The raw read data were deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE328603.

Genes that were differentially expressed (DEGs) between the Ctrl and *h-Foxo3*^{-/-} livers were identified using fold differences of >1.5 or <0.67, and a raw *p* of <0.01. Pathway enrichment analysis was performed using ShinyGO (v.0.82) [17] and these DEGs. KEGG pathway enrichment [18] was assessed using a hypergeometric test against the *Mus musculus* genome background, with correction for the false discovery rate (FDR). Pathways with an FDR < 0.05 were considered to be significantly

enriched. A hierarchical clustering tree of the enriched gene sets was then created using ShinyGO v.0.82.

Because the RNA-seq analysis included only three biological replicates per group, gene-level comparisons using a nominal *p*-value threshold of <0.01 were considered exploratory. No multiple-testing correction was applied at the individual-gene level; therefore, these findings are susceptible to false positives and require independent validation. FDR correction was applied to the pathway-enrichment analyses, and biological interpretation focused primarily on coordinated pathway-level changes rather than on individual transcripts.

Histologic analysis

Six- μ m-thick paraffin sections were subjected to hematoxylin and eosin (HE) or Sirius Red staining according to standard protocols. Immunohistochemistry for F4/80 was used to identify macrophages [19, 20] in paraffin-embedded tissue sections deparaffinized using xylene and rehydrated using graded ethanol. Antigen retrieval was performed by incubation with proteinase K (code: 415231; Nichirei, Osaka, Japan) for 8 min at room temperature. Endogenous peroxidase activity was quenched with 3% H₂O₂ for 15 min. The sections were blocked with Blocking-One Histo (code: 06349-64; Nacalai Tesque, Kyoto, Japan) and incubated with F4/80 primary antibody ($\times 200$; code: ab6640; Abcam, Cambridge, UK) overnight at 4°C. After washing with PBS, the sections were incubated with Histofine Simple Stain Mouse MAX-PO (Rat) (code: 414311; Nichirei), and immunohistochemically positive areas were visualized using Liquid DAB+ Substrate Chromogen (code: K3468; Agilent Technologies). Nuclei were counterstained with hematoxylin. Negative controls were prepared in the same way, with the omission of the primary antibody.

To evaluate the level of macrophage infiltration into the peritesticular adipose tissue depot, the F4/80-positive areas on JPEG images captured using a Nikon microscope equipped with a Digital Sight DS-Fi2 camera system (Nikon Solutions Co., Ltd., Tokyo, Japan), with blinding to the experimental group. Each image was processed using ImageJ (NIH, Bethesda, MD, USA) on a Mac OS X computer (v.1.53a) [21]. DAB-positive areas were randomly selected from three images per mouse, extracted using the ImageJ plug-in “Colour deconvolution2”, and converted to 8-bit images (<https://blog.bham.ac.uk/intellimic/g-landini-software/colour-deconvolution-2/>).

The threshold for the elimination of background staining was determined by analyzing DAB-negative areas in each section immunostained using the F4/80 antibody. The percentage of each image that showed F4/80 staining of an intensity above a threshold was

calculated relative to the total area analyzed for each mouse.

To assess hepatic fibrosis, images of each entire Sirius Red-stained section were captured using an all-in-one fluorescence microscope (BZ-X700, Keyence, Osaka, Japan). The Sirius Red-stained collagen-positive areas and signal intensities in one image from each mouse were automatically quantified using the BZ-X Analyzer software (BZ-H3A, Keyence). Threshold parameters were optimized using the Hybrid Cell Count application (BZ-H3CM, Keyence) and subsequently applied to all the images using the Macro Cell Count application (BZ-H3CM, Keyence) for automated batch analysis, using the manufacturer’s recommended settings.

Quantification of the oxidized and reduced forms of glutathione (GSSG and GSH)

The assay was performed using a GSSG/GSH Quantification Kit II (#G263; Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer’s instructions. Briefly, 50 mg of frozen liver tissue was homogenized in 0.5 mL of ice-cold 5% 5-sulfosalicylic acid (SSA) solution (#192-04571; Fujifilm Wako Pure Chemical Corporation). Following centrifugation at 8,000 $\times$ g for 10 min at 4°C, the supernatant was transferred to a fresh tube and diluted 10-fold with distilled water (final SSA concentration, 0.5%) prior to analysis. The diluted samples were then analyzed by measuring their absorbances at 405 nm using a PHERAstar FS (BMG Labtech, Ortenberg, Germany), with normalization to the protein concentrations determined using the BCA assay.

Induction of steatohepatitis by Gubra amylin nonalcoholic steatohepatitis diet consumption

Male Ctrl and *h-Foxo3*^{-/-} mice were fed the regular CRF-1 diet until 12 wk, then fed the regular CRF-1 diet or Gubra amylin nonalcoholic steatohepatitis diet [GAN diet; 40% energy as fat, including 0% trans-fat and 46% saturated fat by mass, 22% fructose, 10% sucrose, and 2% cholesterol; D09100310N, Research Diets, Inc., New Brunswick, NJ, USA] *ad libitum* [22]. The mice were euthanized by exsanguination under isoflurane anesthesia at 31–33 wk or 44–46 wk, then plasma and liver samples were immediately collected, as described above.

Statistical analysis

The data are expressed as the mean and standard error (SE). The BW, FC, EE, RQ, and FAO data were analyzed using a linear mixed model with repeated measures design for the effects of genotype (G, Ctrl vs. *h-Foxo3*^{-/-}) and time, and the genotype $\times$ time interaction (G $\times$ T). The EE

was evaluated using analysis of covariance (ANCOVA), with adjustment for the effect of BW as a covariate. The normality of the data distributions was assessed by the visual inspection of normal quantile plots, together with a formal goodness-of-fit test, the Shapiro–Wilk test ($p > 0.05$) for residuals. Data were log-transformed to satisfy the assumption of normality if necessary. Although Shapiro–Wilk testing of FAO dataset indicated a statistically significant deviation from normality of the residuals after standard data transformation procedures,

such as log-transformation ($p = 0.0263$), the visual inspection of normal quantile plots showed no marked skewness or systematic deviation from normality. Given the relatively large number of repeated measurements, this small p -value was interpreted as reflecting the high sensitivity of the Shapiro–Wilk test to minor deviations from normality, rather than a serious violation of the assumptions for the model. Therefore, a mixed-effects model was also considered appropriate for the analysis of FAO.

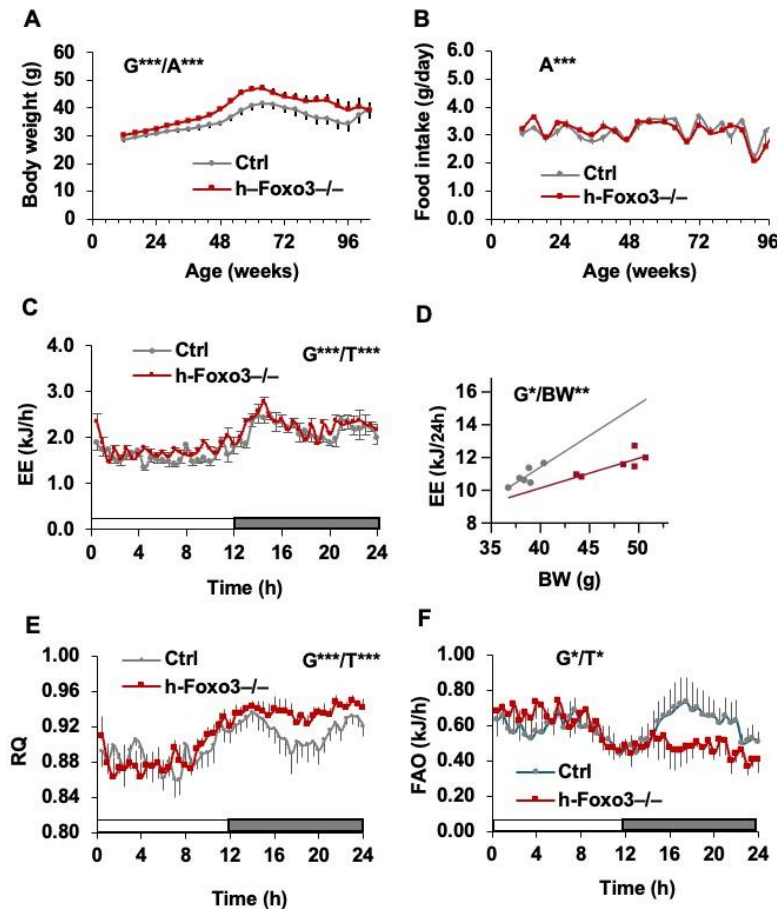


Figure 1. *h-Foxo3*^{-/-} mice develop obesity in middle age as a result of altered energy metabolism and substrate use. (A) Body weights of the mice consuming a regular diet (CRF-1) during aging. The data are the mean and SE at each age. There were initially 18 mice in the control (Ctrl) group and 17 in the *h-Foxo3*^{-/-} group; 9 mice remained in both groups at 96 wk. (B) Food intake (FI) during aging. The data are the mean and SE at each age ($n = 6$ cages per group until 51 wk of age; $n = 3$ or 4 cages at 96 wk of age). Three mice were housed in each cage, and the FI was calculated per mouse. (C) Energy expenditure (EE; kJ/h) of middle-aged Ctrl and *h-Foxo3*^{-/-} mice across the 24-h cycle. (D) Scatterplots of body weight and total EE over 24 h ($n = 6$ mice per group). $R^2 = 0.673$ for Ctrl and $R^2 = 0.623$ for *h-Foxo3*^{-/-}. (E) Respiratory quotient (RQ) over 24 h. (F) Fatty acid oxidation (kJ/h). In Figure 1C, E, and F, the bars and lines represent the mean and SE ($n = 6$ mice per group). G, A, and T, in panels A, B, C, E, and F, represent significant effects of genotype, aging, and time in a mixed model with repeated measures (** $p < 0.001$, * $p < 0.05$). G and BW in Figure 1D represent the significant effects of genotype and BW in the ANCOVA (G* $p < 0.05$; BW: ** $p < 0.01$).

The fat percentage on micro-CT images was evaluated using the Wilcoxon two-sample test, with exact two-sided probability correction for two independent groups. Blood, histologic, and mRNA datasets were compared using aligned-rank transformation (ART) two-way ANOVA for the effects of genotype (G) and fasting (F; fasted (+) vs. fed (-)), and the genotype $\times$ fasting interaction (G $\times$ F). The GAN diet datasets were also analyzed using ART ANOVA for the effects of genotype and age (A; 31–33 wk vs. 44–46 wk), and the genotype $\times$ age interaction (G $\times$ A). *Post hoc* pairwise comparisons were also performed using the Wilcoxon method. The prevalence of steatosis was evaluated using Fisher's exact test. Statistical significance was accepted at $p < 0.05$, and $p < 0.10$ was reported as borderline significance. Mixed model analysis with repeated measures, ANCOVA, and nonparametric statistical analyses for pairwise comparisons were performed in JMP® v.19.0.5 (Cary, NC, USA), and ART ANOVA was performed using Real Statistics (<https://real-statistics.com/>).

Results

Hepatocyte-specific deletion of the Foxo3 gene results in moderate obesity and insulin resistance in middle-aged mice

The BW of both the Ctrl- and *h-Foxo3*^{-/-} mice increased until 64 wk of age, then declined (age, $p < 0.0001$, mixed model with repeated measures; Fig. 1A). Throughout the period of aging, the BW of the *h-Foxo3*^{-/-} mice was higher than that of the Ctrl mice (genotype, $p < 0.0030$, mixed model with repeated measures), with a 10%–15% difference during middle age (60–76 wk). However, the overall change in BW with age did not differ significantly between the two groups. The food intake of the mice changed with age (age effect, $p < 0.0001$, mixed model with repeated measures; Fig. 1B), but did not differ between the groups, suggesting that the higher BW of the *h-Foxo3*^{-/-} mice was not driven by hyperphagia, but rather reflected a difference in energy balance.

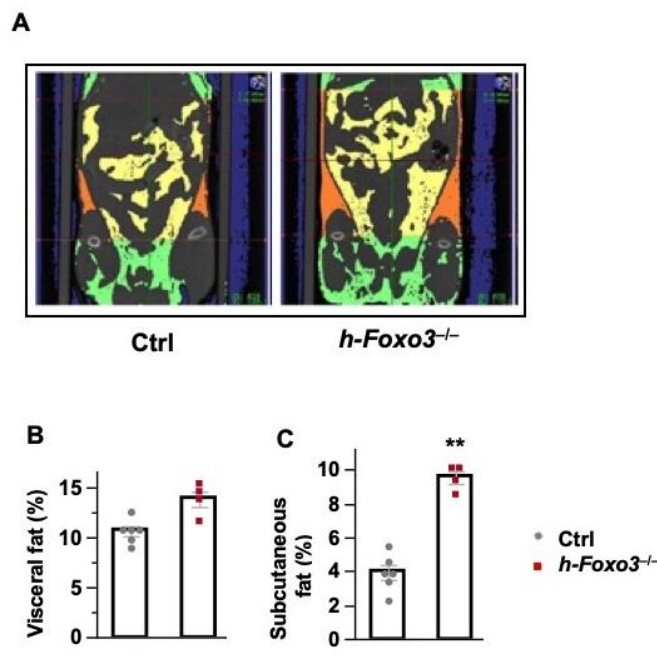


Figure 2. Body fat, measured using micro-CT during middle age. (A) The left and right panels show micro-CT images of a Ctrl mouse and an *h-Foxo3*^{-/-} mouse, respectively. The yellow and orange areas represent the visceral and subcutaneous fat depots, respectively. (B, C) The visceral and subcutaneous fat areas, shown as a percentage of the total area ($n = 6$ mice for the Ctrl group, $n = 4$ mice for the *h-Foxo3*^{-/-} group). Each point represents a measurement for an individual mouse. Gray circles represent Ctrl mice; red squares represent *h-Foxo3*^{-/-} mice. In panels C, ** $p < 0.01$ represent the significance level obtained for pairwise comparisons using the Wilcoxon two-sample test.

EE (kJ/h) displayed a pronounced diurnal rhythm, with higher values during the dark phase for both genotypes (time, $p < 0.0001$, mixed model with repeated measures; Figure 1C). The mean EE over 24 h was

slightly higher in the *h-Foxo3*^{-/-} mice than in the Ctrl mice (+ 6.5%, genotype, $p < 0.0001$, mixed model with repeated measures). However, when this was adjusted for BW using ANCOVA (15), the mean EE of the *h-Foxo3*^{-/-}

mice was significantly lower. The total EE over 24 h (kcal/24 h) positively correlated with the BW ($p = 0.0099$, ANCOVA: Fig. 1D), and the EE of the *h-Foxo3*^{-/-} mice was lower (genotype, $p = 0.0471$, ANCOVA). These findings indicate that hepatic FoxO3 deficiency is associated with lower EE relative to BW.

There was also a clear diurnal pattern in RQ (time, $p < 0.0001$, mixed model with repeated measures: Fig. 1E). This was higher in the *h-Foxo3*^{-/-} mice than in the Ctrl mice (genotype, $p = 0.0004$, mixed model with repeated

measures), particularly during the dark phase, indicating a shift toward the use of carbohydrates as an energy source [23]. Consistent with this, the *h-Foxo3*^{-/-} mice undertook less FAO (kcal/h) (genotype, $p = 0.0132$, mixed model with repeated measures; Fig. 1F), especially during the active phase. Collectively, these findings suggest that hepatic FoxO3 deficiency promotes the reprogramming of substrate use, with less lipid oxidation and greater reliance on carbohydrates.

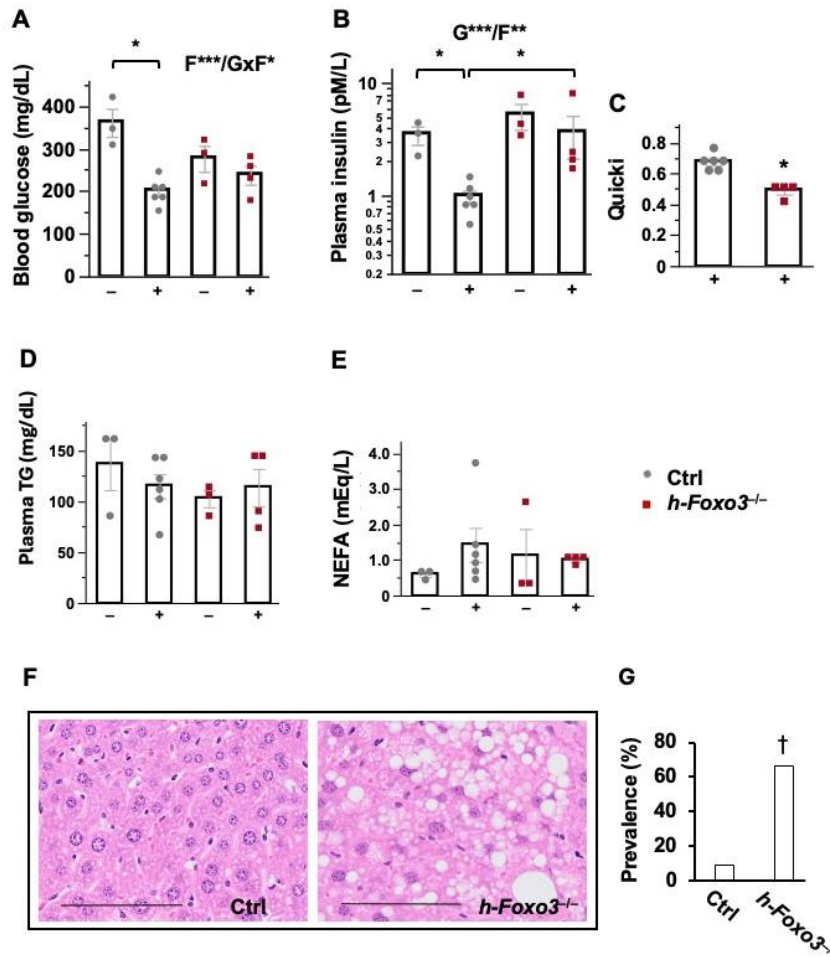


Figure 3. *h-Foxo3*^{-/-} mice show insulin resistance and hepatic steatosis in middle age. (A) Blood glucose concentration (mg/dL) under overnight fasted (+) or non-fasted (-) conditions. (B) Plasma insulin concentration (pmol/L). (C) Quantitative insulin sensitivity check index (QUICKI). (D) Plasma triglyceride concentration (TG, mg/dL). (E) Plasma non-esterified fatty acid concentration (NEFA, mEq/L). (F) Hepatic steatosis in *h-Foxo3*^{-/-} liver. Left panel: an HE-stained section of the liver without steatosis in a Ctrl mouse during middle age. Right panel: modest steatosis in a *h-Foxo3*^{-/-} mouse. (G) Prevalence of steatosis (n = 11 mice for Ctrl, n = 9 mice for *h-Foxo3*^{-/-}). The data for middle and old age are combined (Supplementary Figure 2). In panels A–E, the bars and lines represent the mean and SE (n = 3, 4, or 6 mice), and each point represents a measurement for an individual mouse. Gray circles represent Ctrl mice; red squares represent *h-Foxo3*^{-/-} mice. G, F, and G×F in panels A and B represent significant effects of genotype, fasting, and the genotype × fasting interaction by ART ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). In panels A, B, and C, * $p < 0.05$ represents the significance level obtained for pairwise comparisons using the Wilcoxon method. In panel G, † $p < 0.05$ by Fisher's exact test.

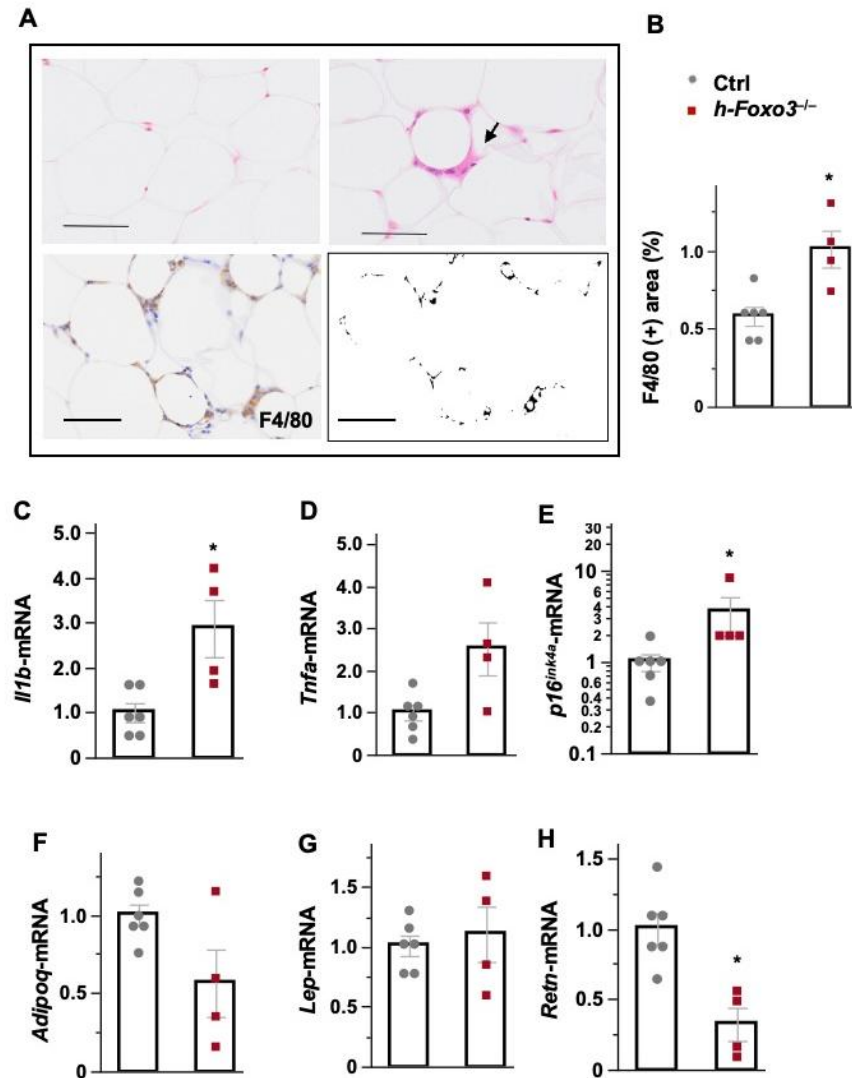


Figure 4. The peritesticular fat tissue in *h-Foxo3*^{-/-} mice has an insulin resistance-associated phenotype. (A) Microscopic images of the epididymal fat tissue of the mice during middle age. Upper panels: HE-stained sections; left, fat tissue without inflammation; right, infiltration of inflammatory cells into fat tissue (arrow). Lower panels: left, a section immunostained for F4/80 (the brown areas are the immunostained regions); right, an image processed by ImageJ for computer-assisted morphometry. The black areas are the immunostained regions. (B) The percentage of the adipose area that was F4/80-positive. (C) Interleukin 1beta (*Il1b*) mRNA expression. (D) Tumor necrosis factor α (*Tnfa*) mRNA expression. (E) *p16^{ink4a}* mRNA expression. (F) Adiponectin (*Adipoq*) mRNA expression. (G) Leptin (*Lep*) mRNA expression. (H) Resistin (*Retn*) mRNA expression. Target mRNA expression was normalized to that of *Rps18* and is expressed relative to that of the Ctrl group. The bars and lines represent the mean and SE ($n = 6$ or 4 mice), and each point represents a measurement for an individual mouse. Gray circles represent Ctrl mice; red squares represent *h-Foxo3*^{-/-} mice. * $p < 0.05$ by the Wilcoxon two-sample test.

Micro-CT at 68 weeks of age revealed significantly greater adiposity, particularly the subcutaneous fat, in the *h-Foxo3*^{-/-} mice (subcutaneous fat, $p = 0.0043$; Wilcoxon two-sample test; Fig. 2A-C). Consistent with this, the body, liver, and adipose tissue weights of the *h-Foxo3*^{-/-} mice were significantly higher during middle age

(Supplementary Table 2), indicating greater fat accumulation and organ enlargement.

Metabolic assessments revealed dysregulation of glucose–insulin homeostasis. Fasting significantly reduced the blood glucose and plasma insulin concentrations of the Ctrl mice, but not the *h-Foxo3*^{-/-}

mice (glucose: fasting, $p = 0.0004$; genotype $\times$ fasting, $p = 0.0331$; insulin: fasting, $p = 0.0046$; genotype, $p = 0.0007$; ART ANOVA; Fig. 3A, B). Notably, the fasting plasma insulin concentrations of the *h-Foxo3*^{-/-} mice were higher than those of the Ctrl mice ($p = 0.0142$, Wilcoxon method for pairwise comparisons), which was accompanied by a significantly lower QUICKI ($p = 0.0095$, Wilcoxon two-sample test; Fig. 3C), suggesting systemic insulin resistance. The plasma TG and NEFA concentrations did not differ between the two groups under fed or fasting conditions (Fig. 3D, E), suggesting that the circulating lipid status is not the primary determinant of the identified shift in substrate use.

Histologic examination of liver sections revealed modest steatosis in the *h-Foxo3*^{-/-} mice during middle age (in 3 of 4 mice), whereas no steatosis was observed in the Ctrl mice (in 0 of 6 mice; Fig. 3F; Supplemental Fig. 2A). During old age, modest steatosis was also identified in 3 of 5 *h-Foxo3*^{-/-} mice and 1 of 5 Ctrl mice (Supplementary Fig. 2B). When the data for both ages were combined, the prevalence of hepatic steatosis was significantly higher in the *h-Foxo3*^{-/-} mice than in Ctrl mice ($p = 0.017$, Fisher's exact test; Fig. 3G), suggesting greater susceptibility to hepatic lipid accumulation.

Given the established link between adipose tissue inflammation and systemic insulin resistance [24], we next characterized the phenotype of the peritesticular white adipose depot. Histologic examination demonstrated inflammatory cell infiltration in the peritesticular fat of the *h-Foxo3*^{-/-} mice during middle age (Fig. 4A). Quantification of the F4/80-positive area confirmed the presence of significantly greater inflammatory cell infiltration into *h-Foxo3*^{-/-} fat ($p = 0.0190$, Wilcoxon two-sample test; Fig. 4B). Consistent with this, the expression of genes encoding a pro-inflammatory cytokine and a senescence marker was higher in *h-Foxo3*^{-/-} adipose than in Ctrl adipose (*Il1b*, $p = 0.0190$; *p16*^{ink4a}, $p = 0.0190$; Wilcoxon two-sample test; Fig. 4C–E), suggesting higher levels of inflammation and senescence-associated remodeling.

Whole-body metabolism and insulin sensitivity are also affected by adipokines [25, 26]. The *Adipoq* and *Lep* expression in the adipose tissue of *h-Foxo3*^{-/-} mice did not differ significantly between the genotypes. By contrast, *Retn* expression was significantly lower ($p = 0.0190$, Wilcoxon two-sample test; Fig. 4F–H). These findings do not directly support a causal relation of adipokines with insulin resistance. By contrast, the presence of adipose inflammation is consistent with the notion that hepatic FoxO3 deficiency induces adipose tissue dysfunction, which may contribute to the systemic metabolic impairment.

Possible contribution of hepatokines to the systemic metabolic alterations in *h-Foxo3*^{-/-} mice

Hepatic FoxO3 deficiency led to systemic alterations in energy metabolism and blood chemical indices suggestive of insulin resistance, which may be mediated at least in part by altered hepatokine signaling [11]. To explore this possibility, we measured the hepatic expression of candidate hepatokines, which is known to be regulated by FoxOs [27–33].

Pck1 expression was measured as an index of hepatic insulin responsiveness. *Pck1* mRNA expression was significantly induced by fasting (fasting, $p = 0.0002$, ART ANOVA; Fig. 5A), consistent with the activation of gluconeogenesis. However, this response was attenuated in *h-Foxo3*^{-/-} liver, with a higher tendency for expression under the fed condition (genotype $\times$ fasting, $p = 0.0181$, ART ANOVA; Ctrl-fed vs. *h-Foxo3*^{-/-}-fed, $p = 0.0809$, Wilcoxon method for pairwise comparisons), suggesting impaired suppression of gluconeogenesis.

Of the hepatokines evaluated, *Igf1*, *Sepp1*, *Sulf2*, *Serpinb1*, and *Gdf15* expression did not show significant effects of genotype, although *Sepp1*, *Sulf2*, and *Serpinb1* expression was induced by fasting (fasting, $p = 0.0017$, $p = 0.0260$, and $p = 0.0034$; ART ANOVA; Fig. 5B–E). Only *Fst* mRNA expression tended to be higher in *h-Foxo3*^{-/-} liver than in Ctrl liver, particularly in the fed condition (genotype $\times$ fasting, $p = 0.0241$, ART ANOVA; Ctrl-fed vs. *h-Foxo3*^{-/-}-fed, $p = 0.0809$, Wilcoxon method for pairwise comparisons; Fig. 5G). The effect of hepatic FoxO3 deficiency on the systemic metabolic phenotype may be mediated by FoxO3-regulated hepatokines, but the measurement of the plasma concentrations of selected hepatokines, including *Fst*, revealed no significant effect of genotype or a genotype $\times$ fasting interaction (Supplementary Fig. 3).

RNA-seq analysis reveals that hepatic FoxO3 regulates lipid metabolism and xenobiotic/detoxification pathways

To further explore the molecular basis of the metabolic phenotype of the *h-Foxo3*^{-/-} mice, we performed RNA-seq analysis of the livers of middle-aged mice under fed and fasting conditions. In Ctrl liver, fasting induced appropriate metabolic adaptation, characterized by the upregulation of PPAR α -driven FAO, gluconeogenesis, and inflammatory signaling pathways, and the suppression of glycolysis, lipogenesis, and xenobiotic/glutathione metabolism (Supplementary Fig. 4A, B; Supplementary Table 3A, B).

Under fasting conditions, genes involved in xenobiotic metabolism and detoxification pathways in *h-Foxo3*^{-/-} liver were significantly enriched (Fig. 6A;

Supplementary Fig. 4C; Supplementary Table 4A). These included genes encoding phase II conjugation enzymes (*Gstm2/3*, *Gstp3*, *Ugt1a5*, and *Sult2a7*), cytochrome P450 enzymes (*Cyp2c55* and *Cyp2c70*), and glutathione-dependent antioxidant systems (e.g., *Gpx6*), suggesting

coordinated activation of detoxification and antioxidant responses. Furthermore, the enrichment of retinol metabolism and bile secretion pathways may imply a broader remodeling of hepatic metabolic and excretory functions.

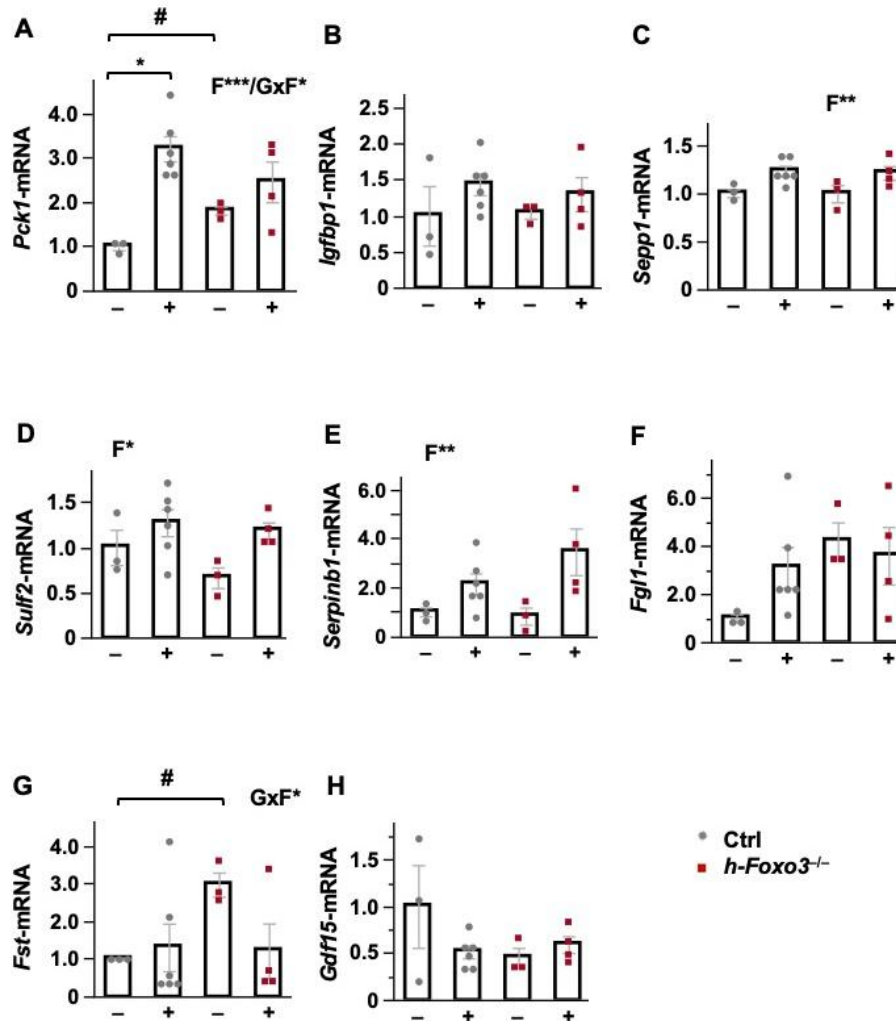


Figure 5. Hepatokine mRNA expression in *h-Foxo3^{-/-}* liver. (A) Phosphoenolpyruvate carboxykinase 1 (*Pck1*) mRNA expression, a marker of gluconeogenesis and insulin resistance. (B) Igf-binding protein 1 (*Igf1bp1*) mRNA expression. (C) Selenoprotein (*Sepp1*) mRNA expression. (D) Sulfatase 2 (*Sulf2*) mRNA expression. (E) Serine peptidase inhibitor, clade b, member1 (*Serpinb1*) mRNA expression. (F) Fibrinogen-like protein 1 (*Fgl1*) mRNA expression. (G) Follistatin (*Fst*) mRNA expression. (H) Growth and differentiation factor 15 (*Gdf15*) mRNA expression. Target mRNA expression was normalized to that of *Atp5f1*. In panels A–H, the bars and lines represent the mean and SE (n = 3, 4 or 6 mice), and each point represents a measurement for an individual mouse. In panels A–H, F and G×F indicate a significant effect of fasting and a significant genotype × fasting interaction, respectively, by ART ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). *** $p < 0.001$ and # $p < 0.10$ indicate significance and borderline significance, according to pairwise comparisons using the Wilcoxon's method after ART ANOVA.

By contrast, genes encoding components of immune system-related pathways were overrepresented among those that were downregulated in fasted *h-Foxo3^{-/-}* liver (Fig. 6A; Supplementary Table 4B), including genes

involved in the hematopoietic cell lineage, cytokine–cytokine receptor interactions, and T-cell differentiation. There was lower expression of genes encoding immune and cytokine signaling proteins (e.g., *Cd3e*, *Cd4*, *Il7r*,

Il2rg, *Ccl5*, and *Ccl8*), and modest suppression of those involved in amino acid metabolic pathways. Collectively, these findings indicate that FoxO3 deficiency during fasting shifts hepatic transcription to favor detoxification over immune signaling and amino acid metabolism.

In the fed state, complement and inflammatory pathway genes were well represented among those that were upregulated in *h-Foxo3*^{-/-} liver (Fig. 6A; Supplementary Fig. 4E; Supplementary Table 4C),

including the complement components *C3*, *C4b*, and *C6*–*C9*, coagulation factors, and the cytokine signaling mediators *Il1b*, *Il6ra*, and *Stat3* (Fig. 6B). The concurrent enrichment of genes encoding proteins involved in adipocytokine signaling and insulin resistance (*G6pc*, *Pck1*, and *Irs2*; Fig. 6C; Supplementary Fig. 4E), suggests the inappropriate activation of gluconeogenesis and insulin resistance under the fed condition.

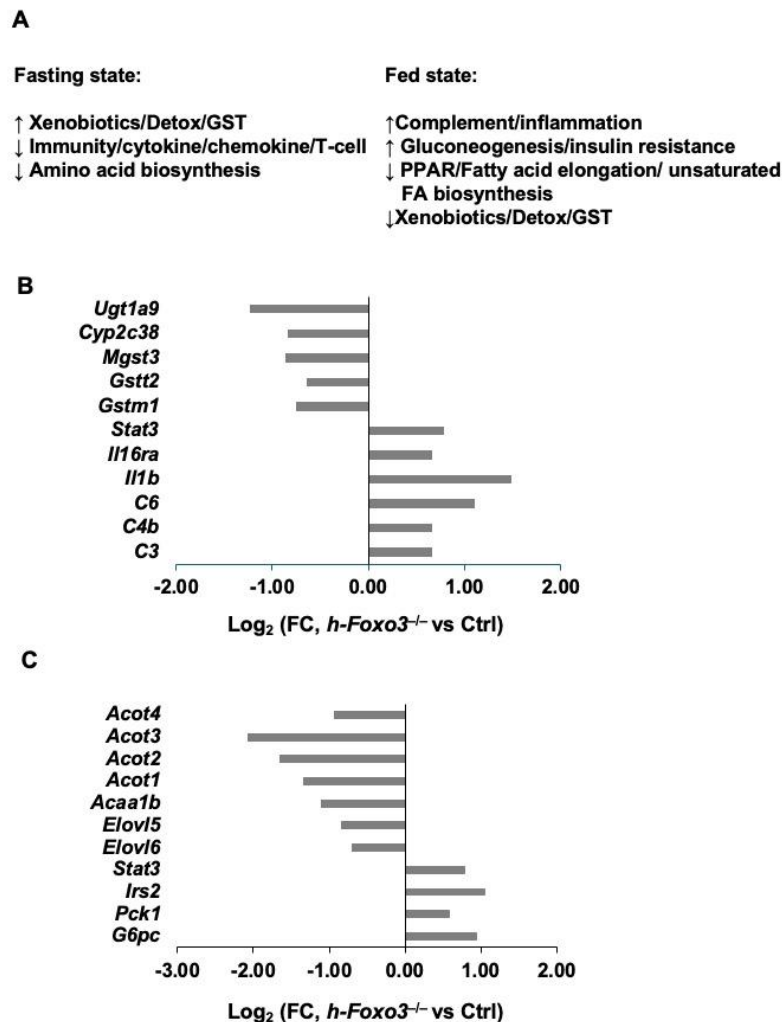


Figure 6. Pathway enrichment for genes that were differentially regulated in Ctrl and *h-Foxo3*^{-/-} liver under fasting and fed conditions. (A) Summary of the pathway enrichment analysis. ↑ and ↓ represent pathways enriched in upregulated genes and downregulated genes, respectively, in *h-Foxo3*^{-/-} liver. **(B)** Fold differences (FC) in the expression of differentially expressed genes in complement and inflammatory pathways, xenobiotics, detoxification, and glutathione-related redox pathways in *h-Foxo3*^{-/-} vs. Ctrl mice. **(C)** Fold differences (FC) in the expression of differentially expressed genes in lipid processing and gluconeogenic pathways or insulin resistance in *h-Foxo3*^{-/-} vs. Ctrl mice.

Conversely, the genes that were downregulated in *h-Foxo3*^{-/-} liver during feeding were representative of lipid metabolic pathways (Fig. 6A; Supplementary Fig. 6F;

Supplementary Table 4D), and particularly PPAR signaling. These included genes involved in lipid uptake, processing, and oxidation (*Plin2*, *Cd36*, *Cyp4a10/14*,

Acaa1b, and *Ehhadh*), as well as fatty acid elongation and remodeling (*Elovl6*, *Elovl5*, and *Acot* family genes; Fig. 6C). The expression of genes involved in peroxisomal metabolism and xenobiotic/drug metabolism pathways, as well as those encoding cytochrome P450 and conjugation enzymes, was also suppressed. These differences indicate a broad impairment in lipid handling and detoxification

capacity in the fed state. The fold differences in the expression of representative genes in the “biosynthesis of unsaturated fatty acids”, “fatty acid elongation”, and “insulin resistance” pathways between the *h-Foxo3*^{-/-} and the Ctrl groups are depicted in Figure 6C.

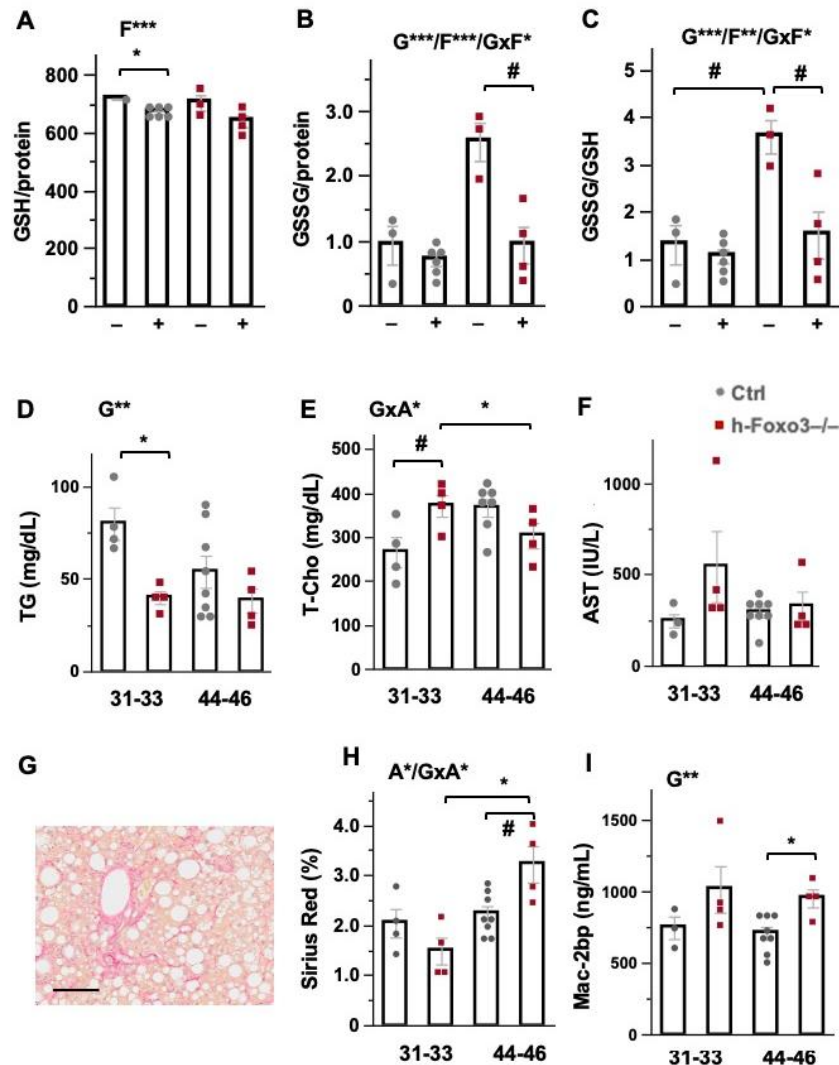


Figure 7. A fat, fructose-, cholesterol-rich diet (GAN diet) induces more severe steatohepatitis in *h-Foxo3*^{-/-} liver. (A) Reduced glutathione (GSH) content of the liver, normalized to the total protein content. (B) Oxidized glutathione (GSSG) content of the liver, normalized to the total protein content. (C) GSSG: GSH ratio. (D) Plasma triglyceride (TG) concentration. (E) Plasma total cholesterol (T-Chol) concentration. (F) Plasma AST activity. (G) A microscopic image of a liver section stained with Sirius Red, showing moderate fibrosis. Bars represent 100 μm. (H) Percentage of the liver showing fibrosis, indicated by Sirius Red staining. (I) Plasma Mac-2bp concentration (ng/mL). In panels A–F, H, and I, gray circles represent Ctrl mice; red squares represent *h-Foxo3*^{-/-} mice. The bars and lines represent the mean and SE (n = 3, 4, or 8 mice), and each point represents a measurement for an individual mouse. G, A, and G×A represent significant effects of genotype and age and the genotype × age interaction, determined using ART ANOVA (**p* < 0.05, ***p* < 0.01). The results of pairwise comparisons using the Wilcoxon’s method are indicated using #*p* < 0.10 and **p* < 0.05.

Overall, these data suggest that FoxO3 exerts context-dependent control over hepatic metabolism; during fasting, it restrains detoxification and maintains immune signaling, whereas during feeding, it supports lipid processing and suppresses the activation of gluconeogenic and inflammatory pathways. Therefore, a deficiency of FoxO3 compromises these metabolic functions and protects against toxic and oxidative stress (Fig. 6A). These findings are consistent with persistent gluconeogenesis despite feeding, along with the suppression of lipid processing, when hepatic FoxO3 is deficient.

Accelerated progression of steatohepatitis in *h-Foxo3*^{-/-} mice

The *h-Foxo3*^{-/-} mice exhibited a higher prevalence of modest, rather than severe, hepatic steatosis when consuming a regular diet (Fig. 2F), and overt steatohepatitis was not observed, even during old age (Supplementary Fig. 2B). Consistent with the RNA-seq data suggesting impairments in the xenobiotic/detoxification/GST pathways, the oxidized glutathione (GSSG) content and the GSSG: reduced glutathione (GSH) ratio were significantly higher in *h-Foxo3*^{-/-} liver (genotype, $p = 0.0007$ for GSSG; $p = 0.0007$ for the GSSG: GSH ratio; ART ANOVA), particularly in the fed state during middle age (genotype $\times$ fasting interaction, $p = 0.0105$ for GSSG; $p = 0.0256$ for GSSG: GSH; ART ANOVA; Fig. 7A–C). However, the GSH content, which was reduced by fasting (fasting, $p = 0.0002$, ART ANOVA), did not differ between the genotypes.

Given the RNA-seq evidence of impairments in lipid metabolism and the GSH redox regulation described above, we hypothesized that hepatic FoxO3 deficiency predisposes mice toward steatohepatitis in the presence of metabolic stress. To test this hypothesis, mice were challenged with a fat-, fructose-, and cholesterol-rich GAN diet [22] from 12 weeks of age, and the effects were analyzed at 31–33 and 44–46 weeks. The BWs and dietary intakes of the Ctrl and *h-Foxo3*^{-/-} mice were similar, although both were greater than those of regular diet-fed controls (Supplementary Fig. 5A, B). The liver and adipose depot masses were similarly high in mice of both genotypes that consumed the GAN diet (Supplementary Fig. 5C, D).

After GAN diet consumption, the plasma TG concentrations of the *h-Foxo3*^{-/-} mice were significantly lower than those of the Ctrl mice (genotype, $p = 0.0074$, ART ANOVA; Fig. 7D), whereas the plasma cholesterol concentrations tended to be higher at earlier time points (0.0606, Wilcoxon method for pairwise comparisons; Fig. 7E) and there was a significant genotype $\times$ age interaction ($p = 0.0308$, ART ANOVA). These findings are

consistent with impaired hepatic lipid handling and export in *h-Foxo3*^{-/-} mice. The plasma AST activity did not differ between Ctrl and *h-Foxo3*^{-/-} mice (Fig. 7F).

Histologic analysis confirmed that the GAN diet induced steatohepatitis in mice of both genotypes, and this was characterized by steatosis, inflammatory infiltration, and fibrosis (Fig. 7G). Quantitative analysis of Sirius Red-stained sections revealed a significant age-related increase in hepatic fibrosis, which was more pronounced in the *h-Foxo3*^{-/-} mice (age, $p = 0.0102$; genotype $\times$ age, $p = 0.0308$; ART ANOVA; Fig. 7H). At 44–46 weeks of age, the fibrotic area tended to be larger in *h-Foxo3*^{-/-} mice than in Ctrl mice ($p = 0.0508$, Wilcoxon method for pairwise comparisons). Consistent with this, the circulating concentrations of Mac-2bp, a marker of hepatic fibrosis [34], were higher in *h-Foxo3*^{-/-} mice (genotype, $p = 0.0027$, ART ANOVA; Fig. 7I), also consistent with an increase in fibrogenesis.

Collectively, these findings indicate that hepatic FoxO3 deficiency does not initiate steatohepatitis in mice while they are consuming a regular diet, but accelerates progression of fibrosis under conditions of nutritional stress, suggesting the attenuating effect of FoxO3 against diet-induced liver injury and fibrosis.

DISCUSSION

In the present study, we demonstrated that the hepatocyte-specific deletion of *Foxo3* leads to obesity, metabolic dysregulation, and hepatic steatosis in middle-aged mice, revealing a previously underappreciated role for hepatic FoxO3 in systemic metabolic homeostasis during aging. Previous studies of liver-specific *Foxo1/3/4* or *Foxo1/3* knockout models [4, 5, 7] have primarily involved the analysis of young animals and focused on hepatic glucose and lipid metabolism. By contrast, we have shown that the loss of FoxO3 alone is sufficient to disrupt whole-body energy balance in an age-related manner. These findings are consistent with a model in which FoxO3 is not essential for basal metabolic regulation in younger animals, but becomes critical for the maintenance of metabolic resilience during aging.

Indirect calorimetry revealed lowered EE and a shift in substrate use in the *h-Foxo3*^{-/-} mice; specifically, a preference for carbohydrate over fatty acid oxidation, particularly during the active (dark) phase. This phenotype is consistent with lower metabolic flexibility and is likely to contribute to the progression of adiposity and insulin resistance.

Although locomotor activity, lean body mass, and thermogenic traits were not measured, the lowered EE, the high dark-cycle RQ, and low levels of FAO in *h-Foxo3*^{-/-} mice may reflect impaired whole-body metabolic flexibility. In obesity-associated insulin resistance,

skeletal muscle often has lower lipid oxidative capacity and demonstrates impaired switching between carbohydrate and fatty acid utilization [35, 36]. Therefore, impaired skeletal-muscle FAO could be one factor contributing to the greater reliance on carbohydrates identified in the *h-Foxo3*^{-/-} mice during the dark period, when feeding-related activity is normally high. In this regard, future study will be needed to investigate energy metabolism by concomitantly measuring those parameters that affect whole body energy metabolism, including skeletal muscle lipid oxidation, in the *h-Foxo3*^{-/-} mouse model.

Given the role of the liver as an endocrine organ, these systemic alterations may imply that dysregulated hepatokine signaling links hepatic FoxO3 deficiency to peripheral metabolic dysfunction. Hepatokines have been shown to coordinate substrate use, insulin sensitivity, and inflammatory responses across tissues, thereby linking the hepatic metabolic state with whole-body energy homeostasis [11].

We measured the expression of genes encoding hepatokines that have previously been reported to be regulated by FoxOs and identified *Fst* (encoding follistatin) as a candidate mediator of the phenotype of the *h-Foxo3*^{-/-} mice. The plasma *Fst* concentration is also high in patients with type 2 diabetes [37, 38], and this has been hypothesized to inhibit insulin signaling in white adipose tissue, thereby promoting lipolysis, directing NEFA to the liver, and leading to the development of NAFLD [38]. In a model of hepatocyte-specific insulin resistance, high-fat diet-induced obesity is attenuated by an increase in whole-body energy expenditure in response to FoxO1-induced hepatic *Fst* expression [39]. In this model, the overexpression of *Fst* activates mTORC pathways that regulate nutrient uptake and energy expenditure in skeletal muscle [39]. These findings indicate that *Fst*-mediated communication between the liver and skeletal muscle regulates energy expenditure and obesity under conditions of hepatic insulin resistance and imply that FoxO1 is involved in the induction of *Fst* expression. However, the effects of *Fst* in metabolic diseases, insulin resistance, and obesity may be context-dependent.

The higher *Pck1* and *Fst* mRNA expression in the fed *h-Foxo3*^{-/-} mouse livers may be the result of the high expression level or the activation of FoxO1 or FoxO4 or both, and have a compensatory effect. Western blotting for the nuclear FoxO1 protein did not show high expression in *h-Foxo3*^{-/-} livers, but the S256-phosphorylation of nuclear FoxO1, which indicates FoxO1 deactivation, tended to be higher in *h-Foxo3*^{-/-} livers (Supplementary Fig. 6). The abundance of hepatic FoxO4 in the nuclear fraction also did not differ between Ctrl and *h-Foxo3*^{-/-} livers (Supplementary Fig. 6).

Therefore, it is unlikely that the high *Pck1* and *Fst* mRNA expression is the result of relatively high FoxO1 or FoxO4 expression in the *h-Foxo3*^{-/-} liver. Instead, insulin resistance resulting from h-FoxO3 deficiency may reduce the suppression of FoxO1 activity through a mechanism distinct from S256 phosphorylation, leading to higher expression of these genes. The exact nature of the post-translational regulation of FoxO1 activity under conditions of FoxO3 deficiency remains to be elucidated.

Altered hepatic production of secreted factors represents one possible mechanism through which hepatocyte FoxO3 deficiency could influence extrahepatic metabolism. However, the plasma hepatokines examined in the present study did not show clear genotype-dependent differences (Supplementary Fig. 3). Thus, an endocrine contribution remains speculative and will require broader proteomic analyses and functional validation. Emerging evidence indicates that nonprotein hepatocyte-derived factors, including microRNAs and lipid species, also participate in inter-organ communication [11]. Therefore, comprehensive analyses of liver-derived factors that affect the insulin sensitivity of key tissues are needed to clarify the potential effects of hepatic FoxO3 on whole-body metabolic resilience.

The role of FoxO3 in hepatic lipid metabolism appears to be context-dependent and cannot be fully explained by canonical models of the regulation of lipogenesis. Previous studies have shown that multiple FoxOs coordinately suppress lipogenesis, while promoting gluconeogenesis, during fasting. In contrast, the insulin-induced inactivation of FoxOs in the fed state redirects glucose flux toward lipid synthesis [4, 5, 7]. The deletion of the *Foxo3* gene alone has been shown to have minimal effects on hepatic lipid metabolism [4], but the present data suggest that FoxO3 specifically maintains lipid processing and flux, rather than directly regulating lipogenic gene expression. Specifically, RNA-seq analysis revealed the downregulation of *Acot* family genes, the products of which hydrolyze acyl-CoA to regulate intracellular lipid use and CoA availability [40]. The suppression of *Acot* expression would be predicted to increase the accumulation of acyl-CoA intermediates and exacerbate the shortage of CoA, thereby promoting β -oxidation and mitochondrial stress. Consistent with this model, the hepatic knockdown of *Acot1* increases oxidative stress [41], whereas mitochondrial *Acot2* and related matrix *Acots* have been proposed to buffer the accumulation of acyl-CoA and preserve mitochondrial FAO [42]. In parallel, the lower expression of *Elovl5*, *Elovl6*, and *Acaa1b* suggests impaired fatty acid elongation and peroxisomal or mitochondrial lipid processing, potentially favoring the accumulation of lipid intermediates. Consistent with this, *Elovl5*^{-/-} mice show

altered hepatic fatty-acid composition and greater hepatic steatosis [43]. Elov16 is required for the elongation of C16 to C18 fatty acids and influences hepatic ceramide acyl-chain composition and insulin sensitivity [44]. The deficiency of peroxisomal 3-ketoacyl-CoA thiolase B, encoded by *Acaalb*, alters the composition of the hepatic fatty-acid pool, consistent with impaired peroxisomal

lipid processing. Notably, the expression of genes encoding canonical lipogenic enzymes, including *Srebp1c*, *Chrebp*, *Gck*, *Fasn*, and *Acc*, was slightly higher in *h-Foxo3*^{-/-} liver (Supplementary Fig. 7), consistent with greater glucose-driven lipogenic signaling in *h-Foxo3*^{-/-} liver.

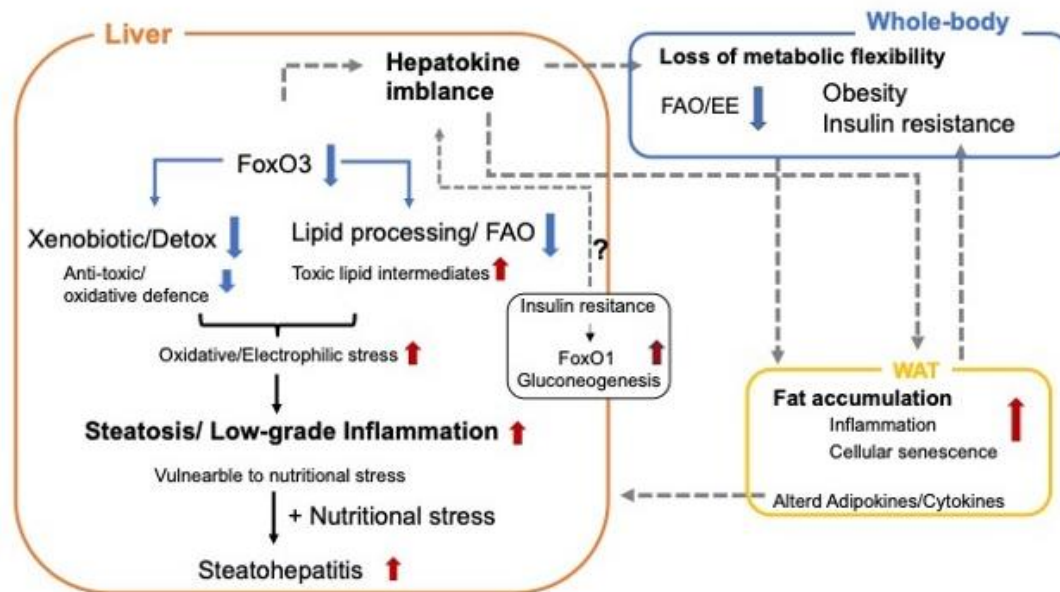


Figure 8. Hepatic FoxO3 deficiency drives systemic metabolic inflexibility via liver/adipose/whole-body crosstalk: a hypothetical scheme. Hepatic FoxO3 deficiency is associated with lower detoxification/redox capacity and impaired lipid metabolism, including less fatty acid oxidation and dysregulated lipid processing (blue downward arrows). These changes favor the accumulation of lipotoxic intermediates and hepatic metabolic stress. The liver dysfunction may be associated with altered circulating hepatokine concentrations (dashed arrows), which affect systemic energy metabolism. At the organismal level, these changes are associated with obesity and insulin resistance, accompanied by lower energy expenditure and fatty acid use (blue downward arrow). This systemic metabolic imbalance promotes white adipose tissue (WAT) expansion and dysfunction. In WAT, lipid accumulation and inflammatory signaling are upregulated (red upward arrow), contributing to altered adipokine secretion. Adipose-derived signals feed back to the liver, further aggravating the hepatic metabolic dysfunction (endocrine amplification loop). Systemic and adipose-derived signals contribute to hepatic insulin resistance. Hepatic insulin resistance leads to FoxO1 activation, which increases gluconeogenesis (red upward arrow) and exacerbates the hyperglycemia and metabolic imbalance. Together, these abnormalities are consistent with a model in which hepatic FoxO3 deficiency disrupts lipid processing and detoxification, alters inter-organ endocrine signaling, and induces metabolic inflexibility. This drives a feed-forward cycle linking liver dysfunction, adipose inflammation, and systemic insulin resistance, ultimately leading to obesity and metabolic disease.

Consistent with the dysregulation of lipogenic pathways, *h-Foxo3*^{-/-} mice developed modest hepatic steatosis on a regular diet but exhibited pronounced susceptibility to progression of fibrosis when challenged with a steatohepatitis-inducing GAN diet. RNA-seq analysis revealed the coordinated downregulation of genes involved in xenobiotic and glutathione-dependent detoxification pathways, implying impaired hepatic defense against oxidative and electrophilic stress. The present GSH and GSSG analysis supported an increase of oxidative stress in *h-Foxo3*^{-/-} livers. These alterations likely exacerbate the toxicity of the accumulated lipid intermediates, thereby promoting liver injury and

subsequent fibrogenic response under conditions of nutrient excess. The finding that the plasma TG concentrations of *h-Foxo3*^{-/-} mice were lower when they were consuming the GAN diet is consistent with impaired lipid processing and export, resulting in the hepatic retention of lipotoxic species, rather than their efficient redistribution to peripheral tissues.

Collectively, the present findings support a unified model in which hepatic FoxO3 contributes to metabolic resilience by coordinating intracellular metabolism and inter-organ communication (Fig. 8). The loss of FoxO3 disrupts lipid processing and detoxification pathways, leading to impaired metabolic flexibility, less fatty acid

use, and blood chemical changes suggesting systemic insulin resistance. These defects become increasingly consequential with age, ultimately predisposing the organism to diet-induced metabolic disease. Although the findings predict the presence of hepatokines causing systemic metabolic disturbance, the present study could not identify them.

Limitations of this study: 1) Interpretation of the energy-expenditure phenotype is limited by the absence of simultaneous measurements of locomotor activity, body composition, and thermogenesis in Ctrl and *h-Foxo3^{-/-}* mice; 2) Although elevated fasting insulin and reduced QUICKI were consistent with reduced insulin sensitivity, insulin resistance was not directly evaluated by glucose-tolerance testing, insulin-tolerance testing, hyperinsulinemic–euglycemic clamp analysis, or tissue-specific insulin-signaling assays; 3) Several analyses were based on relatively small groups of three to six animals. These sample sizes limit statistical power, increase uncertainty in the estimated effect sizes, and may reduce the generalizability of individual findings. Accordingly, findings based on these comparisons should be interpreted cautiously.

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Conflict of Interest Statement

Isao Shimokawa is now employed by SAGL, a limited liability company (SAGL, LLC). SAGL, LLC had no role in the study design, data collection, analysis, interpretation, manuscript writing, or decision to publish the results. The remaining authors declare that the research was conducted without commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' contributions

IS served as the research supervisor, provided funding, analyzed the data, and contributed to the writing and editing of the manuscript. TK supervised the animal

procedures; performed the blood chemical analyses, qPCR, immunoblot, indirect calorimetry, micro-CT, RNA-seq analyses, and the GAN diet study; and obtained funding. SK performed metabolic analyses. SP and HH served as advisors regarding the animal, biochemical, and pathologic analyses.

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2026.0477.

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