Effects of low-fat diet and aging on metabolic profiles of Creb3l4 knockout mice

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INTRODUCTION
Western societies have adopted a lifestyle with low physical activity and overconsumption of energy-rich food, resulting in a substantial increase in the incidence of obesity accompanied by impaired glucose and lipid metabolism. In general, it is accepted that expansion of adipose tissue and lipid accumulation in insulin-sensitive tissues (for example, skeletal muscle and liver) closely relates to the development of metabolic diseases. In particular, increased adipose tissue mass closely associates with the development of insulin resistance and type 2 diabetes mellitus. In addition, increased adiposity is a hallmark of aging and a source of chronic inflammation, which further accelerates the aging process. This vicious cycle of aging, visceral fat accumulation and inflammation often leads to metabolic dysfunction. Thus understanding the molecular mechanism(s) of adipose tissue expansion in the context of transcriptional regulation could be pivotal to combat metabolic syndromes, including obesity and type 2 diabetes mellitus.

The endoplasmic reticulum and Golgi transmembrane protein CREB3L4 (CAMP responsive element binding protein 3-like 4) is mainly expressed in human prostate tissue and mouse testis. Specifically, CREB3L4a, also known as Atce1/Tisp40a, is the major form of CREB3L4 expressed in mouse testis and is highly homologous with mouse CREB3 (identity 62%, similarity 72%). Deletion of the transmembrane domain of CREB3L4 results in nuclear accumulation regulated by endoplasmic reticulum stress. Moreover, whole-body knockout of Creb3l4 (Creb3l4 KO) results in abnormal epididymal sperm nuclei. CREB3L4 is also known to have an important role in the development of prostate cancer and is more highly expressed in cancerous than in non-cancerous prostate cells. However, the functional role of CREB3L4 in insulin-sensitive tissues such as adipose tissue, skeletal muscle or liver is not well known.

Recently, we reported that CREB3L4 could regulate adipocyte differentiation. Upon CREB3L4 downregulation, adipocyte differentiation occurs even when cultured with a minimal hormonal inducer. In addition, we showed that Creb3l4 KO mice fed a high-fat diet (HFD) did not show significant differences in body weight or fat mass, as compared with wild-type (WT) controls. However, KO mice undergo fat cell hyperplasia owing to increased adipogenesis, with improved glucose tolerance and increased insulin sensitivity.

To examine whether the process of aging and low-fat diet (LFD) influences metabolic phenotypes, we explored the effects of an LFD on aging on the metabolic profiles of Creb3l4 KO mice. Both LFD (LFD group) and aged Creb3l4 KO mice fed chow diet (aged group) were prone to body weight gain with increased adiposity. These mice also exhibited impaired glucose tolerance and increased insulin sensitivity, as compared with WT control mice.

MATERIALS AND METHODS
Animal experiments and KO mice
All animal experiments were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (Protocol no. 2011-0199, 2011-0315, 2012-0255). All mice were housed under standardized conditions of humidity, temperature (22–24°C) and a 12-h light/12-h dark cycle. The male mouse (11 weeks, n = 4–5) strains db/m (C57BLKS/J lar-m/Leprdb), db/db (C57BLKS/J lar-Leprdb/Leprdb), lean and ob/ob (C57BL/6J Ham Slc-ob/ob) (Shizuoka laboratory, Hamamatsu, Japan) were fed a regular chow diet. The Creb3l4 KO mouse has been

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described previously, and Creb3l4 KO mouse embryos were purchased from RIKEN (The Institute of Physical and Chemical Research, Ibaraki, Japan) with permission from Dr. Nojima. New born pups of Creb3l4 KO mice and WT littermates were randomly selected by genotyping PCR using specific primer. Creb3l4 KO mice and their WT littermates were 10–11-week old and weighed approximately 23 g; the mice were fed a 10% LFD (D12450B, Research Diets, New Brunswick, NJ, USA) over 16 weeks. The body weight of mice was assessed every week. To observe an effect of aging process, the aged WT and aged Creb3l4 KO mice (n = 3–6) were fed a regular chow diet for up to 85 weeks.

Glucose and insulin tolerance tests
For oral glucose tolerance tests, mice were fasted for 16 h and then orally administered glucose (20% w/v/vol.) using a feeding tube (2 g kg⁻¹ body weight), with blood glucose levels monitored at each time point. For insulin tolerance tests, mice were fasted for 6 h and then given an intraperitoneal injection of insulin (0.75 U kg⁻¹ body weight; Humulin R, Eli Lilly, Indianapolis, IN, USA). Blood glucose levels were monitored (One Touch Sure Step, Life Scan, Milpitas, CA, USA) at the time points indicated in the figure legends. The areas under the curve of glucose were calculated during the course of the tests.

Phenotypic evaluation of mice
WT male and Creb3l4 KO male mice were fed a 10% LFD for 18 weeks. Plasma insulin and adiponectin levels were then measured using enzyme-linked immunosorbent assay kits (ALPCO Immunosassays, Salem, NH, USA). Plasma resistin was measured using MAGPIX (Luminex, Austin, TX, USA) with MILLIPLEX MAP mouse magnetic beads (Merck Millipore Corp., St Charles, MO, USA).

Quantitative real-time PCR
Total RNA was isolated from mouse adipose tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, and cDNA was generated using a reverse transcription system (ImProm-II Reverse Transcription System; Promega, Madison, WI, USA). Quantitative real-time PCR was performed using a real-time PCR system (Step One, Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s protocol. Changes in mRNA levels were calculated using the comparative Ct method. The relative amount of mRNA in each sample was normalized to transcript levels of the RpLp0 (3684) ribosomal protein-encoding housekeeping gene. Primers used for real-time PCR were as follows: Creb3l4-F, 5′-ATATCTTTCCGACGGATCTT-3′; Creb3l4-R, 5′-TCCCTACAGGAGATGTT-3′; Cidec-F, 5′-TCAGGACATCTGAAATT-3′; Cidec-R, 5′-GGCCATGCAGTTTCTGTG-3′; Cd36-F, 5′-TCCAGCACATATCTACCAAA-3′; Cd36-R, 5′-TTGTAACCCCACAAGAGTTC-3′; Fasn-F, 5′-TTTGGAGGGCAGCTCTTTCA-3′; Fasn-R, 5′-ATGGTACAGACACCTTCCGCT-3′; Nr1h3-Lxra-F, 5′-GAGAAGCTGTGCTGGCAACA-3′; Nr1h3-Lxra-R, 5′-AGCTTGAGGAAGCCAGGAG-3′; C361-Fc, 5′-ACACAGTGTCCTGTA-3′; C361-Rc, 5′-GCCAGGATATGCTCAAGC-3′; Emr1-Fb/Fb-04-F, 5′-CTGTGGCTAAGCTGGCTC3′; Emr1-Fb/Fb-04-R, 5′-GGACGGAGACAGTTTACGTG-3′; Rplp0-F, 5′-TGGCAATAAAGGGCAGCTGTA-3′; and Rplp0-R, 5′-CTGCTTCCTGATCTGGACAGC-3′.

Hematoxylin and eosin staining
Liver, brown adipose tissue (BAT), epididymal white adipose tissue (WAT) and subcutaneous WAT were stained with Hematoxylin and eosin staining method. Sections were then performed on these sections.

Body composition analysis
Body compositions of WT and Creb3l4 KO mice were determined using non-invasive quantitative magnetic resonance relaxometry on an EchoMR-900 (Echo Medical Systems, Houston, TX, USA) at the Phenomence Research Center, Woori Jung BSC, Inc., Korea. Scans were then performed by placing animals in a thin-walled plastic cylinder (3-mm thick, 4.5-cm inner diameter, based on mouse body weight), which was inserted into a mouse cylindrical sensory antenna, the A100, to limit movement. All quantitative magnetic resonance measurements were made during the light phase (0700–1900 hours). The accumulation factor was used for extra-high precision (x3), resulting in a scan time of approximately 2.5 min.

Statistical analysis
Data are presented as means ± s.e.m.s. The LFD experiment was repeated at least three times. For aging study, experiment was performed once. All data sets were analyzed for statistical significance using nonparametric Mann–Whitney tests or Student’s T-tests. All P-values < 0.05 were considered significant. Statistical analyses were carried out using SPSS (IBM SPSS statistics ver. 20; IBM Corp., Armonk, NY, USA).

RESULTS
Creb3l4 KO mice fed an LFD show increased adipocyte hypertrophy and obesity
Because Creb3l4 is known to be expressed in WAT,1,4 we examined the mRNA levels of Creb3l4 in WAT of ob/ob and db/db mice to test whether obesity correlated with Creb3l4 gene expression. Creb3l4 mRNA levels in WAT of these mice were lower than those in the WT controls (Figures 1a and b). The reduced Creb3l4 expression in WAT of these mice led us to explore a cause–effect relationship between CREB3L4 and the development of adiposity.

To observe the effect(s) of CREB3L4 on whole body metabolism, we fed Creb3l4 KO mice an LFD and observed body weight changes over 16 weeks. LFD induced a significant gain in body weight (Figures 1c and d) in Creb3l4 KO mice that associated with increased weight of epidymal WAT, as compared with their WT littermates (Figure 1e). Although the food intake was not significantly different between WT and Creb3l4 KO mice, the Creb3l4 KO mice were likely to consume more food (Supplementary Figure S1a). Liver weight was not significantly different between these mice (Figure 1e). The size of subcutaneous and epidymal fat cells in Creb3l4 KO mice were larger than those in WT mice (Figure 1f). In addition, lipid accumulation in the liver and BAT increased in Creb3l4 KO mice (Figure 1g). These results suggest that Creb3l4-null mice fed an LFD incur adipocyte hypertrophy and obesity, with lipid accumulation in the liver and BAT, in addition to increased adipose tissue weight.

Creb3l4 KO mice fed an LFD exhibit impaired glucose tolerance and decreased insulin sensitivity
Because obesity leads to insulin resistance, we assessed glucose tolerance and insulin sensitivity in WT and Creb3l4 KO mice. Creb3l4 KO mice fed an LFD (LFD-Creb3l4 KO) showed impaired glucose tolerance and insulin sensitivity, as compared with WT littermate mice (Figures 2a and b).

Fasting glucose and insulin levels were also increased in LFD-Creb3l4 KO mice, typical signs of insulin resistance (Figure 2c). However, plasma lipid profiles, including triglycerides, total cholesterol and non-esterified free fatty acid levels, were not significantly different between these groups (Supplementary Figure S1b). Because adipose tissue is known to control systemic glucose homeostasis by secreting adipokines, we measured adipokine plasma levels. The LFD-Creb3l4 group showed decreased serum adiponectin, but increased resistin levels, with increased levels in WT mice (LFD-WT). These results suggest that LFD-Creb3l4 KO mice possess an altered adipokine profile that would aggravate insulin sensitivity (Figure 2d).

Obesity is known to be a state of chronic low-grade inflammation with infiltration of adipose tissue macrophages. Adipose tissue macrophages are characterized by the presence of F4/80 and CD11b, with subtypes M1 and M2 classified by the presence (M2) or absence (M1) of CD11c.16 As shown in Figure 2e, adipose tissue macrophages, particularly the M2 subtype, strongly

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infiltrated the epididymal fat in LFD-Creb3l4 KO mice, as compared with LFD-WT mice. These results are consistent with data showing increased fat mass and adipocyte size (hypertrophy) in LFD-Creb3l4 KO mice (Figures 1c and g). These findings further suggest that the WAT of Creb3l4-deficient mice, when fed an LFD, may undergo increased fat mass that impairs glucose tolerance and decreases insulin sensitivity. In addition, lipid accumulation in the liver and BAT may also contribute to defects in glucose tolerance and insulin sensitivity (Figure 1g).

Aged-Creb3l4 KO mice fed chow diet show increased adiposity with impaired glucose tolerance and decreased insulin sensitivity. Aging is an important risk factor for metabolic disorders, including obesity, impaired glucose tolerance and type 2 diabetes. To further investigate an effect of CREB3L4 on age-related adiposity, both WT and Creb3l4 KO mice were fed a chow diet for up to 85 weeks, resulting in significantly increased body weight of the aged-Creb3l4 KO mice, compared with the body weight of aged-WT mice (Figure 3a). The fat composition of Creb3l4 KO mice also increased (Figure 3b), as did fat cell size, in aged-Creb3l4 KO mice, compared with aged-WT mice (Figure 3c). Lipid accumulation in the liver and BAT also increased in aged-Creb3l4 KO mice (Figure 3d). Aged-Creb3l4 KO mice also showed impaired glucose tolerance and decreased insulin sensitivity, compared with their WT littermates (Figures 3e and f), a phenomenon we also observed in LFD-fed mice.

**DISCUSSION**

As accumulation of fat is known to be related to insulin resistance, obesity and metabolic syndrome, studies of associated transcription factor are critical to development of therapy. Based on previous studies which showed that inhibition of Creb3l4 induced adipogenesis, we examined whether Creb3l4 affects fat mass in mice fed an LFD and aged mice with chow diet. Increased fat mass, with hypertrophic WAT, was present in LFD-fed Creb3l4 KO mice and aged-Creb3l4 KO mice, which likewise exhibited impaired glucose tolerance and decreased insulin sensitivity,
presumably caused by upregulated lipogenic genes in their epididymal WAT. Increased expression of lipogenic genes in the WAT of Creb3l4 KO mice could contribute to accumulation of triglyceride in adipocytes. Triglyceride content was also increased in the liver and BAT of LFD-Creb3l4 KO mice. There is no difference of energy expenditure and physical activity between WT and Creb3l4 KO mice (Supplementary Figure S2). But the Creb3l4 KO mice showed tendency to uptake more food (Supplementary Figure S1a), which may contribute to higher body fat contents in LFD-fed Creb3l4 KO mice than LFD-fed WT mice.

Furthermore, because Creb3l4 KO mice used in this study is whole-body KO, we could not rule out indirect effect of the absence of Creb3l4 on lipid homeostasis. Detailed relationship between lipogenesis and Creb3l4 gene needs further research. In contrast, HFD-fed Creb3l4 KO mice showed no significant differences in body weight or fat mass, with adipocyte hyperplasia improving glucose tolerance and insulin sensitivity, as compared with WT controls. However, these findings raise the question of why do adipocyte responses in Creb3l4 KO mice fed an LFD differ from those fed a HFD? Moreover, why do LFD-fed Creb3l4 KO mice showed decreased insulin sensitivity (with adipocyte hypertrophy), whereas HFD-fed Creb3l4 KO mice exhibit increased insulin sensitivity with adipocyte hyperplasia, as compared with WT mice? We speculated that the phenotype of HFD-Creb3l4 KO

Figure 2. Creb3l4 KO mice show impaired glucose tolerance and decreased insulin sensitivity. (a, b) Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) responses (0–120 min) performed after 14 and 15 weeks of LFD feeding, respectively (c, d) Fasting glucose, plasma insulin, adiponectin and resistin levels from WT or Creb3l4 KO mice fed an LFD for 16 weeks. (e) Relative expression of adipogenic genes, macrophage marker genes and lipogenic genes in epididymal white adipose tissue, normalized to the housekeeping gene RpLp0. Values are expressed as means ± s.e.m., n = 6–7 in panels (a) and (c), n = 4–5 in panels (b, d and e), *P < 0.05 for Creb3l4 KO versus WT mice.
mice may arise owing to increasingly generated adipocyte precursors, arising from embryonic stem cells and their self-renewal, being recruited to WAT, resulting in adipocyte hyperplasia that improves glucose tolerance. Indeed, high dietary fat content is known to expand adipose tissue mass by recruiting white adipocytes from progenitor cells.18,19 In addition, HFD-induced adipose tissue expansion is triggered by hypertrophy during the first month of HFD feeding, after which adipose tissue expands by formation of a large number of new fat cells (hyperplasia),20 which are known to increase glucose disposal and insulin sensitivity.21–23 At present, it is not clear whether LFD induces formation of new fat cell. However, as it was shown that chow diet did not induce adipogenesis,20 we speculate that the response and role of Creb3l4 may differ from food (diet) type in terms of adipogenesis, resulting in different body fat content, as manifested by opposite patterns of lipogenesis versus lipolysis, resulting in weight gain in the Creb3l4 KO mice fed HFD versus LFD diets, as compared with WT. Indeed, because Creb3l4 is mainly expressed in the stromal vascular fraction, an adipose tissue subpopulation of various progenitor and immune cells,14 we could not exclude the possibility that Creb3l4 may have a role in the formation and recruitment of adipocyte precursors to WAT, based on diet (for example, LFD or HFD) that affects adipocyte size. These observations, together with the presence of Creb3l4 in the stromal vascular fraction in WAT, might explain another potential role of Creb3l4 in the transition of embryonic stem cells into preadipocytes, leading to the increased numbers of adipocytes observed in HFD-Creb3l4 KO mice. In addition, in contrast to the HFD group, the LFD regimen consisted of 35% sucrose, rather than fat. Fructose, derived from sucrose, can affect carbohydrate metabolism by changing intermediate glycolytic metabolites and gene expression patterns of metabolism-related enzymes.24 Moreover, high consumption of fructose causes hepatic steatosis and obesity, while also eliciting metabolic abnormalities such as insulin resistance, leptin resistance and hypertriglyceridemia.25
Thus it is speculated that different phenotype observed in Creb3l4 KO mice, in response to different diets, might be due to the sucrose disaccharide contained in the LFD diet.

Thus LFD and/or aged Creb3l4 KO mice showed increased adiposity with hypertrophic WAT, which may contribute to the development of impaired glucose tolerance and decreased insulin sensitivity. Because WAT expresses more Creb3l4 mRNA than other metabolic tissues,14 this metabolic phenotype may result from altered CREB3L4 action in WAT. In contrast, the absence of WAT-specific Creb3l4 KO mice, we cannot rule out that Creb3l4 deficiency in other tissues also contributes to the phenotype observed in these mice.

In summary, the aging process, or consumption of an LFD, in Creb3l4 KO mice showed different phenotypes than those fed a HFD. Thus, understanding the molecular mechanism of differential regulation of adiposity in Creb3l4 KO mice fed different diet or aging may provide critical clues in combating metabolic syndromes associated with obesity.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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