Nuclear orphan receptor TAK1/TR4-deficient mice are protected against obesity-linked inflammation, hepatic steatosis, and insulin resistance

**Running title:** TAK1 and metabolic syndrome

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Objective—The nuclear receptor TAK1/TR4/NR2C2 is expressed in several tissues that are important in the control of energy homeostasis. In this study, we investigate whether TAK1 functions as a regulator of lipid and energy homeostasis, and has a role in metabolic syndrome.

Research design and methods—We generated TAK1-deficient (TAK1\(^{-/-}\)) mice to study the function of TAK1 in the development of metabolic syndrome in aged mice and mice fed a high fat diet (HFD). (Immuno)histochemical, biochemical, and gene expression profile analyses were performed to determine the effect of the loss of TAK1 expression on lipid homeostasis in liver and adipose tissues. In addition, insulin sensitivity, energy expenditure, and adipose-associated inflammation were compared in wild type (WT) and TAK1\(^{-/-}\) mice fed a HFD.

Results—TAK1-deficient (TAK1\(^{-/-}\)) mice are resistant to the development of age- and HFD-induced metabolic syndrome. Histo- and biochemical analyses showed significantly lower hepatic triglyceride levels and reduced lipid accumulation in adipose tissue in TAK1\(^{-/-}\) mice compared to WT mice. Gene expression profiling analysis revealed that the expression of several genes encoding proteins involved in lipid uptake and triglyceride synthesis and storage, including Cidea, Cidec, Mogat1, and CD36, was greatly decreased in the liver and primary hepatocytes of TAK1\(^{-/-}\) mice. Restoration of TAK1 expression in TAK1\(^{-/-}\) hepatocytes induced expression of several lipogenic genes. Moreover, TAK1\(^{-/-}\) mice exhibited reduced infiltration of inflammatory cells and expression of inflammatory genes in white adipose tissue, and were resistant to the development of glucose intolerance and insulin resistance. TAK1\(^{-/-}\) mice consume more oxygen and produce more carbon dioxide than WT mice suggesting increased energy expenditure.

Conclusion—Our data reveal that TAK1 plays a critical role in the regulation of energy and lipid homeostasis, and promotes the development of metabolic syndrome. TAK1 may provide a new therapeutic target in the management of obesity, diabetes, and liver steatosis.

Obesity is a major health-care concern in Westernized cultures that affects approximately 30% of the general population in the United States (1; 2). A strong etiological link has been found between obesity and several obesity-associated diseases, including insulin-resistance, type 2 diabetes, cardiovascular disease, and nonalcoholic fatty liver disease (NAFLD). There is considerable evidence indicating that systemic low-grade inflammation associated with obesity plays a pivotal role in the pathogenesis of metabolic syndrome (3-6). In particular, the infiltration of macrophages and T lymphocytes in hypertrophic adipose tissue and the production of proinflammatory cytokines are important early events in the development of obesity-associated complications (6-9).

TAK1 (TR4, NR2C2), together with the closely related transcription factor TR2 (NR2C1), form a subclass of the nuclear receptor superfamily (10-12). TAK1 is highly expressed in several tissues, including the testis, brain, kidney, liver, and adipose tissue. While TAK1 is still considered to be an orphan receptor, recent reports suggest that certain fatty acids and eicosanoids bind to and enhance the transcriptional activity of TAK1; thereby suggesting that TAK1 might function as a lipid sensor (13; 14). Although the precise physiological functions of TAK1 remain poorly understood, characterization of TAK1-deficient mice have suggested a role.
for TAK1 in cerebellar development and reproductive functions (15-18). More recent studies have provided evidence suggesting a role for TAK1 in lipid metabolism and gluconeogenesis (14; 19-21).

In the present study, we used a TAK1-deficient (TAK1−/−) mouse model to obtain further insights into the physiological roles of TAK1 in energy homeostasis. We show, for the first time, that male TAK1−/− mice are resistant to the development of age- and high fat diet (HFD)-induced obesity and are protected against obesity-linked hepatic steatosis, white adipose tissue (WAT)-associated inflammation, and insulin resistance. Our study reveals that the TAK1 signaling pathway plays a critical role in the regulation of lipid and energy homeostasis, and metabolic syndrome. Because TAK1 functions as a ligand-dependent transcription factor, it may provide a novel therapeutic target in the management and prevention of obesity and associated pathologies.

RESEARCH DESIGN AND METHODS

TAK1−/− mice. A schematic view and detailed information on the knockout strategy and mice are provided in Supplementary Fig. 1 in the online appendix available at http://diabetes.diabetesjournals.org. TAK1−/− mice were bred into a C57BL/6 background for >8 generations. Mice were supplied ad libitum with NIH-A31 formula and water. 8-12 week-old mice were fed a high fat diet (HFD; D12492, Research Diets Inc., New Brunswick, NJ) for 6 weeks, unless indicated otherwise. All animal protocols followed the guidelines outlined by the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the NIEHS.

Cell culture and viral infection. Primary hepatocytes were isolated using a Hepatocyte Isolation System (Worthington Biomedical Corp., Lakewood, NJ). To generate adenovirus, TAK1WT and TAKΔAF2, a mutant lacking the AF2 domain, were cloned to pShuttle-IREShrGFP-1 vector and then transferred into AdEasy-1 (Stratagene, La Jolla, CA). Adenovirus was then generated according to the manufacturer’s protocol. Hepa1-6/Emp, Hepa1-6/TAK1, and Hepa1-6/TAKΔAF2 cells were generated by infection with retrovirus containing the empty vector pLXIN, pLXIN-TAK1, or pLXIN-TAK1ΔAF2, respectively. Following selection in G418, separate clones were isolated. All cells were maintained in DMEM containing 10% FBS.

Histology and immunostaining. Adipose and liver specimens (n=6) were fixed in 4% paraformaldehyde, paraffin-embedded, and tissues sections (5 µm) stained with hematoxylin-eosin. The average diameter of white adipocytes was calculated from 20-30 cells/field and three fields/section. For the detection of macrophages, sections of white adipose tissue (WAT) were stained with an F4/80 antibody (Santa Cruz, CA) and avidin-biotin-peroxidase detection system.

RNA isolation, microarray analysis, and QRT-PCR. RNA isolation, microarray analysis, and QRT-PCR were carried out as described previously (22). Total RNA from individual mice (n=4-10) in each group was analyzed as indicated. Details are listed in Supplementary Table 1.

Biochemical assays. Blood levels of free fatty acids (FFA), β-hydroxybutyrate, glucose, cholesterol, triglycerides, and high-density lipoprotein (HDL) were determined using the Cobas Mira Classic Chemistry System (Roche Diagnostics Systems Inc., Montclair, NJ). The chemical reagents for all assays were purchased from Equal Diagnostics (Exton, PA). Serum insulin levels were analyzed with an insulin radioimmunoassay (RIA) kit (Millipore, St. Charles, MO). To measure liver lipid content, tissues were homogenized and lipids extracted as previously described (23). Triglyceride and cholesterol levels were measured with Stanbio
TAK1 and metabolic syndrome

assay kits (Stanbio Laboratory, Boerne, TX). Total ketones were analyzed with an Autokit (Waco Chemical GmbH, Neuss, Germany).

**Metabolic analysis.** WT and TAK1^−/−^ mice were fed either a normal diet or HFD for 18 weeks and their oxygen consumption, CO₂ production, and respiratory exchange ratio (RER) were analyzed with a LabMaster system (TSE systems INC, Chesterfield, MO). All values were measured every 5 minutes for 3 days. The average of the values during the circadian time (CT) or light period and dark period were calculated and presented. P-values were calculated using Student’s t-test.

**Isolation of the stromal-vascular fraction (SVF) and flow cytometry analysis.** SVF was isolated from epididymal white adipose tissue (eWAT) of mice fed with a HFD for 18 weeks and analyzed by flow cytometry with anti-F4/80 antibody (Invitrogen, Camarillo, CA), and anti-CD3, CD4, CD8, and CD11b antibodies (BD Biosciences, San Jose, CA) as described (6). Cells were co-stained with 7-amino-actinomycin D (7-AAD) or propidium iodine (PI) to exclude dead cells. Cells were analyzed with a BD LSR II Flow cytometer (Becton Dickinson) using FACSDiVa software as described (6).

**RESULTS**

**Generation of TAK1^−/−^ mice.** To obtain further insights into the role of TAK1 in vivo, we generated TAK1^−/−^ mutant mice in which TAK1 was functionally inactive (Supplementary Fig. 1). Increased mortality of TAK1^−/−^ embryos was noted (Supplementary Table 2). Although at 2-3 months the surviving TAK1^−/−^ mice were slightly underweight, they were healthy and had a normal appearance and lifespan. Analysis of multiple organ tissues did not identify any gross anatomical or histological abnormalities in TAK1^−/−^ mice.

**TAK1^−/−^ mice are resistant to age-induced hepatic steatosis.** TAK1 is highly expressed in several tissues that are critical in lipid and energy homeostasis (Supplementary Fig. 2). To study the role of TAK1 in lipid homeostasis, we examined first whether loss of TAK1 function has any effect on age-induced hepatic steatosis. As shown in Figure 1A, in contrast to aged male WT mice (24), one year-old male TAK1^−/−^ mice were protected against age-induced hepatic steatosis (Fig. 1A). Heterozygous male TAK1^+/−^ mice developed steatosis to a similar degree as WT littermates (data not shown).

One year-old male TAK1^−/−^ mice weighed approximately 30% less (Fig. 1B) and the size of epididymal and abdominal WAT, when measured as percentage of total body weight, was markedly reduced (respectively, 50% and 70% less than in WT littermates) (Fig. 1C). Histochemical analysis showed reduced lipid accumulation in WAT and BAT of TAK1^−/−^ mice (Fig. 1A). Furthermore, adipocytes in WAT of TAK1^−/−^ mice were dramatically smaller than those of WT mice (Fig. 1D) suggesting that the reduced adiposity observed in TAK1^−/−^ mice may be due to a large extent to reduced triglyceride accumulation.

Consistent with our histological observations, biochemical analysis showed that the triglyceride level was greatly reduced in the liver of TAK1^−/−^ mice compared to those of WT mice (Fig. 2A). Levels of hepatic cholesterol were slightly, but not significantly, decreased in TAK1^−/−^ mice. Blood triglyceride and cholesterol levels were significantly lower in TAK1^−/−^ mice compared to WT, whereas there was no change in blood glucose levels (Fig. 2A). Examination of the food intake over a 5-day period indicated that TAK1^−/−^ mice displayed a modest, but significant, increased food intake relative to WT mice suggesting that the reduced fat mass in these mice was not due to reduced food intake (Fig. 2B).

**Gene expression profiling.** To understand the mechanism by which loss of TAK1
prevented age-induced hepatic steatosis, we analyzed and compared the gene expression profiles in liver from WT and TAK1\(^{-/-}\) mice by microarray analysis (http://www.ncbi.nlm.nih.gov/geo; accession number GSE21903). Loss of TAK1 function affected the expression of many genes that are implicated in lipid, fatty acid, and carbohydrate metabolism (Table 1). Cell death-inducing DFFA-like effector c (Cidec), also termed fat-specific protein (FSP27), and cell death-inducing DFFA-like effector a (Cidea), two proteins that play a critical role in triglyceride accumulation (25-27), monoacylglycerol O-acyltransferase 1 (Mogat1), which is part of an alternative pathway of triglyceride synthesis, and CD36, which plays a role in lipid transport and steatosis (28), were among the genes most strongly suppressed in TAK1\(^{-/-}\) liver. Thus, these observations suggest that TAK1 positively regulates the expression of several genes encoding proteins involved in promoting lipid uptake and triglyceride accumulation.

Among other notable changes, the expression of a number of Phase I and Phase II enzyme, and drug transporter genes was affected in TAK1\(^{-/-}\) livers, including several cytochrome p450 enzymes, sulfotransferase Sult1c2, and several ATP-binding cassette (Abc) transporters (Table 1). These observations suggest that TAK1 may also play a role in the regulation of the transport and metabolism of various drugs and xenobiotics. Several transcription factors, including Srebf1 and Ppar\(\gamma\), were expressed at significantly lower levels in TAK1\(^{-/-}\) liver compared to WT liver, while Onecut1 and Foxa1 were expressed at higher levels in the liver of TAK1\(^{-/-}\) mice.

The repression of hepatic expression of Cidea, Cidec, Gpcre5b, Mogat1, resistin (Retn), CD36, Srebf1, acetyl-CoA carboxylase a (Acca), and fatty acid binding protein 2 (Fabp2), in TAK1\(^{-/-}\) mice was confirmed by QRT-PCR (Fig. 2C). The expression of the co-repressor RIP140, which has been reported to regulate Cidea (29), was not significantly different between TAK1\(^{-/-}\) and WT mice. The repression of PPAR\(\gamma\) in TAK1\(^{-/-}\) liver was confirmed by QRT-PCR, whereas the expression of estrogen-related receptor \(\alpha\) (ERR\(\alpha\)), pregnane X receptor (PXR), and liver X receptor \(\alpha\) (LXR\(\alpha\)) was not changed in the liver of TAK1\(^{-/-}\) mice (Fig. 2C).

We next examined whether the changes in gene expression in aged mice could be detected at an earlier age. Although histologically no significant differences were observed between liver of 4-5 month old WT and TAK1\(^{-/-}\) mice (Fig. 3A, B), the expression of Cidea, Cidec, Mogat1, Cd36, and Retn was significantly reduced in TAK1\(^{-/-}\) liver compared to WT liver (Fig. 3C). Moreover, analysis of gene expression in primary hepatocytes showed that Cidea, Cidec, Ppar\(\gamma\), Cd36, and Mogat1 were expressed at significantly lower levels in TAK1\(^{-/-}\) primary hepatocytes than in WT hepatocytes (Fig. 3D). Next, we examined, whether the expression of genes down-regulated in TAK1\(^{-/-}\) hepatocytes could be restored by exogenous TAK1 expression. Infection of TAK1\(^{-/-}\) hepatocytes with Ad-TAK1 adenovirus restored TAK1 expression and induced Cidea and Mogat1 expression several fold and that of Cidec by 70%, while infection with Ad-Empty or Ad-TAK1\(\Delta\)AF2, in which the activation domain of TAK1 was deleted, had little effect on the expression of these genes (Fig. 3E). Expression of Ppar\(\gamma\) was not significantly altered by Ad-TAK1, suggesting that the increase in Cidea, Cidec, and Mogat1 mRNA occurred independently of the increased Ppar\(\gamma\) mRNA expression. TAK1\(^{-/-}\) mice are resistant to HFD-induced hepatic steatosis. TAK1\(^{-/-}\) mice were also protected against HFD-induced hepatic steatosis and obesity. Eight-to-ten week-old TAK1\(^{-/-}\) mice fed a high-fat diet for 6 weeks
gained less weight than their WT littermates (Fig. 4A). By the end of the feeding period, the average body weight of WT mice increased by 55%, while TAK1−/− mice gained only 12% body weight. TAK1−/−(HFD) mice also exhibited a reduced fat mass compared to WT(HFD) controls. In fact, the relative weight of epididymal and abdominal WAT in TAK1−/−(HFD) mice was, respectively, 40% and 50% less compared to WT(HFD) mice, while no significant difference in kidney weights was observed (Fig. 4B).

Histological analysis revealed that TAK1−/−(HFD) mice showed significantly smaller WAT adipocyte size, as well as less accumulation of hepatic lipid droplets than their WT(HFD) littermates (Fig. 4C-F). The latter was supported by biochemical data showing that the significantly lower hepatic triglyceride accumulation in TAK1−/−(HFD) mice than in WT(HFD) mice (Fig. 5A). The serum concentrations of triglycerides and high-density lipoprotein (HDL) were not significantly changed, but total cholesterol, low-density lipoprotein (LDL), and glucose levels were significantly reduced in TAK1−/− mice compared to WT mice (Fig. 5B). Together, these observations indicate that TAK1−/− mice were significantly protected against HFD-induced obesity and hepatic steatosis. The protective effect cannot be attributed to increased level of secreted lipid in the feces, because no appreciable difference was found in that regard between WT and TAK1−/− mice (Fig. 5C). Analysis of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), markers of hepatocytotoxicity, showed that ALT and AST levels were significantly elevated in WT(HFD) mice compared to TAK1−/−(HFD) mice (Fig. 5D). Hepatic expression of Cidea, Mogat1, Cidec, CD36, and Retn was significantly lower in TAK1−/−(HFD) mice than in WT(HFD) mice (Fig. 5E), consistent with observations in aged TAK1−/− mice.

**TAK1+ mice have an increased energy expenditure.** Although their relative food consumption was higher (Fig. 6E), TAK1+ mice were leaner than WT mice, which suggested that TAK1+ mice might have an increased energy expenditure. Using indirect calorimetry, oxygen consumption (VO2) and CO2 production (VCO2) rates were measured in TAK1+−(HFD) and WT(HFD) mice over a period of 2 days. In both WT(HFD) and TAK1−/−(HFD) mice, VO2 and VCO2 were significantly increased during the dark phase compared to light phase (Fig. 6A, B). Moreover, TAK1+−(HFD) mice exhibited elevated VO2 and VCO2 in both the light and dark phase as compared to WT(HFD) mice, and an increased respiratory exchange ratio (RER) (Fig. 6A-C). These observations are consistent with a higher rate of energy expenditure by TAK1−/−(HFD) mice that might be partly due to the observed increase in heat generation (Fig. 6D). The increased expression of uncoupling protein 1 (Ucp1), CoxIV, and Pgc-1α in BAT of TAK1−/−(HFD) mice compared to that of WT(HFD), is consistent with the notion of increased energy expenditure (Fig. 6F).

**Inflammation was significantly reduced in WAT of TAK1−/−(HFD) mice.** WAT-associated inflammation plays a critical role in the development of obesity-related complications (6-8; 30). Consistent with this, WAT of WT(HFD) mice showed an increase in crown-like structures (CLS) representing aggregated F4/80-positive macrophages (Fig. 7A). In contrast, F4/80-positive cells were infrequently observed in WAT from TAK1−/−(HFD) mice (Fig. 7A). This was substantiated by quantitative analysis showing that the percentage of stromal vascular fraction (SVF)-associated macrophages (F4/80+/Cd11b+) was significantly reduced in TAK1−/−(HFD) mice compared to WT(HFD) (Fig. 7B). Furthermore, the percentage of CD3+ T lymphocytes in TAK1−/−(HFD) mice was 45% lower than in WT mice; however, the ratio between CD4+ and CD8+ T cells was
not different, indicating that both CD4+ and CD8+ cell populations are decreased in the WAT of TAK1−/−(HFD) mice (Fig. 7B). Together, these results suggest that loss of TAK1 greatly reduced HFD-responsive inflammation in WAT. The inhibition of inflammation in WAT of TAK1−/−(HFD) mice was supported by decreased expression of the macrophage markers, F4/80 and Mac-2, and several other inflammation-related genes, including serum amyloid 3 (Saa3), matrix metallopeptidase 12 (Mmp12), interleukin 1 receptor antagonist (Il1rn), and the Toll-like receptor 8 (Tlr8) compared to WT(HFD) WAT (Fig. 7C). In addition, as observed in TAK1−/−(HFD) mice, the expression of Mmp12, Saa3, Mac-2, and F4/80 was also significantly reduced in WAT of one year-old TAK1−/− mice compared to their age-matched WT mice (Supplementary Fig. 3). These data support the hypothesis that TAK1−/− mice are protected against obesity-associated inflammation of adipose tissue.

TAK1−/− mice are protected against insulin-resistance. It is well established that obesity greatly enhances the risk of type 2 diabetes as indicated by the development of insulin resistance and glucose intolerance (3; 4). As shown in Fig. 8A, blood insulin levels were significantly lower in chow-fed, 4-5 month-old TAK1−/− mice compared to their age-matched WT littermates. Insulin levels increased further in aged WT and WT(HFD) mice, but remained low in corresponding TAK1−/− littermates. Moreover, WT(HFD) mice developed glucose intolerance and insulin resistance as indicated by the GTT and ITT analyses (Fig. 8B, C). In sharp contrast, TAK1−/−(HFD) mice retained their glucose tolerance and insulin sensitivity indicating that TAK1−/− mice are protected against insulin resistance, a common symptom of diabetes.

DISCUSSION

In this study we show, for the first time, that loss of TAK1 protects mice against age- and HFD-induced metabolic syndrome. TAK1−/− mice remain lean and show reduced adiposity and hepatic steatosis during aging or when fed a HFD. Moreover, TAK1−/− mice are protected against the development of age- and diet-induced adipose tissue-associated inflammation, insulin resistance, and glucose intolerance. These observations indicate that the nuclear receptor TAK1 plays a critical role in the control of energy balance and lipid homeostasis.

Livers of TAK1−/− mice showed a reduced lipid accumulation compared to their WT littermates. Hepatic triglyceride accumulation is controlled at several levels, including fatty acid uptake, synthesis and storage of triglycerides, fatty acid oxidation, and lipolysis. Gene expression profiling revealed a great number of differences in gene expression between livers from one year-old WT and TAK1−/− mice, including genes that are critical in the regulation of lipid, fatty acid, carbohydrate, and xenobiotic metabolism, and gene transcription (Table 1). The expression of many of these genes has been reported to be elevated in hepatic steatosis (31; 32). One of these genes is CD36, which encodes a multifunctional protein implicated in angiogenesis, immunity, and in several metabolic disorders, such as obesity, hepatic steatosis, and insulin resistance (28; 33). In several cell types, including adipocytes and hepatocytes, CD36 facilitates long-chain fatty acid uptake. Thus, the reduced CD36 expression observed in TAK1−/− liver may lead to diminished hepatic fatty acid uptake and, at least in part, be responsible for the resistance to hepatic steatosis.

Cidea and Cidec were also among the genes that were the most dramatically down-regulated in TAK1−/− mice. Cide proteins promote triglyceride accumulation within lipid droplets and regulate lipolysis and their expression correlates positively with the
development of obesity and hepatic steatosis (25; 34; 35). Deficiency in Cidea or Cidec in mice resulted in increased energy expenditure and lipolysis, and yielded a lean phenotype in mice and resistance to diet-induced obesity (25; 26; 36). Therefore, the repression of these genes in TAK1^−/− mice may have also contributed to the reduction in hepatic triglyceride levels and resistance to hepatic steatosis in TAK1^−/− mice. Although the expression of Cidea and Cidec, as well as CD36, was greatly repressed in the liver of TAK1^−/− mice, TAK1 did not appear to regulate the expression of these genes in WAT suggesting a tissue-dependent regulation.

Mogat1, another gene that was dramatically down-regulated in TAK1^−/− liver, is part of an alternative, less-studied pathway of triglyceride synthesis. The main pathway of triglyceride synthesis is catalyzed by glycerol-3-phosphate acyltransferase (GPAT), acyl-glycerol-3-phosphate acyltransferases (AGPATs), and diacylglycerol transferase (DGAT) in the final step of synthesis (37). The expression of DGAT1 was not altered; however, the expression of GPAT1 and AGPAT6 was significantly reduced in TAK1^−/− liver. The latter is interesting because AGPAT6-deficiency has been reported to cause lipodystrophy and resistance to obesity (38). Thus, the lower levels of Mogat1, GPAT1, and AGPAT6 expression may be part of the mechanism by which triglyceride synthesis and storage is reduced in TAK1^−/− liver. Thus, the regulation of several genes with functions related to fatty acid uptake (CD36), triglyceride synthesis (Mogat1, GPAT1, AGPAT6), and storage (Cidea, Cidec) suggests that TAK1 affects several aspects of lipid accumulation. In contrast, no significant changes in fatty acid oxidation were observed.

In contrast to aged mice, 4-5 month-old mice fed with a normal diet did not show histological signs of hepatic steatosis; however, the hepatic expression of Cidea, Cidec, Mogat1, CD36, and Retn was significantly lower in TAK1^−/− mice than WT littermates. Consistent with a previous study (19), young TAK1 KO mice were also more glucose tolerant and insulin sensitive than WT mice (Supplementary Fig. 4). These observations suggest that TAK1 affects changes in hepatic gene expression and insulin sensitivity at an early age.

Energy and lipid homeostasis is under the control of a complex network of transcription factors and co-regulators (32; 39-41). Deficiencies in many of these factors have been associated with resistance to diet-induced obesity. For example, mice deficient in the nuclear receptors COUP-TFII and ERRα, or the co-regulator RIP140 exhibit a lean phenotype; however, the expression of these genes was unaltered in TAK1^−/− liver. Because TAK1 itself functions as a transcription factor, one might expect that some of the differentially expressed genes be regulated directly by TAK1. Indeed, a recent report showed that TAK1 regulates CD36 transcription in macrophages by binding to TAK1 response elements in the CD36 gene promoter (14) suggesting that CD36 is a direct TAK1 target gene. CD36 is also a known target of several other nuclear receptors, including PPARγ, LXR, and PXR (42). Although the expression of PXR and LXR was unchanged, the expression of PPARγ was reduced by 50% in liver of TAK1^−/− mice. Therefore, hepatic CD36 expression might be regulated by TAK1 directly as well as indirectly through modulation of PPARγ expression (Fig. 8D). The co-regulators RIP140 and PGC-1α, and the receptor PPARγ have also been implicated in the regulation of Cidec (29; 42; 43). TAK1 might cooperate with these transcriptional modulators to regulate the expression of these genes. Moreover, the down-regulation of the transcription factor Srebf1, which promotes triglyceride synthesis (44), may contribute to
the reduced lipid accumulation in TAK1−/− liver.

Our data also demonstrated that the expression of several lipogenic genes was dramatically decreased in TAK1−/− primary hepatocytes compared to WT hepatocytes. Restoration of TAK1 expression in TAK1−/− hepatocytes by Ad-TAK1 induced the expression of Mogat1, Cidea, and Cidec, while empty virus or expression of an inactive form of TAK1 had little effect on their expression level. Moreover, down-regulation of TAK1 in Hepa1-6 cells by TAK1 siRNAs suppressed Cidec, while stable expression of TAK1 induced Cidec expression. These data indicate that these changes in gene regulation by TAK1 are hepatocyte cell autonomous and not a response to changes in other tissues. Whether these TAK1-responsive genes are direct targets of TAK1 transcriptional regulation needs further study.

Recent studies have provided evidence indicating that TAK1 functions as a ligand-dependent transcription factor. Certain fatty acids, including γ-linoleic acid and γ-linolenic acid, as well as several eicosanoids, have been shown to activate TAK1-mediated transcription suggesting that TAK1 might function as a fatty acid sensor (13; 14). Consistent with this hypothesis, we speculate that during aging or when fed a HFD, elevated levels of fatty acids may result in increased activation of TAK1 and enhanced expression of TAK1 responsive genes, such as CD36, that promote fatty acid uptake and triglyceride accumulation, and subsequent obesity (Fig. 8D). Hence, one could speculate that TAK1-selective antagonists would inhibit the expression of these genes and might be useful for the management of metabolic syndrome.

In addition to hepatic steatosis, adiposity is greatly reduced in aged TAK1−/− and TAK1−/−(HFD) mice compared to WT mice. The adipocytes in TAK1−/− mice were significantly smaller than in WT mice suggesting reduced storage of triglycerides. Obesity is well known to be associated with chronic, low-grade inflammation and there is considerable evidence that inflammation, insulin resistance, and aberrant lipid metabolism are interlinked in metabolic syndrome (3-5; 9). Hypertrophy of adipose tissues and infiltration of inflammatory cells have been recognized as important early events in the development of obesity-linked pathologies. The molecular process of the recruitment and function of macrophage infiltration is not fully understood; however, the release of various cytokines by adipose tissue is likely part of the recruitment of various immune cells (6-8). In contrast to WT mice, TAK1−/− mice are protected against the development of age- and diet-induced adipose tissue-associated inflammation as indicated by reduced infiltration of macrophages and T lymphocytes. Crown-like structures were rarely observed in WAT of TAK1−/− mice and the macrophage markers, F4/80 and Mac-2, were expressed at significantly lower levels. In addition, the expression of several pro-inflammatory genes, including Saa3, Mmp-12, Il1rn, and Tlr8, were also reduced in adipose tissues of TAK1−/− mice. T lymphocytes have also been implicated in the development of obesity-associated complications (6-8; 30). CD8+ effector T cells have been reported to exhibit an essential role in the initiation and maintenance of adipose tissue inflammation, including macrophage recruitment, during obesity. The observed reduction in the number of CD8+ cells in SVF might be linked to the diminished infiltration of macrophages and inflammatory response in TAK1−/− mice. Moreover, the reduced WAT inflammation in TAK1−/− mice may in part be responsible for the preservation of the insulin sensitivity and glucose tolerance observed in TAK1−/− mice. In this regard, the repression of Il1rn expression in TAK1−/− WAT is particularly interesting because up-regulation of this gene has been reported to be associated
with obesity while Il1rn KO mice have been shown to be resistant to obesity (45; 46). Therefore, repression of this gene may contribute to the resistance to obesity observed in TAK1 KO mice.

Finally, two important factors in energy balance are food intake and energy expenditure. Although their relative food intake was slightly higher than their WT littermates, TAK1—/— mice exhibited a lean phenotype compared to WT mice. Furthermore, TAK1—/— mice showed a significant increase in energy expenditure as indicated by increased oxygen consumption and CO2 production rates. The increase in energy expenditure by TAK1—/— mice is consistent with the elevated expression of UCP1 in BAT. UCP1 diverts energy derived from mitochondrial electron transport chain and generation of ATP into heat production. Thus, the elevated energy expenditure observed in TAK1—/—(HFD) mice may at least in part be responsible for the reduced weight gain and resistance to hepatic steatosis and insulin insensitivity.

In summary, in this study we show for the first time that TAK1—/— mice are protected against age- and HFD-induced obesity, hepatic steatosis, adipose tissue-associated inflammation, and insulin resistance. As a ligand-dependent nuclear receptor, TAK1 might provide a novel therapeutic target in the management and prevention of obesity and related pathologies, such as diabetes.

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**Legends**

FIG. 1. TAK1−/− mice are resistant to age-induced hepatic steatosis and display a reduced adiposity. **A:** Representative H&E staining of sections of liver, WAT, and BAT from one year-old WT and TAK1−/− male mice. Scale bar indicates 250 μm. **B:** One year-old male TAK1−/− mice fed a normal diet have a reduced total body weight compared to littermate WT controls. **C:** Relative weights of epididymal (eWAT) and abdominal (AbWAT) WAT of WT and TAK1−/− mice. **D:** Comparison of the cell size of WAT adipocytes from one year-old WT and TAK1−/− male mice. Cell diameters (n=100) were measured and the percentages of different size cells calculated and plotted.

FIG. 2. Reduced lipid accumulation and lipogenic gene expression in liver of aged TAK1−/− mice. **A:** Comparison of cholesterol (Chol), triglyceride (TG), and glucose (levels in liver and serum from one year-old WT and TAK1−/− male mice on a normal diet (WT, n=6; TAK1−/−, n=10). **B:** Relative food intake by WT and TAK1−/− mice. **C:** Several genes with roles in lipid accumulation are expressed at significantly lower levels in livers of one year-old male TAK1−/− mice than those of littermate WT mice (WT, n=6; TAK1−/−, n=10). The level of expression was examined by QRT-PCR. Data represent mean ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001.

FIG. 3. Changes in lipogenic gene expression in liver and primary hepatocytes from 4-5 month-old, chow-fed TAK1−/− mice. **A, B:** Representative H&E stained sections of liver from WT and TAK1−/− male mice. Scale bar indicates 200 μm. **C:** Reduced expression of several lipogenic genes in liver of 4-5 month-old male TAK1−/− mice compared to WT littermates (WT, n=5; TAK1−/−, n=4). Hepatic gene expression was also compared between 1-year old and 4-5 month old WT and TAK1−/− mice. Data represent mean ± SEM. *, p<0.05; **, p<0.01. **D:** Comparison
of gene expression between primary hepatocytes from 4-5 month-old TAK1−/− and WT mice. E: TAK1−/− hepatocytes were infected with Ad-Empty, Ad-TAK1WT, or Ad-TAK1ΔAF2 adenovirus and 72 hrs later analyzed for Cidea, Mogat1, Cidec, and Pparγ expression by QRT-PCR (right panel). The expression of TAK1 and TAK1ΔAF2 was confirmed by Western blot using anti-Flag M2 antibody (left panel).

FIG. 4. TAK1−/− mice are resistant to diet-induced obesity. Ten week-old male mice were fed a HFD for 6 weeks. A: The percent body weight gain was calculated based on the body weight at the start of the HFD. The average body weight gains of WT (n=6) and TAK1−/− (n=6) mice were calculated and plotted. (*, p< 0.05; **, p<0.01; ***, p<0.001). B: Comparison of the relative weights of kidneys, eWAT, and AbWAT were determined after 6 weeks on a HFD. *, p< 0.01. C – F: Representative H&E stained sections of liver and WAT from WT(HFD) and TAK1−/−(HFD) mice.

FIG. 5. Reduced lipid accumulation and lipogenic gene expression in liver of TAK1−/− mice fed a HFD. A: Comparison of hepatic triglyceride and cholesterol levels in WT(HFD) and TAK1−/−(HFD) mice (n=6) fed a HFD for 6 weeks. B: Lipid and glucose levels in serum of WT(HFD) and TAK1−/−(HFD) mice. C: Steatocrit was analyzed from feces of WT(HFD) and TAK1−/−(HFD) mice. D: ALT and AST activity in serum of WT(HFD) and TAK1−/−(HFD) mice. E: Comparison of hepatic gene expression in WT(HFD) and TAK1−/−(HFD) mice. Gene expression was analyzed by QRT-PCR. Data represent mean ± SEM. (*, p<0.05; **, p<0.01; ***, p<0.001).

FIG. 6. TAK1−/− mice have increased energy expenditure. A–D: Oxygen consumption (VO2) and carbon dioxide generation (VCO2) by WT(HFD) and TAK1−/−(HFD) were analyzed by indirect calorimetry during two 12 h light/12 h dark cycles (WT, n=6, TAK1−/− n=5). Respiratory exchange ratio (RER) and heat generation were computed. E: Relative food consumption of WT and TAK1−/− mice during light and dark periods. F: Increased expression of Ucp-1, CoxIV, and Pgc-1α in BAT of TAK1−/−(HFD) mice compared to WT(HFD) littermates. Gene expression was analyzed by QRT-PCR. Data represent mean ± SEM. * p <0.05, ** p<0.01, *** p<0.001.

FIG. 7. WAT-associated inflammatory response is reduced in TAK1−/−(HFD) mice. A: Macrophage infiltration into eWAT was greatly reduced in TAK1−/− mice. F4/80+ macrophages were identified by immunohistochemical staining as “crown-like structures” (arrows). Scale bar indicates 250 µm. B: SVF cells from eWAT of WT(HFD) and TAK1−/−(HFD) mice were examined by FACS analysis. The percent macrophages (F4/80+CD11b+ cells), T lymphocytes (CD3+ cells), and CD8+ and CD4+ T cells were determined (WT, n=6; TAK1−/−, n=4). Data represent mean ± SEM. *, p<0.05. C: Induction of inflammatory genes was greatly decreased in WAT of TAK1−/−(HFD) mice (n=5) compared to WT mice (n=5). Gene expression was analyzed by QRT-PCR. Data represent mean ± SEM. * p <0.05, ** p<0.01, *** p<0.001.

FIG. 8. TAK1−/− mice are protected against HFD-induced insulin resistance and glucose intolerance. A: Blood insulin levels were analyzed in 5-month old mice (WT, n=5; TAK1−/−, n=4), one-year old mice (WT, n=8; TAK1−/−, n=9), and mice fed a HFD (WT, n=10; TAK1−/−, n=7). B. C: Glucose tolerance test (GTT) and insulin tolerance test (ITT) analyses in WT(HFD) and TAK1−/−(HFD) mice (WT, n=5; TAK1−/−, n=4). Blood samples were drawn and glucose levels analyzed every 20 min for up to 2-2.5 hrs. Data represent mean ± SEM. * p <0.05, ** p<0.01,
*** p<0.001. D: Schematic view of the potential role of TAK1/TR4 in lipid homeostasis and hepatic steatosis. Elevated levels of fatty acids during aging and HFD may promote the activation of TAK1 leading to increased transcription of TAK1-responsive genes, such as CD36, Cidec, Cidea, and Mogat1. The induction of these proteins then lead to increased fatty acid uptake and triglyceride synthesis and storage, and promote hepatic steatosis. Induced expression of other transcription factors, such as PPARγ, by TAK1 can also lead to the activation of CD36, Cidec or other lipogenic genes and may provide an alternative way to further enhance hepatic triglyceride accumulation.
Table 1. A partial list of genes up- or down-regulated in the liver of one year-old TAK1−/− mice compared to WT liver. Of the 40,000 transcripts analyzed, the expression of 490 transcripts was decreased by ≥1.5-fold, while the expression of 260 transcripts was enhanced by ≥1.5-fold in livers of TAK1−/− mice compared to WT mice.

| Functional Category | Gene Symbol | GeneBank Accession# | Gene Description | Fold change |
|---------------------|-------------|---------------------|------------------|-------------|
| **Metabolism**      |             |                     |                  |             |
| Lipid               | Acsm2       | NM_146197           | acyl-CoA synthetase medium-chain family member 2 | 3.9         |
|                     | Mgll        | NM_011844           | monoglyceride lipase | -1.4        |
|                     | Dhrs8       | NM_053262           | hydroxysteroid (17-beta) dehydrogenase 11 | -1.5        |
|                     | Adfp        | NM_007408           | adipose differentiation related protein | -1.5        |
|                     | Adipor2     | NM_197985           | adiponectin receptor 2 | -1.5        |
|                     | Lrp4        | NM_172668           | low density lipoprotein receptor-related protein 4 | -1.6        |
|                     | Acox1       | NM_015729           | acyl-Coenzyme A oxidase 1, palmitoyl | -1.7        |
|                     | Lpin1       | NM_015763           | lipin 1/Fatty liver dystrophy protein | -1.8        |
|                     | EhhaA       | NM_023737           | enoyl-Co A, hydratase/3-hydroxyacyl Co A dehydrogenase | -1.8        |
|                     | Acaalb      | NM_146230           | acetyl-Coenzyme A acyltransferase 1B | -1.8        |
|                     | Acad10      | NM_028037           | acyl-CoA dehydrogenase family member 10 | -1.8        |
|                     | Dgat2E      | NM_177746           | acyl-CoA wax alcohol acyltransferase 2 | -1.8        |
|                     | Fabp2       | NM_007980           | fatty acid binding protein 2, intestinal | -1.8        |
|                     | AcaalbA     | NM_130864           | acetyl-Coenzyme A acyltransferase 1A | -1.9        |
|                     | Crat        | NM_007760           | carnitine acyltransferase | -2.0        |
|                     | Acss2       | AK035497            | acyl-CoA synthetase short-chain family member 2 | -2.1        |
|                     | Elolv5      | NM_134255           | ELOVL family member 5, elongation of long chain fatty acids | -2.2        |
|                     | Acot2       | NM_134188           | acyl-CoA thioesterase 2 | -2.2        |
|                     | Gpam        | NM_008149           | glycerol-3-phosphate acyltransferase, mitochondrial | -2.6        |
|                     | Acot11      | NM_025590           | acyl-CoA thioesterase 11 | -3.9        |
|                     | Cd36        | NM_007643           | CD36 antigen | -3.9        |
|                     | Mogat1      | NM_026713           | monoacylglycerol O-acyltransferase 1 | -14.6       |
|                     | Cidec       | NM_178373           | cell death-inducing DFFA-like effector c (FSP27) | -18.0       |
|                     | Cidea       | NM_007702           | cell death-inducing DFFA-like effector A | -94.3       |
| **Carbohydrate**    | Car2        | NM_009801           | carbonic anhydrase 2 | -1.6        |
| **Steroid**         | Osbp3       | AK040984            | oxysterol binding protein-like 3 | -4.2        |
| **Glutathione**     | Mgst3       | NM_025569           | microsomal glutathione S-transferase 3 | -1.5        |
|                     | Gst1        | NM_008185           | glutathione S-transferase, theta 1 | -1.6        |
|                     | Gst2        | NM_010361           | glutathione S-transferase, theta 2 | -1.7        |
|                     | Gst3        | NM_133994           | glutathione S-transferase, theta 3 | -2.2        |
| **Cytochrome c**    | Cox7a1      | NM_009944           | cytochrome c oxidase, subunit VIIa 1 | -1.6        |
| oxidase VIIb        | Cox8b       | NM_007751           | cytochrome c oxidase, subunit VIIb | -4.4        |
| **Cytochrome P450** | Cyp2c70     | NM_145499           | cytochrome P450, family 2, subfamily c, polypeptide 70 | 2.0         |
|                     | Cyp2c40     | NM_010004           | cytochrome P450, family 2, subfamily c, polypeptide 40 | 2.0         |
|                     | Cyp39a1     | NM_018887           | cytochrome P450, family 39, subfamily a, polypeptide 1 | 1.8         |
|                     | Cyp51       | NM_020010           | cytochrome P450, family 51 | 1.7         |
|                     | Cyp5b       | NM_025558           | cytochrome P450, family 5 type B | -1.5        |
|                     | Cyp2a5      | NM_007812           | cytochrome P450, family 2, subfamily a, polypeptide 5 | -1.7        |
|                     | Cyp2a4      | NM_009997           | cytochrome P450, family 2, subfamily a, polypeptide 4 | -2.0        |
|                     | Cyp4a10     | NM_010011           | cytochrome P450, family 4, subfamily a, polypeptide 11 | -2.7        |
### TAK1 and metabolic syndrome

| Others          | Asns   | NM_012055 | asparagine synthetase | 26.8 |
|-----------------|--------|-----------|-----------------------|------|
| Arsa            | NM_009713 | arylsulfatase A |                  | -1.6 |
| Aldh3a2         | NM_007437 | aldehyde dehydrogenase family 3, subfamily A2 | -1.9 |
| Uck1            | NM_011675 | uridine-cytidine kinase 1 | -2.0 |
| Wwox            | NM_019573 | WW domain-containing oxidoreductase | -2.0 |
| Rdh16           | NM_009040 | retinol dehydrogenase 16 | -2.3 |

### Transcription

| Others          | Onecut1 | BC023444 | one cut domain, family member 1 (Hnf6) | 3.0  |
|-----------------|---------|----------|---------------------------------------|------|
| Foxa1           | NM_008259 | forkhead box A1 (Hnf3a) | 2.0 |
| Sreb2f          | AF374267 | sterol regulatory element binding factor 2 | 1.4  |
| Rxrg            | NM_009107 | retinoid X receptor gamma | -1.4 |
| Ppargc1b        | NM_133249 | peroxisome proliferative activated receptor, gamma, coactivator 1 beta | -1.5 |
| Ar              | NM_013476 | androgen receptor | -1.5 |
| Nfe2l2          | AK029360 | nuclear factor, erythroid derived 2, like 2 | -1.6 |
| Pparg           | NM_011146 | peroxisome proliferator activated receptor gamma | -1.9 |
| Sreb1f          | NM_011480 | sterol regulatory element binding transcription factor 1 | -2.1 |

### Transport

| Others          | Apom    | NM_018816 | apolipoprotein M | 2.0  |
|-----------------|---------|-----------|------------------|------|
| Abcb9           | NM_019875 | ATP-binding cassette, sub-family B (MDR/TAP), member 9 | -1.7 |
| Abcb1a          | NM_011076 | ATP-binding cassette, sub-family B (MDR/TAP), member 1A | -2.1 |
| Abcd3           | AK031611 | ATP-binding cassette, sub-family D (ALD), member 3 | -2.3 |

### Solute carrier

| Others          | Slc25a14 | NM_011398 | solute carrier family 25 | -1.4 |
|-----------------|---------|-----------|--------------------------|------|
| Slc27a4         | NM_011989 | solute carrier family 27 (FATP4) | -1.5 |
| Slc5a6          | NM_177870 | solute carrier family 5 | -1.9 |
| Slc13a4         | NM_172892 | solute carrier family 13 | -4.2 |

### Growth/differentiation factors

| Others          | Fgfr1   | NM_010206 | fibroblast growth factor receptor 1 | 3.3  |
|-----------------|---------|-----------|------------------------------------|------|
| Ctgf            | NM_010217 | connective tissue growth factor | 2.1 |
| Bmp7            | NM_007557 | bone morphogenetic protein 7 | -1.5 |
| Vegfb           | NM_011697 | vascular endothelial growth factor B | -1.6 |
| Gdf15           | NM_011819 | Growth differentiation factor 15 (Mic-1) | -2.4 |
| Fgf9            | NM_013518 | fibroblast growth factor 9 | -4.1 |

### G-protein coupled receptor protein signaling

| Others          | Avpr1a  | NM_016847 | arginine vasopressin receptor 1A | 3.6  |
|-----------------|---------|-----------|----------------------------------|------|
| Adra1a          | NM_013461 | adrenergic receptor, alpha 1a | -2.0 |
| Gpcre5b         | NM_022420 | G protein-coupled receptor, family C, group 5, member B | -10.9 |

### Sulfotransferase

| Others          | Sult1c2 | NM_026935 | sulfotransferase 1C, member 2 | -2.3 |

### Immune response

| Others          | Tff3    | NM_011575 | trefoil factor 3, intestinal | 4.2  |
|-----------------|---------|-----------|-----------------------------|------|
| Th5             | NM_016928 | toll-like receptor 5 | -1.8 |
| Cxcl7           | NM_023785 | chemokine (C-X-C motif) ligand 7 | -2.2 |
| Raet1a          | NM_009016 | retinoic acid early transcript 1, alpha | -3.0 |

### Miscellaneous

| Others          | Sqle    | NM_009270 | squalene epoxidase | 2.6  |
|-----------------|---------|-----------|--------------------|------|
| Fbln2           | NM_007992 | fibulin 2 | 2.6 |
| Inhba           | NM_008380 | inhibin beta-A | 2.0 |
| Fbxo7           | AK082146 | F-box protein 7 | -1.9 |
| Ins6            | NM_013754 | insulin-like 6 | -2.5 |
| Adam11          | BC054536 | a disintegrin and metallopeptidase domain 11 | -3.6 |
| Retn            | NM_022984 | resistin | -3.7 |
Figure 1

A

Liver

WAT

BAT

WT

WT

WT

TAK1<sup>−/−</sup>

TAK1<sup>−/−</sup>

TAK1<sup>+</sup>

B

C

D

Body weight (g)

Relative tissue weight %

% cells

eWAT

AbWAT

μm diameter

WT

TAK1<sup>−/−</sup>
Figure 2

A

![Graph A showing liver cholesterol and triglyceride levels in WT and TAK1-/- mice.]

B

![Graph B showing serum concentration in WT and TAK1-/- mice.]

C

![Graph C showing relative expression of genes in the liver of 1-year-old mice.]

TAK1 and metabolic syndrome
Figure 5

A. Liver lipid level (mg/dl)

B. Serum concentration (mg/dl)

C. Serum concentration (mg/dl)

D. ALT and AST activity from Serum (U/L)

E. Relative gene expression
Figure 6

A  VO2  

B  VCO2  

C  RER  

D  Heat  

E  Relative food consumption  

F  Relative expression
Figure 7

**A**  
WT vs. TAK1⁻/⁻ cellular structures

**B**  
Bar graphs showing the percentage of SVF cells and the percentage of cells from CD3⁺ populations in WT vs. TAK1⁻/⁻ conditions.

**C**  
Bar graph depicting relative gene expression levels for various genes in WT and TAK1⁻/⁻ conditions.
Figure 8