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Deletion of TNF-Like Weak Inducer of Apoptosis (TWEAK) Protects Mice From Adipose and Systemic Impacts of Severe Obesity

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Abstract

Objective—To investigate the role of TNF-like weak inducer of apoptosis (TWEAK) in pathological adipose tissue (AT) remodeling and complications of obesity.

Design and Methods—Wild type (WT) and TWEAK knockout (KO) mice were fed normal diet (ND) or a high fat diet (HFD) for up to 17 weeks. Adipocyte death was induced using an established transgenic mouse model of inducible adipocyte apoptosis (FAT-ATTAC). Metabolic, biochemical, histologic and flow cytometric analyses were performed.

Results—TWEAK and its receptor, fibroblast growth factor-inducible molecule 14 (Fn14) were upregulated in gonadal (g)AT of WT mice after HFD week 4 and 24 h after induction of adipocyte apoptosis. Phenotypes of KO and WT mouse were indistinguishable through HFD week 8. However, at week 17 obese KO mice had ~30% larger gAT adipocytes and gAT mass than WT mice, coincident with reduced adipocyte death, enhanced insulin signaling, Th2/M2 immune skewing, fewer thick collagen fibers, and altered expression of extracellular matrix constituents and modulators that is consistent with reduced fibrosis and larger adipocytes. KO mice were less

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GB performed all experiments and wrote the initial draft. KJS planned and supervised flow cytometry. JD assisted with metabolic studies JW. and DW performed splenocyte and T cell assays. LCB assisted with the experimental plan, data interpretation and writing. MSO supervised all aspects of the research and manuscript preparation.

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steatotic and became more insulin sensitive and glucose tolerant than WT mice after HFD week 12.

Conclusion—TWEAK constrains ‘healthy’ gAT expansion and promotes metabolic complications in severe obesity.

Keywords

TWEAK; adipose tissue; obesity; fibrosis; inflammation; insulin resistance

Introduction

Adipose tissue (AT) expands by a program of homeostatic remodeling involving extracellular matrix (ECM) turnover, angiogenesis, adipogenesis and adipocyte hypertrophy (1; reviewed in 2). This program can become dsyregulated in hypertrophic obesity, transitioning to a maladaptive, pathologic process characterized by proinflammatory skewing of immunity, adipocyte insulin resistance (IR), increased adipocyte turnover and excessive ECM deposition (fibrosis) (1–5). These events impair triglyceride sequestration in AT and are therefore implicated in ectopic lipid deposition and metabolic complications of obesity (1, 3, 4, 6, 7). We model this transition in the gonadal adipose tissue (gAT) of male C57BL/6 mice fed a high saturated fat diet (HFD) for ≥ 10 weeks (16). In our laboratory, maximal adipocyte hypertrophy and gAT mass are attained after 12 weeks of HFD. However by week 16, increases in adipocyte turnover in an increasingly proinflammatory and fibrotic milieu reduce adipocyte size and gAT mass by >30% to values measured at HFD week 8 (1). Additional metrics of this pathologic remodeling between HFD weeks 12 and 16 include the negative correlation of gAT mass with whole body mass and the acceleration of hepatic and skeletal muscle steatosis (1).

Tumor necrosis factor (TNF)-related weak inducer of apoptosis (TWEAK) is a Type II membrane protein of the TNF superfamily that is expressed primarily on leukocytes and cleaved to a soluble form by furin in response to inflammatory stimuli (8,9). Pleiotropic actions of membrane-bound (m)TWEAK) and soluble (s)TWEAK) are mediated by its signaling receptor, fibroblast growth factor-inducible molecule 14 (Fn14), a Type I membrane protein upregulated by inflammation, injury or disease on diverse cell types. Receptor engagement activates NF-κB, ERK1/2 and p38 pathways underlying numerous processes, including inflammation and ECM remodeling (8–10). TWEAK exerts pleiotropic actions depending on context. In acute injury or inflammation, TWEAK coordinates inflammatory and proliferative responses that facilitate regeneration and repair. However, in settings of chronic insult characterized by persistent Fn14 upregulation, TWEAK can amplify inflammation and promote tissue damage, fibrosis and aberrant remodeling (8).

TWEAK’s roles in adipose tissue biology and obesity are controversial. Pathologic impacts in obesity are suggested by upregulated TWEAK and Fn14 gene expression in subcutaneous and visceral AT depots of morbidly obese subjects (11–13) and by association of Fn14 levels with type 2 diabetes (T2D) in morbidly obese women (11). However, exogenous TWEAK does not impair insulin signaling, alter adipokine production or significantly increase proinflammatory cytokines in cultured adipocytes, although it can reportedly

downregulate C/EBPα and PPARγ (14). To the contrary, an emerging view suggests that sTWEAK is cardiometabolically protective (reviewed in 10). This view is based on the ability of sTWEAK to antagonize TNF-α actions *in vitro* and by the association of low levels of circulating sTWEAK with poorer cardiometabolic profiles in obese and T2D patients (10).

Here, using TWEAK-deficient (KO) mice we directly test the hypothesis that TWEAK promotes aberrant gAT remodeling and metabolic complications of chronic HFD-induced obesity.

Methods

Materials

Animal care—TWEAK KO mice (backcrossed 10 generations to C57BL/6J) (15) and WT mice derived from mating of TWEAK heterozygotes were obtained from Biogen Idec, Inc.. Mice were housed at the Comparative Biology Unit of the JMUSDA-HNRCA at Tufts University (16). Individually-caged, 9–12 week old weight-matched male WT and TWEAK KO mice were fed a normal diet (ND; 17% fat kcal; Harlan Teklad #7012)) or a HFD (60% fat kcal; Research Diets #D12492) for up to 17 weeks. Mice were euthanized by CO_2 narcosis/cervical dislocation. Harvested tissues were snap frozen, fixed for histology or used immediately for FACS analysis. FAT-ATTAC mice (17) were from Dr. Philipp Scherer (Texas Southwest Medical Ctr.) and maintained on ND. At 8 weeks of age male FAT-ATTAC mice received (IP) either 0.1 μg/kg FK1012 analog to initiate caspase-8 dependent adipocyte apoptosis or saline control (17). Mice were euthanized after 24 h and gAT was harvested. All procedures adhered to the JMUSDA-HNRCA Institutional Animal Care and Use Committee guidelines under protocols OB-29 and OB-30.

Procedures

Collagen analysis (18), tissue triglyceride determination (19), insulinogenic index (20) and IFN-γ and IL-4 production by splenocytes and CD4+ T cells (21) were by established procedures. All other procedures were as per our published methods (1, 16, 22).

Primer sequences (Table S1), antibodies and flow cytometry gating (Figure S1) are described in Supporting Information.

Statistical analysis

Data are presented as mean ± SEM. Cell sizes were log- transformed and percentage data were arcsin√- transformed (1). Differences between treatments were assessed by PROC TTEST or PROC GLM (SAS v9.2.l Cary, NC) using Tukey's HSD procedure or Bonferroni-protected t-tests. $P \leq 0.05$.

Results

TWEAK and Fn14 are upregulated by HFD feeding and by adipocyte death

In WT mice made obese (> 38 g) by 17 weeks of HFD, TWEAK gene expression was greatest in gAT as compared to retroperitoneal (rpAT), mesenteric (mAT) or subcutaneous (scAT) depots (Fig. 1A). Fn14 gene expression was 7–9 fold greater in gAT than in other depots examined (Fig. 1A). A gene expression time course in gAT indicated that TWEAK and Fn14 transcripts increased progressively after HFD week 4 (Fig. 1B), but remained unchanged in mice fed ND (data not shown). These results demonstrate HFD-induced upregulation and greater transcript levels of TWEAK and F14 in gAT relative to other AT depots in obese mice.

TWEAK was expressed by ATMφs, notably MGL1−/CD11c+ ATMφs (Fig. 1C, left panel), a subset expressing a mixed M1/M2 phenotype that is recruited to sites of obesity-associated adipocyte death and implicated in the immunopathogenesis of IR (22–24). Fn14 transcripts were upregulated in adipocytes and ATMφs, with greater expression in adipocytes (Fig. 1C, right panel). These results link TWEAK expression in gAT with ATMφ infiltration and render it plausible that ATMφ-derived TWEAK engages Fn14 on adipocytes.

TWEAK and FN14 gene expression were upregulated in response to adipocyte death in lean FAT-ATTAC mice (Fig. 2) (17). Fn14 mRNA was elevated 8-fold following induction of adipocyte apoptosis (Fig. 2B), at which time ~20% of adipocytes had formed crown-like structures (Fig. 2A). These results suggest that adipocyte death *per se* can elicit an Fn14-mediated injury response.

Increased adipocyte size and gAT mass in obese TWEAK KO mice

WT and KO mice gained comparable body mass on both ND and HFD (Fig. 3A), reflecting similar energy balance and respiratory exchange ratios in WT and KO mice (Fig. S2). After 17 weeks of diet, AT depots in mice fed ND and mAT, rpAT and scAT in mice fed HFD did not differ between WT and KO mice (Fig. 3B). However, among mice fed HFD, gAT mass was significantly (33%; 800 mg) greater in KO mice (Fig. 3B). Greater gAT mass reflected proportionally more of the largest adipocytes and proportionally fewer of the smallest, resulting in ~30% greater mean adipocyte size (Fig. 3C, D). Accordingly, leptin and mesoderm-specific transcript (MEST) mRNAs were more highly expressed in gAT of obese KO mice (Table S2). TWEAK abrogation had no significant effect on adipocyte number (WT: $6.31 \pm 0.65 \times 10^6$ cells; KO: $6.35 \pm 0.91 \times 10^6$ cells, n = 6, 8, $p = 0.99$), or on transcript levels of C/EBPα, PPARγ or additional genes regulating lipid metabolism (Table S2).

Notably, adipocyte size (Fig. 3D). and gAT mass (data not shown) were indistinguishable between WT and KO mice after the initial 8 weeks of HFD. Adipocyte size in obese KO mice increased thereafter from ~ 5,800 μm^2 to ~7,800 μm^2 (compare Fig. 3D with Fig. 3C) and gAT mass and body mass remained positively- correlated ($r^2 = 0.70$, $p \leq 0.01$, data not shown). In contrast, adipocyte size in obese WT mice (~5,800 μm^2) was no greater at week 17 than at week 8 (compare Fig. 3D with Fig. 3C), and gAT mass and body mass were negatively- correlated at week 17 ($r^2 = -0.40$, $p = 0.07$, data not shown). Overall, these

results suggest that TWEAK deficiency prevented or substantially attenuated gAT regression after HFD week 12.

Larger gAT adipocytes in obese KO mice are insulin sensitive

Despite greater adipocyte size, expression of ER stress-related transcripts was not significantly elevated in gAT of obese KO mice (Table S2), and the frequency of crown-like structures was reduced ($p = 0.10$; Fig. 4A,B). In addition, *in vivo* insulin signaling (AKT phosphorylation) was enhanced in gAT of obese KO mice (Fig. 4C). These results link greater triglyceride storage in gAT of obese KO mice (Fig. 3B–D) with enhanced adipocyte viability and insulin sensitivity.

Altered immune activation in gAT of obese TWEAK KO mice

Th1/M1 immune activation promotes AT and systemic complications of obesity (24, 25). Expression of select Th1/M1 genes was attenuated in gAT of obese KO mice, including interferon gamma (IFNγ), regulated upon activation normal T cell expressed (RANTES/CCL5), the IFN-γ target gene F locus-associated transcript (FAT)10 (26), and monokine induced by IFNγ (MIG/CXCL9) (Fig. 5A). Transcript levels of other proinflammatory genes were not significantly reduced in obese KO mice, including TNF-α, inducible nitric oxide synthase, and interleukins -1α, -6, -8 and -12 (Table S2), The Th2/M2 genes IL-13 and its target, YM-1/Chitinase 3-like 3 were upregulated in gAT of obese KO mice, potentially reflecting ~ two-fold higher levels of transcripts for IL-25 (Table S2), which stimulates IL-13 expression in innate immune cells (27). However, levels of IL-4, IL-10 and arginase-1 transcripts were not altered by TWEAK deficiency (Table S2), and transcript levels of the M2 Mφ markers, found in inflammatory zone (Fizz)-1 and CD163 were downregulated (Fig. 5C). Despite no statistically significant change in IL-10 or IL-12 gene expression (Table S2), the ratio of the two transcripts was increased >2-fold in obese KO mice (Fig. 5D), indicating M2 skewing (28). These results suggest that TWEAK deletion promotes a selective Th2/M2 skewing of immune activation in gAT of HFD-fed mice that is characterized by attenuated expression of IFNγ-associated genes and enhanced expression of some IL-13- associated genes.

Numbers of ATMφs ($F4/80^+$ cells) were essentially identical in gAT of obese KO and WT mice ($p < 0.97$; data not shown). However, consistent with reduced Th1 gene expression and enhanced IL-10:IL-12 ratio, gAT of obese KO mice contained fewer $MGL1^-/CD11c^+$ (IL-12 expressing) ATMφs and proportionally more $MGL1^+/CD11c^-$ (IL-10 expressing) ATMφs (Fig. 5E) (21). Similar results were obtained using CD206 (rather than MGL1) as a marker of M2 ATMφs (data not shown). T cells ($CD3^+$) and $CD4^+$ subtypes, including T regulatory (Treg) and T helper 1 (Th1) cells were more plentiful in gAT of obese KO as compared with WT mice (Fig. 5F,G). However, the ratio of Tregs to Th1 cells was not increased in TWEAK-deficient mice (Fig. 5G). Moreover, we detected no significant differences in the number of NK or $CD8^+$ cells in obese KO as compared to WT mice (Fig. 5F) or intrinsic alterations in the capacity for Th1 or Th2 activation (Fig. S3) that would account for reduced Th1 gene expression in gAT of obese KO mice.

Reduced gAT fibrosis in obese TWEAK KO mice

Picrosirius staining viewed under visible light suggested no reduction in total collagen in gAT of obese KO mice (Fig. 6A), consistent with comparable or greater expression of collagen genes (Fig. 6C). However, polarized light revealed dramatically fewer thick (cross-linked) collagen fibers throughout gAT of obese KO mice (Fig. 6B). This was associated with increased transcript levels of the ECM constituents, lumican and elastin (Fig. 6D). TWEAK deficiency also altered MMP expression in gAT (Fig. 6E). HFD-induced expression of MMP-3 and MMP-13 was attenuated in KO mice (Fig. 6E). MMP-7 was downregulated in KO mice on ND, and subsequently increased with HFD- i.e., a pattern opposite to that in WT mice. In addition, MMP-14 transcripts were significantly upregulated in obese KO but not in obese WT mice (Fig. 6E), and levels in KO tended to be greater than in WT mice ($p = 0.09$, Fig. 6E). Levels of MMP -2, -9 and 12 transcripts were not altered by TWEAK deletion (data not shown). TIMP-1 transcripts were modestly increased in gAT of obese KO mice, whereas transcripts for TIMPs 2, 3 and 4 were modestly (1.3–1.7 fold) reduced (Fig. 6F). As TIMPs 2–4 inhibit MMP-14, but TIMP-1 does not (29), the ratio of MMP-14 transcripts to endogenous TIMP inhibitors was 2.5 to 3.6- fold greater in gAT of obese KO mice as compared with obese WT mice ($p \leq 0.01$).

Transcript levels of other molecules implicated in fibrosis pathology were not significantly altered by TWEAK deficiency, including transforming growth factor beta-1, the collagen cross-linking enzyme lysyl oxidase, decorin, hypoxia-inducible factor 1α, plasminogen activator inhibitor -1, and vascular endothelial growth factor-1 (Table S2). Overall, these results suggest that the formation or stability of thick collagen fibers is reduced in gAT of obese KO mice, coincident with altered expression of several ECM components and MMPs.

Metabolic protection in obese TWEAK KO mice

After 17 weeks of HFD, KO mice were protected from dyslipidemia and ectopic fat deposition a (Figs. 7A, B). In addition, they exhibited enhanced whole body insulin action and glucose tolerance (Figs. 7C,D), and a lower insulinogenic index (Fig. 7E), a characteristic of enhanced beta cell function (20). In accord with these results, insulin-signaling was enhanced in quadriceps and liver of obese KO mice (Fig. 7F). Notably, GTT and ITT responses of obese WT and KO mice were comparable when measured at HFD weeks 8 and 12 (Fig. 7G). Thus, enhanced glucose tolerance and insulin sensitivity emerged in obese KO mice relative to obese WT mice after 12 weeks of HFD.

Enhanced insulin signaling in liver and skeletal muscle of obese KO mice (Fig. 7F) were associated with attenuated expression of TWEAK, TNF-α and FAT10 mRNA in liver (Fig. 8A) and TNF-α and FAT10 mRNA in quadriceps (Fig. 8B). Mice deficient for TWEAK (present study), TNF-α (30) and FAT10 (31) are more insulin sensitive than WT mice both in peripheral tissues and systemically. Moreover, TWEAK deletion reduced skeletal muscle upregulation of musclin (Fig. 8B), a myokine implicated in obesity-associated increases in glycolytic (Type IIb) fibers and impaired insulin action (32). These results link enhanced insulin signaling in liver and quadriceps of obese KO mice with reduced expression of genes that promote insulin resistance. HFD did not alter TWEAK gene expression in quadriceps of WT mice or Fn14 expression in liver or quadriceps of WT and KO mice (data not shown).

Discussion

The present study provides the first *in vivo* demonstration that the TWEAK/FN14 pathway regulates adipocyte, AT and whole body metabolism. We conclude that TWEAK constrains adipocyte hypertrophy and promotes maladaptive gAT remodeling, AT dysfunction and the systemic metabolic complications of HFD-induced obesity in mice. This conclusion derives from the time course of TWEAK and Fn14 upregulation in gAT of WT mice (Fig. 1) and our demonstration that obese TWEAK-deficient mice develop larger and more insulin-sensitive gAT adipocytes coincident with mitigation of systemic dyslipidemia, glucose intolerance and IR.

TWEAK deletion did not affect 1) 'normal' AT development, 2) adipocyte hypertrophy in mice fed HFD for 8 weeks, or 3) AT expansion in depots (mAT, rpAT, scAT) with relatively low frequencies of HFD-induced adipocyte death (1) and Fn14 gene expression (Fig. 1). These observations suggest that TWEAK/Fn14 signaling inhibits adipocyte and gAT expansion specifically during the maladaptive gAT remodeling associated with prolonged HFD feeding (1). Adipocyte death is a salient feature of this remodeling in mice and is linked to fibrosis and poor metabolic outcomes in both mice and humans (1, 5, 33). Although TWEAK itself is not cytotoxic to adipocytes (12), we show that Fn14 is expressed by adipocytes, is induced by adipocyte death in gAT of lean (FAT-ATTAC) mice and that CD11c+ ATMφs, which preferentially localize to- and remodel areas surrounding moribund adipocytes (1,2) express TWEAK. These results suggest that TWEAK/Fn14 signaling is a feature of adipocyte-macrophage crosstalk at sites of adipocyte death and subsequent remodeling. Autocrine effects of adipocyte-derived TWEAK could also regulate adipose expansion and gAT remodeling.

A less rigid ECM can facilitate adipocyte expansion while minimizing the cell stress, tissue inflammation and IR associated with extreme adipocyte hypertrophy (3, reviewed in 4) The larger, insulin sensitive adipocytes in gAT of obese TWEAK KO mice developed within a 'permissive' ECM containing less cross-linked collagen fibers, which impart tensile strength and rigidity to the ECM. This occurred despite no reductions in collagen gene expression and upregulation of the profibrotic Th2/M2 genes IL-13 and YM-1. However, transcript levels of FIZZ-1 and CD163, markers of fibrogenic wound healing Mφs (28), were reduced, suggesting less fibrogenic phenotype(s) of M2-polarized ATMφs in gAT of obese KO mice. The dearth of thick (mature) collagen fibers in obese KO mice may reflect elevated expression of lumican, a collagen-binding proteoglycan that can interfere with fibrilogenesis, resulting in reduced fibril thickness (34). Reduced mature collagen may also reflect enhanced collagen breakdown potentially resulting from the three-fold increase in the transcript ratio of MMP-14 to its inhibitors, TIMPs 2–4. Degradation of pericellular collage by MMP-14 is essential for adipocyte hypertrophy and maximal AT expansion in response to HFD (35). Other noteworthy changes in MMPs in obese KO mice include increased expression of MMP-7 and decreased expression of MMPs 3 and 13, as these changes were reported in the AT of hyper-obese but metabolically-protected collagen VI deficient mice (3). Moreover, mice deficient in MMP-3 (36) or MMP-3 activation (38) have larger adipocytes and are insulin sensitive. Lastly, increased expression of elastin is predicted to increase gAT distensibility in obese KO mice, potentially allowing the pericellular matrix to

accommodate larger changes in adipocyte volume (38). Zymography studies and analyses of ECM constituents will be required to confirm which proteolytic activities, collagen-modulating enzymes and ECM substrate levels are altered in obese KO mice to promote adipocyte expansion. Notwithstanding this limitation, our results implicate TWEAK in the AT fibrosis of obesity, consistent with its fibrogenic actions in other settings of chronic inflammation and persistent Fn14 upregulation (8, 9).

Reduced expression of IFNγ and IFNγ-associated genes may also contribute to the enhanced gAT and metabolic phenotype of obese KO mice, as IFN-γ promotes IR and RANTES is a recruitment and survival factor for inflammatory ATMφs (25, 39). Our data appear to contradict a prior study of immune activation in TWEAK KO mice distinct from those employed here (40). That study reported enhanced production of IFNγ and IL-12 and an accelerated transition to adaptive Th1 immunity in KO mice acutely challenged with LPS. The discrepancy between that study and the current one may reflect TWEAK's ability to promote different outcomes in settings of chronic as opposed to acute inflammation (i.e., obesity vs LPS challenge).

AT expansion in the relative absence of fibrosis and inflammation maintains metabolic homeostasis in response to energy surfeit (6, 7) and is a hallmark of metabolically 'healthy' obesity (33). Protection from glucose intolerance and IR emerged in obese KO mice after 12 weeks of HFD coincident with depot regression in gAT of obese WT mice, but continued gAT expansion and adipocyte insulin sensitivity in obese KO mice, These salutary effects in gAT of obese TWEAK KO mice were manifest in reduced circulating and ectopic lipids and potentially in the enhanced insulin signaling observed in liver and muscle. Our data suggest that direct effects of TWEAK deletion on liver and skeletal muscle (Fig. 8) also promote enhanced tissue and systemic insulin sensitivity in obese KO mice.

In conclusion, we note that TWEAK's pleiotropic actions partially overlap those of its more notorious sibling, TNFα, yet mice lacking TNF- α or its receptor (TNFR1) do not develop greater adipocyte size or gAT mass (30). This observation suggests that specific molecular actions and/or localization unique to TWEAK could be targeted to achieve therapeutic benefit in obesity. However, we must first determine the key cell types, form(s) of TWEAK (membrane or soluble), and mode(s) of signaling (autocrine, juxtacrine, paracrine) that contribute to pathologic AT remodeling and obesity complications. A related unresolved issue is the proposed cardiometabolic benefits of circulating sTWEAK (10). Although the suppression of TNF-α gene expression and the improved metabolic profile of obese TWEAK-deficient mice reported here fail to support such benefits, it does not rule them out. Despite this complexity therapeutic 'TWEAKing' of obesity and its complications remains a compelling translational goal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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What Is Already Known

1. TNF-related weak inducer of apoptosis (TWEAK) is a Type II membrane protein of the TNF superfamily that is expressed primarily on leukocytes and participates with its signaling receptor Fn14 in inflammatory tissue remodeling.
2. TWEAK and Fn14 are upregulated in subcutaneous and visceral AT depots of morbidly obese subjects, and Fn14 levels have been associated with Type 2 diabetes in morbidly obese women.
3. However, levels of circulating (s)TWEAK decline with obesity, are restored by bariatric surgery, and are inversely associated with cardiometabolic pathology, perhaps reflecting the reported *in vitro* ability of sTWEAK to antagonize TNF-α-induced insulin resistance.

What This Study Adds

1. Using mice globally-deficient in TWEAK, we provide the first *in vivo* demonstration that the TWEAK/FN14 pathway regulates adipocyte, adipose tissue and whole body metabolism.
2. We demonstrate that TWEAK's fibro-inflammatory actions constrain adipocyte hypertrophy and promote maladaptive adipose remodeling and the peripheral and systemic metabolic complications of high fat diet--induced obesity in mice.
3. The improved adipose and metabolic profile and the suppression of TNF-α gene expression in peripheral tissues of obese TWEAK-deficient mice do not support the hypothesis that declines in circulating sTWEAK promote cardiometabolic complications of obesity.

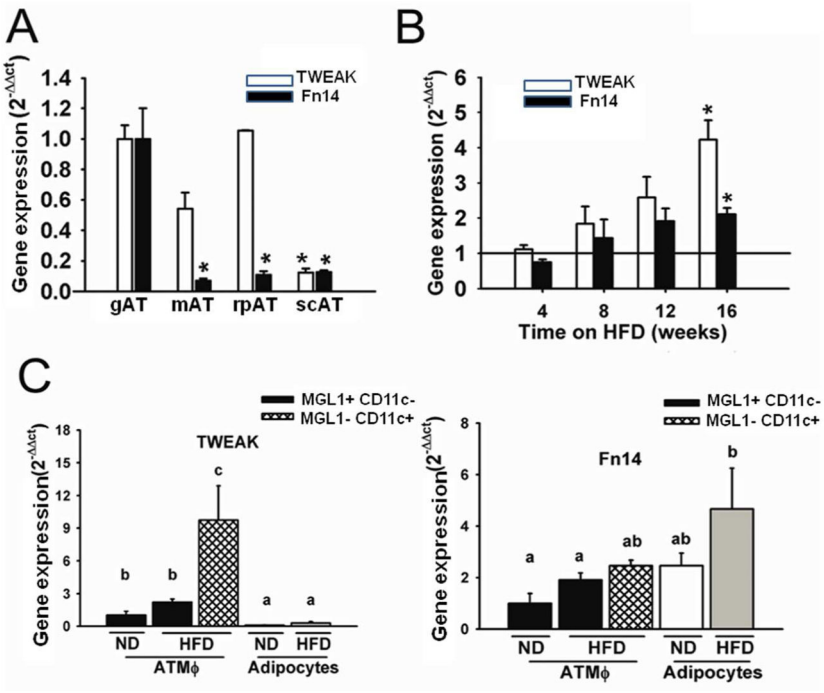

Figure 1. TWEAK and Fn14 gene expression in mouse AT

A) TWEAK and Fn14 gene expression in AT depots of WT mice fed high fat diet (HFD) for 17 weeks. Data are presented as mean ± SEM. *, significantly different from gonadal AT depot ($p \leq 0.05$), Student's t-test with Bonferroni adjustment (n=4/group). gAT, gonadal adipose tissue; mAT, mesenteric adipose tissue; rpAT, retroperitoneal adipose tissue; scAT, subcutaneous adipose tissue. **B**) TWEAK and Fn14 gene expression from gonadal AT in WT animals fed HFD for 4–17 weeks. *, significantly different from baseline values determined at the start of HFD (depicted by horizontal line) ($p<0.05$), Student's t-test with Bonferroni adjustment, (n=4/group). **C**) TWEAK (*left panel*) and Fn14 (*right panel*) gene expression in 'M2' (CD11c−/MGL1+) ATMφs (solid bars), 'M1/M2' (CD11c+/MGL1−) ATMφs (checked bars) and adipocytes of WT mice fed either normal diet (ND) or HFD for 17 weeks. Values designated by different letters are significantly different ($p<0.05$), one-way ANOVA with Tukey's HSD (n=6–8/group). Gene expression data are calculated as fold difference by the $2^{-\Delta\Delta CT}$ method. Primer sequences are provided in Table S1.

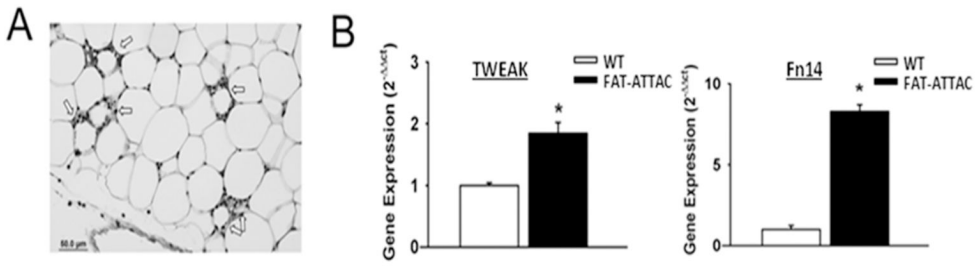

Figure 2. TWEAK and Fn14 gene expression increase with adipocyte death

A) Fixed sections of gAT from FAT-ATTAC mouse 24 after induction of adipocyte apoptosis by chemical dimerization of an aP2-driven caspase-8 fusion protein (17). Note crown-like structures (arrows). **B)** Gene expression of TWEAK (*left panel*) and Fn14 (*right panel*) in gAT 24 h after injection of dimerizer into WT (open bars) and FAT-ATTAC (shaded bars) mice. Data are presented as mean ± SEM fold difference calculated by the $2^{-\Delta\Delta CT}$ method. *, $p \leq 0.05$; Student's t test (n=6/group).

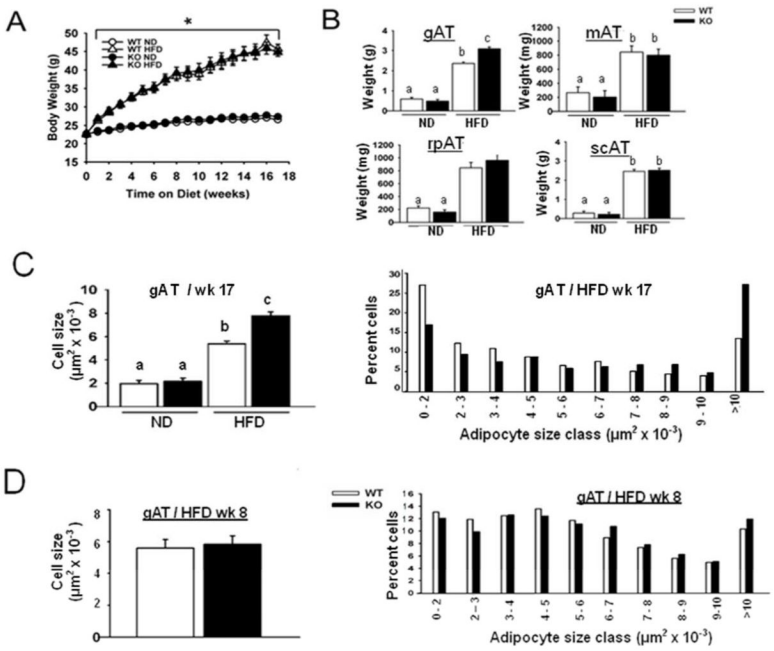

Figure 3. TWEAK KO mice develop larger gAT adipocytes and depot mass

A) Body weights during 17 weeks of ND (circles) and HFD (triangles) of WT (open symbols) and TWEAK KO mice (shaded symbols). Data are presented as mean ± SEM. *, significant effect of HFD vs. ND at each time point ($p \leq 0.05$), two-way ANOVA with Tukey's HSD (n=6–8/group). **B)** Weights of AT depots after 17 weeks of ND or HFD. gAT, gonadal; mAT, mesenteric; rpAT, retroperitoneal; scAT, subcutaneous. Data are presented as mean ± SEM. Means designated by different letters are significantly different ($p \leq 0.05$), ANOVA with Tukey's HSD (n=10/group). **C)** Adipocyte size in gAT of WT (open bars) and TWEAK KO (shaded bars) mice after 17 weeks of HFD. *left panel*: Mean (± SEM) cell size determined from H&E- stained tissue sections (> 600 adipocytes from three histologic sections per mouse, three microscopic fields per section). Values designated by different letters are significantly different ($p \leq 0.05$), one-way ANOVA with Tukey's HSD (n=8/group). *right panel*: Size distribution of gAT adipocytes used to calculate mean cell size. **D)** Adipocyte size in gAT of WT (open bars) and TWEAK KO (shaded bars) mice after 8 weeks of HFD, determined as in panel C.

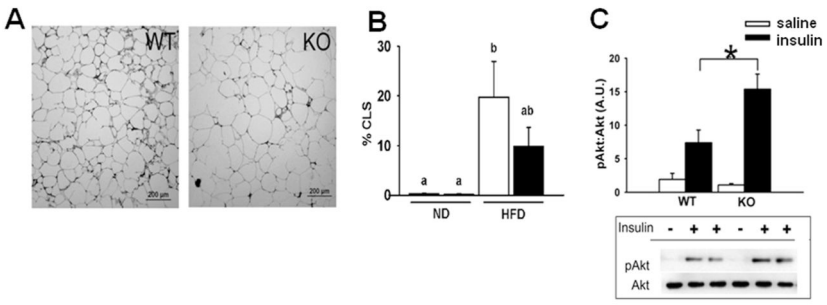

Figure 4. Enhanced insulin signaling in gAT of obese TWEAK KO mice

A) H&E-stained sections of gAT from WT (left panel) and KO (right panel) mice fed HFD for 17 weeks showing crown like structures. **B)** Percentage of crown like structures (CLS) in gAT of WT (open bars) and KO mice (shaded bars) fed ND or HFD for 17 weeks. CLS were counted in three histologic sections per mouse, three microscopic fields per section). Data are presented as mean ± sem. Values designated by different letters are significantly different ($p \leq 0.05$), one-way ANOVA with Tukey's HSD (n=6/group). **C)** *In vivo* insulin signaling in gAT of WT and KO mice fed HFD for 17 weeks. Insulin signaling was assessed by Western blotting as the ratio of phosphorylated Akt (pAkt) to total Akt 10 minutes after intraperitoneal injection of insulin or saline control. *upper panel*: summary of densitometry data from saline-injected (open bars) and insulin-injected (shaded bars) mice. *, significantly different from WT value ($p \leq 0.05$), Student's t test (n=8/group). *lower panel*: a representative Western blot is shown.

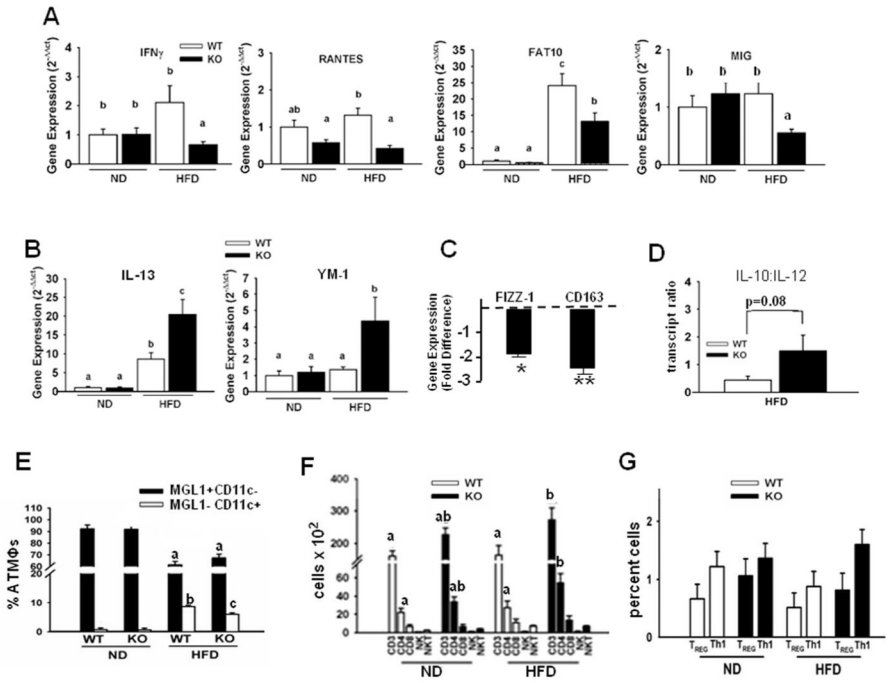

Figure 5. TWEAK deletion alters HFD-induced immune activation in gAT

(**A**) Expression of Th1/M1 transcripts in WT (open bars) and KO (shaded bars) mice. IFNγ, Interferon gamma; RANTES, Regulated upon Activation and Normally Expressed by T cells and Secreted; FAT10, F Locus-Associated Transcript 10; MIG, Myokine Induced by IFNγ. **B**) Expression of Th2/M2 transcripts: IL-13, Interleukin -13; YM-1/Chi3L3, Chitnase 3-like 3. Data are presented as mean (± sem) fold difference relative to WT mice fed ND. Groups designated by different letters are significantly different ($p \leq 0.05$), ANOVA with Tukey's HSD (n=6–8/group). **C)** Reduced transcript levels of Found in Inflammatory Zone (Fizz)-1 and CD163 in obese TWEAK KO mice. Gene expression was calculated by the $2^{-\Delta\Delta CT}$ method and expressed as fold difference relative to obese WT mice. *, $p < 0.05$; **, $p < 0.01$ Student's t test (n=8/group). **D**) Ratio of IL-10 and IL-12 transcripts in gAT of obese WT (open bars) and TWEAK KO mice (shaded bars), (n=6/group). **E**) Flow cytometric analysis of ATMφ subtypes (21) reveals proportionally fewer $MGL1^{-}CD11c^{+}$ ATMφs in gAT of obese KO mice. Cell counts (mean ± SEM) are from 50,000 events/mouse; n=6/group. Means designated by different letters are significantly different within each diet ($p \leq 0.05$), ANOVA with Tukey's HSD. **F)** Flow cytometric analysis of gAT showing Mean (±SEM) frequencies of total T cells ($CD3^{+}$), CD4 ($CD3^{+}CD4^{+}$); CD8 ($CD3^{+}CD8^{+}$); NK ($CD3^{-}NK1.1^{+}$) and NKT ($CD3^{+}NK1.1^{+}$) cells (50,000 events/mouse collected; n=6/group). Gating scheme is in Figure S1). Means designated by different letters are significantly different within each cell type ($p \leq 0.05$), ANOVA with Tukey's HSD. **G**) T-regulatory (Treg) ($CD3^{+}CD4^{+}CD25^{+}Foxp3^{+}$) and T-helper 1 (Th1) ($CD3^{+}CD4^{+}CD45^{+}T\text{-}bet^{+}$) cells as a percentage of total viable cells (25,000 events/mouse collected; n=4–6/group).

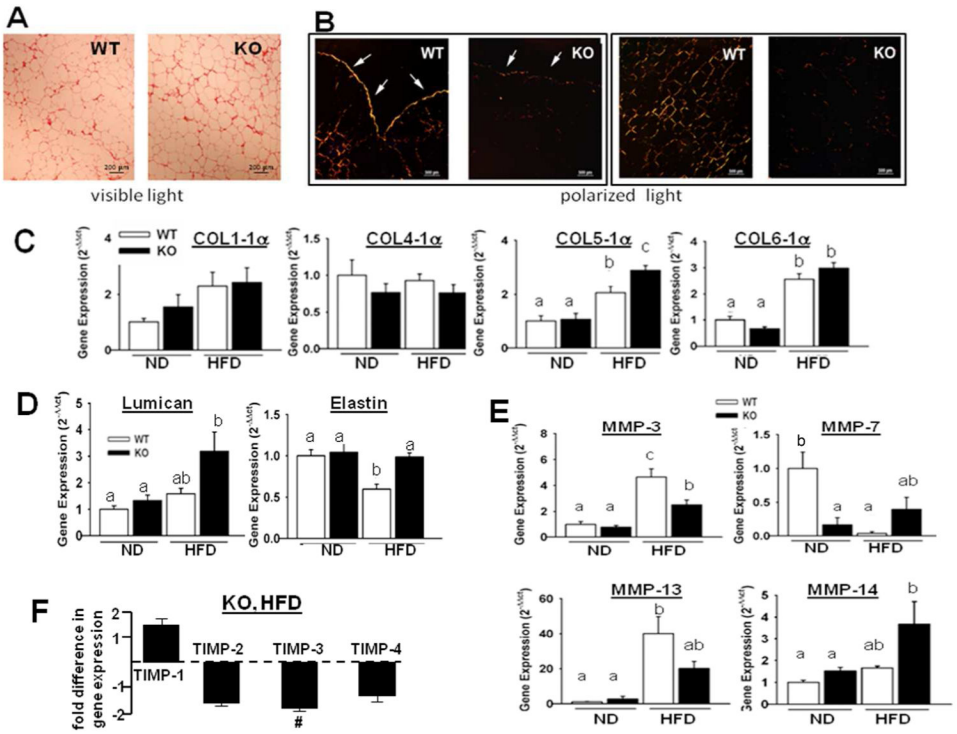

Figure 6. Reduced fibrosis in gAT of obese TWEAK KO mice is associated with altered expression of ECM components and MMPs

Shown are representative fields of gAT sections stained with picrosirius red and visualized under **A**) visible light, showing total collagen and other ECM components as red or **B**) polarized light revealing mature cross-linked collagen as thick yellow and red fibers. bar = 500 μm. Arrows point to the leading edge of gAT expansion **C)** Expression of collagen genes 1- 1α, 4-1α, 5-1α and 6-1α in WT (open bars) and KO (shaded bars) mice fed either ND or HFD for 17 weeks. Data are presented as fold difference calculated by the $2^{-\Delta\Delta CT}$ method, using ND-fed WT mice as the comparer group. Treatments designated by different letters are significantly different ($p \leq 0.05$) ANOVA with Tukey's HSD, (n=6–8/group). **D**) Increased expression of lumican and elastin in gAT of obese TWEAK KO mice. Transcript levels were calculated by the $2^{-\Delta\Delta CT}$ method and are expressed relative to levels in ND-fed WT mice. Means designated by different letters are significantly different ($p \leq 0.05$) ANOVA with Tukey's HSD, (n=6–8/group). E) Altered expression of matrix metalloproteinases (MMPs) in gAT of TWEAK KO mice. Transcript levels were calculated by the $2^{-\Delta\Delta CT}$ method and are expressed relative to levels in ND-fed WT mice. Means designated by different letters are significantly different ($p \leq 0.05$) ANOVA with Tukey's HSD, (n=6–8/group). F) Expression of tissue inhibitors of metalloproteinases (TIMPs) in gAT of obese TWEAK KO mice. Gene expression data are calculated by the $2^{-\Delta\Delta CT}$ method and expressed as fold difference relative to obese WT mice. #, $p < 0.06$; Student's t test, n = 6/group.)

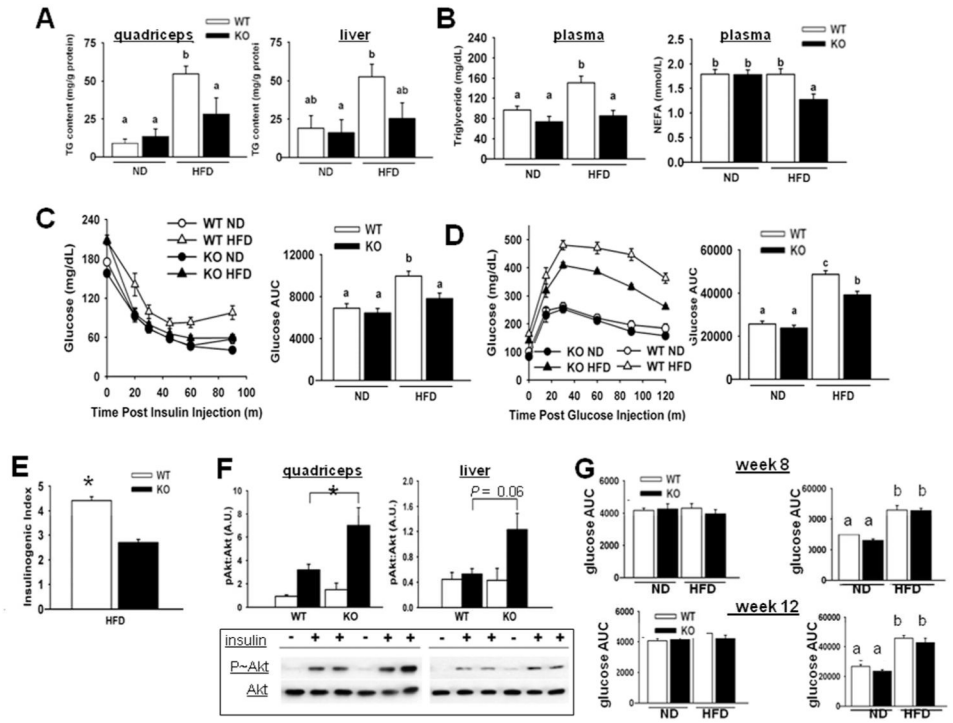

Figure 7. Protection from dyslipidemia, insulin resistance and glucose intolerance in obese KO mice after 16 weeks of HFD

A) Triglyceride content (mg/g protein) in quadriceps and liver of WT (open bars) and KO (shaded bars) mice after 17 weeks of diet. **B)** Concentrations of plasma lipids. left panel: triglycerides (md/dL); right panel, non-esterified fatty acids (NEFA) (mmol/L). **C)** Insulin tolerance tests after 16 weeks on diet and calculated glucose area under curve (AUC). **D)** Glucose tolerance tests conducted after 16 weeks on diet and calculated glucose AUC (n=8/group. **E)** Insulinogenic Index measured as the ratio of insulin AUC to glucose AUC during the GTT (20). *, $p \leq 0.05$, Student's t test (n=8/group). **F)** *In vivo* insulin signaling in quadriceps and liver of mice fed HFD for 17 weeks measured as the ratio of phosphorylated Akt (pAkt) to total Akt. Mice were injected (IP) with either insulin or saline and tissues were harvested after 10 minutes for Western blotting. *upper panel*: summarized densitometry data (arbitrary units) for WT and KO mouse responses to insulin (shaded bars) and saline (open bars). *, $p<0.05$, Student's t test (n=8/group). *lower panel*: representative Western blots are shown. **G)** Insulin sensitivity and glucose tolerance after 8 weeks (*upper panes*) or 12 weeks (*lower panels)* of diet. Glucose AUCs are shown for ITTs (*left panels*) and GTTs (*right panels*) from WT (open bars) and KO (shaded bars) mice. In panels A-D, F and G, means designated by different letters are significantly different ($p \leq 0.05$), ANOVA with Tukey's HSD (n=8/group).

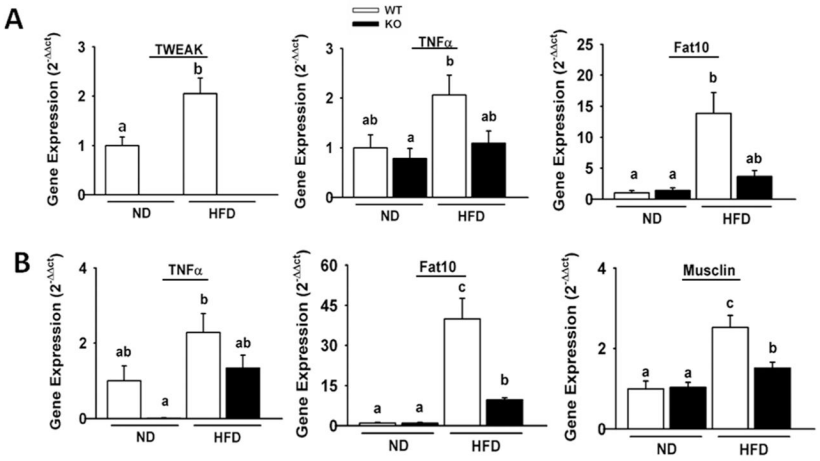

Figure 8. Expression of genes associated with insulin resistance is attenuated in liver and skeletal muscle of TWEAK KO mice

Transcript levels of **A)** TWEAK, TNF-α and FAT10 in liver and **B)** TNF-α, FAT10 and musclin in quadriceps were determined after 17 weeks of HFD. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method and expressed relative to levels in WT mice (open bars) fed ND. Means designated by different letters or symbols are significantly different ($p \leq 0.05$), ANOVA with Tukey's HSD, (n=6–8/group).