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A Role for TNMD in Adipocyte Differentiation and Adipose Tissue Function: A Dissertation

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A ROLE FOR TNMD IN ADIPOCYTE DIFFERENTIATION AND ADIPOSE TISSUE FUNCTION

A Dissertation Presented

By

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This work was undertaken in the Graduate School of Biomedical Sciences Interdisciplinary Graduate Program

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Dedication

I would like to dedicate this thesis to Dr. Guldal Kirkali, my beloved advisor for my Master's thesis. I thank her always for teaching me innumerable lessons, especially how important being influential in other people's life. I thank her for the countless hours of conversations, fun times and valuable mentorship. Meeting her has been one of the luckiest things that ever happened to me.

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Abstract

Adipose tissue is one of the most dynamic tissues in the body and is vital for metabolic homeostasis. In the case of excess nutrient uptake, adipose tissue expands to store excess energy in the form of lipids, and in the case of reduced nutrient intake, adipose tissue can shrink and release this energy. Adipocytes are most functional when the balance between these two processes is intact. To understand the molecular mechanisms that drive insulin resistance or conversely preserve the metabolically healthy state in obese individuals, our laboratory performed a screen for differentially regulated adipocyte genes in insulin resistant versus insulin sensitive subjects who had been matched for BMI. From this screen, we identified the type II transmembrane protein tenomodulin (*TNMD*), which had been previously implicated in glucose tolerance in gene association studies. *TNMD* was upregulated in omental fat samples isolated from the insulin resistant patient group compared to insulin sensitive individuals. *TNMD* was predominantly expressed in primary adipocytes compared to the stromal vascular fraction from this adipose tissue. Furthermore, *TNMD* expression was greatly increased in human preadipocytes by differentiation, and silencing *TNMD* blocked adipogenic gene induction and adipogenesis, suggesting its role in adipose tissue expansion.

Upon high fat diet feeding, transgenic mice overexpressing *Tnmd* specifically in adipose tissue developed increased epididymal adipose tissue (eWAT) mass without a difference in mean cell size, consistent with elevated *in*

vitro adipogenesis. Moreover, preadipocytes isolated from transgenic epididymal adipose tissue demonstrated higher BrdU incorporation than control littermates, suggesting elevated preadipocyte proliferation. In *TNMD* overexpressing mice, lipogenic genes *PPARG*, *FASN, SREBP1c and ACLY* were upregulated in eWAT as was *UCP-1* in brown fat, while liver triglyceride content was reduced. Transgenic animals displayed improved systemic insulin sensitivity, as demonstrated by decreased inflammation and collagen accumulation and increased Akt phosphorylation in eWAT. Thus, the data we present here suggest that TNMD plays a protective role during visceral adipose tissue expansion by promoting adipogenesis and inhibiting inflammation and tissue fibrosis.

Table of Content

List of Tables

List of Figures

List of Frequently Used Abbreviations

BMI - Body mass index

TAG (TG)- triacylglyceride (triglyceride)

FFA - free fatty acid

VAT- visceral adipose tissue

SAT- subcutaneous adipose tissue

WAT- white adipose tissue (epididymal [eWAT], inguinal [iWAT])

BAT- brown adipose tissue

SVF - stromal vascular fraction

SGBS - Simpson-Golabi-Behmel syndrome

GTT - glucose tolerance test

ITT- insulin tolerance test

TNMD - tenomodulin

PPARG - peroxisome proliferator activated receptor gamma

C/EBP - CCAAT/enhancer-binding protein

HFD - high fat diet

CHAPTER I

INTRODUCTION

Obesity as an epidemic

The obesity epidemic is a major health burden that humanity faces in modern times. Given the virtually unlimited food availability in developed countries, obesity has been thought to emerge in economically rich countries. In the United States obesity prevalence among adults was 34.9% in 2011-2012 (1). However, low and middle-income countries have also been suffering from obesity and related disorders. According to WHO, increase rate of overweight and obese children in these countries is 30% higher than in developed countries. The prevalence of obesity has doubled worldwide since 1980, which signifies that it is indeed a global problem (2).

Obesity is excess fat accumulation in the body by definition. The most common tool to estimate obesity in adults is body mass index (BMI), which is calculated by dividing weight by square of height (kg/m^2) and first described by Adolphe Quetelet in 1832 (3-5). According to World Health Organization (WHO) guidelines, individuals with a BMI between 25-29.9 are considered to be overweight and those who have a BMI over 30 are categorized as obese. Even though it is debated whether BMI, fat distribution or total body fat mass is the predictor, it is now well established that obesity is associated with plethora of metabolic complications such as type II diabetes, cardiovascular disease, nonalcoholic fatty liver disease and cancer (6, 7).

Adipose tissue biology

Energy storage is vital for all organisms, and lipids serve as an efficient way to store excess energy. Regardless of their complexity and the type of lipids to be stored, most organisms have specialized compartments to deposit fat (8). *Caenorhabditis elegans,* for example, stores lipid as triacylglyceride (TAG) in intestinal epithelial cells (9, 10) whereas *Drosophila melanogaster* has a specialized organ termed the 'fat body' (11). In mammals, adipose tissue, the body's fat reservoir, is a connective tissue that is composed of adipocytes, blood vessels, neurons, extracellular matrix and immune cells (12). Adipocytes occupy most of the space in adipose tissue, and similar to the lower organisms, take up and convert nutrients into triglycerides (13). However, over the last decade, adipose tissue has become recognized as a dynamic endocrine organ that is a major regulator of energy homeostasis rather than an inert storage organ (14-16). Therefore, much effort has been devoted to fully understand its role in metabolic diseases such as type II diabetes.

Despite excess accumulation of fat, which is associated with many morbidities, individuals lacking adipose tissue also display extreme insulin resistance, similar to metabolically unhealthy obese subjects. Mutations in genes such as 1-acylglycerol-3-phosphate acyltransferase (*AGPAT2)* and Berardinelli-Seip congenital lipodystrophy 2 (seipin) (*BSCL2)* causes lipodystrophy, a congenital disease characterized by partial or total loss of adipose tissue (17-19).

People with this rare condition are insulin resistant and have high levels of circulating glucose, hypertriglyceridemia and hepatic steatosis. Mouse models of lipodystrophy also mimic the human phenotype (20). Dominant-negative AZIP/F-1 inhibits DNA binding of BZIP transcription factors such as CCAAT/enhancer-binding protein alpha (C/EBP) and Jun. These mice are lipodystrophic and exhibit a similar phenotype to lipodystrophic humans (21, 22). Moreover, *Agpat2 null* mice were insulin resistant and also had fatty liver and hyperglycemia (23). Thus, adipose tissue is essential for metabolic health.

Energy storage by adipose tissue

Adipocytes are specialized to store energy in the form of fat. They respond to several nutritional cues and hormones to manage pathways involved in lipid storage (24). After feeding, insulin stimulates glucose uptake in adipose tissue by binding its receptor on the plasma membrane of adipocytes (25). Insulin binding to its receptor, a tyrosine kinase, triggers a signaling cascade that results in activation of phosphoinositide 3-kinase (PI3K) and the AKT protein kinase (also known as protein kinase B) (26, 27). AKT activation mediates many of the metabolic effects of insulin as it is the upstream kinase for many cellular metabolic pathways including *de novo* lipogenesis (28). AKT activates mTORC1, which phosphorylates S6 kinases (S6K1 and S6K2) and sterol regulatory element binding protein-1 (SREBP1) (29). Another transcription factor,

carbohydrate-responsive element-binding protein (ChREBP), is also activated by cellular glucose uptake (30). SREBP1 and ChREBP regulate the expression of various lipogenic enzymes (31-33). In peripheral insulin target tissues, activated AKT is required for insulin-mediated glucose uptake. It achieves this function through activation of Akt substrate of 160 kDa (AS160) (34). Phosphorylated AS160 then binds to glucose transporter type 4 (GLUT4) vesicles and stimulates their translocation to the plasma membrane (25, 35-37). Glucose that is taken up by adipocytes is not only utilized for *de novo* fatty acid synthesis, but it also provides the glycerol backbone for triacylglyceride (TAG) (38). Fatty acid synthase (FASN) is an enzyme complex that along with acetyl-coA carboxylase (ACC) converts acetyl-coA into fatty acids (39). Free fatty acids, which are augmented in the circulation upon feeding, are also taken up by adipocytes. Along with passive diffusion, cluster of differentiation 36 (CD36) and fatty acid binding protein 1 (FABP1) are involved in free fatty acid uptake in adipocytes (40-43). Moreover, insulin increases the expression of lipoprotein lipase, the enzyme that breaks down triglycerides bound in very low-density lipoprotein (VLDL) or chylomicrons and promotes fatty acid uptake into adipocytes (44). Fatty acids within the cell are activated and finally esterified into TAG and stored in lipid droplets.

As previously mentioned, adipocytes are also capable of breaking down TAG into free fatty acids and glycerol by lipolysis, which is a multistep process. Catecholamine signaling is the main pathway that stimulates lipolysis (45, 46).

The beta-adrenergic receptor is stimulated by catecholamines upon fasting, which in turn elevates cAMP levels and activates protein kinase A (PKA) (46, 47). Consecutive breakdown of TAG into free fatty acids and glycerol involves three enzymes; adipocyte triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglcerol lipase (MGL), the first two of which require phosphorylation for activation (48, 49). Similar to HSL, Perilipin 1 (PLIN1), an adipocyte-specific lipid droplet protein, is also phosphorylated by PKA and facilitates HSL localization on lipid droplets (50). Conversely, insulin strongly inhibits lipolysis by activating phosphodiesterase 3 (PDE3), which degrades cAMP. Hence, abated cAMP levels in insulin-stimulated cells inhibit lipolysis (51, 52).

Adipose tissue as an endocrine organ

Adipocytes and other cell types residing in the adipose tissue secrete a variety of molecules, termed adipokines that act as endocrine and paracrine hormones. Leptin was the first known adipokine, discovered in 1994 (15). It binds to the leptin receptor, which is expressed in many tissues including the hypothalamus (53, 54). The metabolic function of leptin has been widely studied, and a significant correlation between circulating leptin levels and fat storage has been shown (55, 56). Leptin not only suppresses food intake through hypothalamic signaling, and mice lacking leptin or its receptor are hyperphagic and develop extreme obesity and insulin resistance. Leptin also activates fatty

acid oxidation in muscle and improves glucose metabolism in streptozotocin (STZ), β-cell toxicant, treated mice (57, 58). Another adipokine secreted from both brown and white adipocytes is adiponectin, which has insulin sensitizing effects (59). Mice lacking adiponectin were first generated by Nawrocki et al. and demonstrated severe hepatic insulin resistance in liver but not in other peripheral tissues. Adiponectin null mice exhibit a dampened response to thiazolidinediones (TZDs), a class of insulin sensitizing drugs that function via PPARγ activation (60). On the other hand, despite excessive weight gain, transgenic mice overexpressing adiponectin on the leptin-deficient ob/ob background have a vastly improved metabolic profile (61). Adipocytes also release pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein 1 (MCP1) upon various conditions, and these factors regulate adipose tissue metabolism (62-64). Adipose tissue TNF-α expression is increased in obese states, and inhibition of this cytokine using specific antibodies improved glucose uptake in obese rodents (65). Similarly, circulating MCP1 levels are decreased by weight loss, and insulin-stimulated glucose uptake was reduced in MCP1-treated adipocytes (66, 67). Recently, studies demonstrated systemic metabolic effects of brown adipose tissue transplantation, which attracted attention to endocrine functions of brown adipose tissue (68-70). For instance, brown adipose tissue (BAT) contributes to plasma levels of fibroblast growth factor 21 (FGF21), a factor that stimulates thermogenic gene expression (70, 71).

Adipose tissue depots

In mammals, white adipose tissue (WAT) is distributed in various anatomical depots (72, 73). Subcutaneous depots store more than 80 % of total body fat and constitute the largest portion of WAT (74). In humans, subcutaneous adipose tissue (SAT) is found in the thighs, hips and abdomen, whereas visceral adipose tissue (VAT), also known as intra-abdominal fat, is located around vital organs in the peritoneum (75). Similarly, rodents have subcutaneous fat depots of which inguinal and axillary fat represent the majority, whereas VAT is mostly distributed in the mesenteric, perigonadal and retroperitoneal areas (76, 77).

Differences in lipid handling, gene signatures, precursor cells and physiology between subcutaneous and visceral fat have been broadly studied (78-81). Location of WAT depots is associated with some metabolic outcomes. Growing evidence suggests that VAT is correlated with metabolic disorders to a greater extent than SAT (82-84). According to the prevailing “portal vein hypothesis’, VAT is in close proximity to vital organs in the abdomen and lipids released from VAT reach the liver directly through the portal vein (85-87). Given that ectopic lipid deposition in liver may contribute to systemic insulin resistance, one possibility is that due to its location VAT expansion is a major risk factor for metabolic disease (74, 88). However, a transplantation study performed by Tran et al. demonstrated that SAT, when transplanted into the intra-abdominal area,

can improve insulin sensitivity. This suggests that differences between SAT and VAT are not only related to the anatomical location, but also other inherent mechanisms play a role in metabolic function of these depots (77). In fact, studies focusing on the gene signature differences between depots revealed that expression of developmental genes such as T-box (TBX) and HOX genes varies between SAT and VAT (89, 90). In humans and mice, Tbx15 is expressed more highly in subcutaneous depots than in VAT. These findings suggest that different developmental programs play role in formation of these depots. Adipogenic genes such as PPARγ and C/EBPα, however, were uniformly distributed between depots in mice (91). Moreover, receptor composition is distinct between SAT and VAT.

Glucocorticoid and adrenergic receptors display regional variation in expression and have prominent density in visceral depots (92, 93). Even though the exact molecular mechanisms still remain unclear, increased central obesity after glucocorticoid exposure may be explained by high expression of glucocorticoid receptor in VAT (94). Lipid handling also varies between depots. VAT stores dietary fat more efficiently than SAT (95) and in hyperinsulinemia, postprandial free fatty acid release and lipolysis is greater in obese people with upper body/visceral obesity versus subjects with lower body obesity (78, 96). Finally, when the secretome was analyzed in AT explants, VAT displayed a greater abundance of secreted proteins (97).

BAT differs from WAT not only by location but also by metabolic and physiological function (98). Until it was detected in adults, BAT had long been thought to appear only in babies to regulate the body temperature (99, 100). Many recent studies have focused on approaches to activating the thermogenic function of BAT, thereby utilizing the excess caloric energy to a tissue to produce heat rather than contributing to excess fat storage (101, 102). Brown adipocytes are characterized by high expression of uncoupling protein 1 (UCP-1), high mitochondrial density and the presence of multiple small (multilocular) lipid droplets, distinct from the single, large (unilocular) droplet typical of white adipocytes (103). During cold stress, β-adrenergic receptors on brown adipocyte membranes are stimulated by catecholamines. Subsequently, down stream molecules such as cAMP/PKA are stimulated, which in turn increase UCP1 expression and lipolysis (104, 105). Brown adipocytes can also take up high amounts of glucose and free fatty acids from the circulation, which can be utilized in thermogenesis (106). It has recently been realized that BAT is not the only depot where thermogenesis occurs. Even though the origin of these thermogenic cells is controversial, it is now well established that brown-like adipocytes are present in white adipose depots (107). These so-called “Brite” or “beige” cells express UCP1 and can activate the thermogenic network upon cold exposure or direct stimulation of the β-adrenergic receptor (108, 109). These cells can increase energy expenditure and potentially protect against obesity (110).

Therefore, strategies aiming to increase browning within WAT have been attractive for therapeutic purposes (111).

Adipose tissue formation

In vivo adipose tissue formation

Studies that used microscopic imaging in human embryos and fetuses showed that AT first appears in the 14th week of gestation. Even though there is extensive AT growth between the 14th and 24th weeks of gestation, elevated nutrient uptake also gives rise to rapid postnatal expansion of adipose depots (112-114). Adipocytes are believed to originate from mesenchymal stem cells (MSCs), which can also transform into osteoblasts, myocytes and chondrocytes (115). WAT contains various types of cells including adipocyte precursors, endothelial cells, neurons, immune cells and mature adipocytes (116). Using collagenase digestion and centrifugation, lipid-laden adipocytes can be separated from other cells in the AT, and the remaining cells are called the stromal vascular fraction (SVF). Some cells from the SVF fraction can differentiate into cells of mesenchymal origin including adipocytes, myocytes and osteoblasts *in vitro* (117, 118). These multipotent cells are termed adipose tissue-derived stromal cells (ADSC). Even though these cells have successfully been differentiated into adipocytes *in vitro*, their adipogenic ability is limited *in vivo* (119).

Much effort has been devoted to find the precise white adipocyte precursors that are also able to form a fat pad *in vivo*. Approaches include sorting the SVF fraction into subpopulations using specific cell surface markers and flow cytometry. Of these cells, those that are CD24+ not only have *in vitro* adipogenic potential, but they are also capable of forming a fat pad in the lipodystrophic A-ZIP mice but not in wild type mice (120). Using a similar method and a genetically labeled mouse model, Graff and colleagues demonstrated the presence of a committed cell population that resides in adipose tissue, expresses mural cell markers such as PDGFRβ and can develop into a fat pad even in wild type mice (121). These data were supported by several others, who demonstrated that cells within the AT vasculature can convert into mural cells, which can proliferate and differentiate into mature adipocytes (116, 122-124). Some studies also indicated that when transplanted into mice and treated with PPARγ agonists, hematopoietic stem cells can develop into adipocytes (125). Even though studies based on cell surface markers shed light on the field of adipocyte progenitors, the specificity of these markers is a challenge, especially for *in vivo* studies.

Despite some limitations, *in vivo* tracing studies based on the Cre/Lox reporter system has become a gold standard to elucidate adipocyte progenitors (103, 126). Using this method, it has been discovered that white and brown adipocytes do not arise from the same precursors. Instead, brown adipocytes are derived from progenitor cells expressing myogenic factor 5 (Myf5) and paired

box-7 (Pax7), which also differentiate into myocytes (127, 128). However, these findings were challenged by a study that combined Myf5-Cre with 'LoxP–membrane targeted dTomato–LoxP–membrane targeted GFP'-also known as mT/mG- dual fluorescence reporter mice. *Myf5Cre-mT/mG* mice revealed that Myf5+ cells are not the only source of brown adipocytes. Instead mature white and brite/beige adipocytes in specific WAT depots also arise from these progenitors (129). *In vivo* tracing studies have also initiated an interesting debate on whether the origin of brite/beige cells that arise in WAT. One of the hypotheses suggests that beige adipocytes in WAT emerge through a transdifferentiation or interconversion mechanism. In other words, unilocular white adipocytes undergo some phenotypic changes to develop into brite/beige adipocytes with multiple lipid droplets and high UCP1 expression. This hypothesis has been supported by studies from two different groups (130-132). On the other hand, findings from a study that used a permanent adipocyte labeling approach argued that brite/beige adipocytes are formed via *de novo* differentiation from progenitor cells (133). Further studies will provide a better understanding of the *in vivo* origin of white, brown and beige adipocytes (Figure 1.1).

Figure 1.1

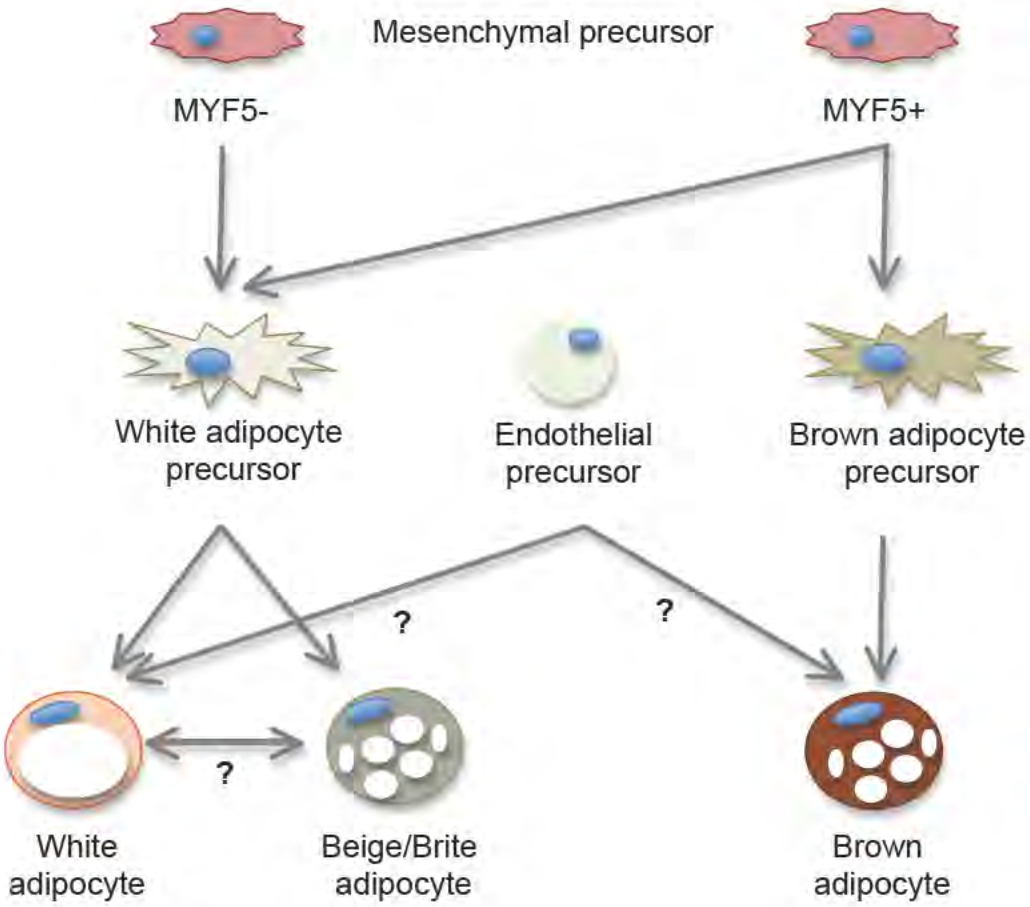


Figure 1.1: Origin of adipocytes
White adipocyte precursors are derived from both MYF5- and MYF+ mesenchymal cells. White adipocyte and endothelial precursors differentiate into white or beige/brite adipocytes upon different stimuli. Endothelial precursors are able to form brown adipocytes, which are also derived from brown adipocyte precursors originating from MYF5+ cells. Question marks indicate controversial results in the field. Modified from Peirce et.al, 2014 (134).

Adipogenesis

Adipocyte precursor cells undergo a process called adipogenesis, which is critical for adipose tissue development and expansion (135-137). Hence, forming adipocytes requires strict regulation that involves many intra and extracellular pathways. This section will focus on the molecular mechanisms that are implicated in adipocyte differentiation and the cellular models that have been used to elucidate these mechanisms.

In vitro and *in vivo* models of adipocytes

Adipogenesis is a well-studied developmental program, and availability of preadipocyte cell culture models has contributed a great deal to studies that investigate adipocyte differentiation (138). 3T3-L1 cells are the most established cell line through which many aspects of adipogenesis have been examined. These cells are already committed preadipocytes, and they were originally derived from Swiss 3T3 mouse embryos (139). 3T3-L1 cells are responsive to adipogenic stimuli and can easily convert into adipocytes in culture. 3T3-F442A preadipocytes were also isolated from Swiss 3T3 cells (140) and serve as a useful model for adipogenesis. They are considered to be in the later stage of commitment (138). Furthermore, multipotent stem cell lines that can differentiate into multiple lineages are frequently utilized as *in vitro* models. C3H10T1/2 cells were derived from C3H mouse embryos (141, 142). Interestingly, these cells

commit to an adipogenic lineage upon induction with bone morphogenic protein 4 (BMP4) prior to stimulus with an adipogenic cocktail including glucocorticoids, cyclic AMP agonists and insulin (143). There has also been much effort to establish human adipocyte models (144-147). The Simpson-Golabi-Behmel-Syndrome (SGBS) cell strain was first isolated from the subcutaneous adipose tissue of an infant with the syndrome of the same name. Even though they are not immortalized, they have a high capacity to proliferate and differentiate into adipocytes, even at later passages (148). SGBS cells differentiate into adipocytes under very similar conditions as 3T3-L1 cells; however, they require an absence of serum and the presence of PPARγ agonists in the differentiation media. SGBS cells acquire multiple lipid droplets, even in the early time points of differentiation. Once they become adipocytes, glucose uptake is stimulated and lipolysis is inhibited upon insulin treatment in these cells (149). Finally, human embryonic and inducible pluripotent stem cells have been utilized as *in vitro* adipocyte model (150-155). These multipotent cells are useful to elucidate the molecular mechanisms implicated in adipogenesis, especially because cells isolated from patients may give insight into the role of adipocytes in the pathogenesis of various metabolic diseases such as lipodystrophy. However, monolayer cell culture models have some limitations for unveiling the role of physical properties like cell shape and tension, and extracellular matrix interactions in adipogenesis and adipocyte function. Three dimensional adipocyte culture models that utilize extracellular matrix gels or adipose tissue organoid

models in which tissue fragments are embedded in collagen scaffolds will be of great use in exploring these aspects of adipose tissue formation (138).

Transcriptional regulation of adipogenesis

Adipogenesis is a two-step process that is regulated by many factors. In the first step, multipotent mesenchymal stem cells are committed to the adipocyte lineage. Committed fibroblast-like preadipocytes then undergo several changes in morphology and gene expression to form lipid droplet-containing mature adipocytes. Therefore, this step can be called terminal differentiation (137, 156, 157). Developmental pathways, DNA modifiers, extracellular matrix (ECM) composition and transcription factors are implicated in both phases of adipogenesis (136, 158, 159).

Several lines of evidence suggest that fate of MSCs can be determined by the stiffness of the milieu in which they reside (160-163). Dense or stiff ECM has been shown to favor osteogenic and myogenic differentiation but not adipogenesis (162). Cells displayed greater attenuation in adipogenesis when placed in dense gels compared to softer matrices (164). Matrix metalloproteinases (MMP) are enzymes that cleave ECM proteins (165). Several studies demonstrate that MMP activity is required for adipogenesis and *in vivo* adipose tissue formation (166-168). In cultured cells, cell shape and confluence are also important determinants of the cell fate. Both of these factors affect adipogenesis by modulating the RHO signaling pathway and the cytoskeleton

(169, 170). Once cells are committed to the adipogenic lineage, further events are required to transform preadipocytes into adipocytes. Many inhibitory and stimulatory molecules modulate this process (114). β-catenin-mediated canonical Wnt signaling has been shown to inhibit adipogenesis in various cell culture models whereas activating non-canonical Wnt5B signaling stimulated adipogenesis in 3T3-L1s (171-174). Different transforming growth factor beta (TGFβ) family members have been implicated in adipocyte differentiation. TGFβ inhibited adipogenesis in NIH3T3 cells that stably overexpressed adipogenic transcription factors such as C/EBPα or PPARγ (175). Furthermore, overexpression of Smad3 and Smad2 inhibited 3T3-F442A preadipocyte differentiation (176). These results, however, were not supported by *in vivo* studies demonstrating that Smad3 knockout mice did not gain weight upon high fat diet (HFD) feeding (177).

The major transcription factors involved in early differentiation include cAMP response element binding protein (CREB), C/EBPδ, C/EBPβ, signal transducer and activator of transcription 5A (STAT5A), krüppel-like factor (KLF4) and early growth response 2 (EGR2 or KROX20) (178-183). These transcription factors respond to different stimuli in adipogenic media. 3-isobutyl-1-methylxanthine (IBMX), for example, stimulates protein kinase A (PKA) activation. PKA then phosphorylates CREB, and this event is followed by elevated C/EBPβ expression (184). KROX20 and KLF4 expression are also increased 2-4 hours after stimulation, which up-regulates C/EBPβ expression

(182, 183). C/EBPβ activation depends on phosphorylation by various kinases such as mitogen activated protein kinase (MAPK), cyclin dependent kinase 2 (CDK2) and glycogen synthase kinase 3 beta (GSK3β) (185, 186). Conformational changes in phosphorylated C/EBPβ facilitate its DNA binding (187). Acetylation is also involved in C/EBPβ activation, whereas ubiquitination and SUMOlation inhibit C/EBPβ via proteasomal degradation (187, 188). Mitotic clonal expansion is a characteristic event in early adipogenesis. Even though it is controversial previous studies suggest that 3T3-L1 cells undergo one or two rounds of cell cycle before they start expressing the adipocyte phenotype (189, 190). C/EBPβ has been shown to positively regulate some cell-cycle genes to enable mitotic clonal expansion (191).

In terminal differentiation, C/EBPβ directly binds to the C/EBPα and peroxisome proliferator activated receptor-γ (PPARγ) promoters (192, 193). C/EBPα and PPARγ are the major transcription factors that not only promote adipogenesis but also maintain differentiated adipocyte function (194). PPARγ is considered to be the ‘master regulator’ of adipogenesis mostly because it is both necessary and sufficient to drive adipocyte differentiation (Figure 1.2) (195). Alternative PPARγ promoters result in two isoforms of this transcription factor: PPARγ1 and PPARγ2, the latter of which is exclusively expressed in adipocytes (196-198). PPARγ is a nuclear hormone receptor that binds to the DR-1 sequence on DNA subsequent to heterodimer formation with retinoid X receptor (RXR) (199). Similar to other nuclear receptors, PPARγ is also activated upon

ligand binding. Even though it has been suggested that some polyunsaturated fatty acids and eicosanoids bind to and activate PPARγ, endogenous ligands with higher binding affinities remain unknown (200). Synthetic ligands such as thiazolidinediones (TZDs) have been used to promote adipogenesis in some *in vitro* cell culture models such as SGBS cells. They have also been utilized as drugs in type II diabetes patients to enhance PPARγ activity and its insulin sensitizing effects (201). PPARγ is also regulated by C/EBPα and PPARγ and C/EBPα reciprocally activate each other (193, 202-204). Interestingly, studies by Lazar and Mandrup also showed that C/EBPα and PPARγ share many targets and cooperate to activate important adipogenic genes (205, 206). Both C/EBPα and PPARγ target genes that are involved in mature adipocyte function such as adiponectin, HSL and fatty acid binding protein 4 (FABP4) (207-210). PPARγ also regulates lipid metabolism, glucose homeostasis and adipokine secretion (211).

Figure 1.2

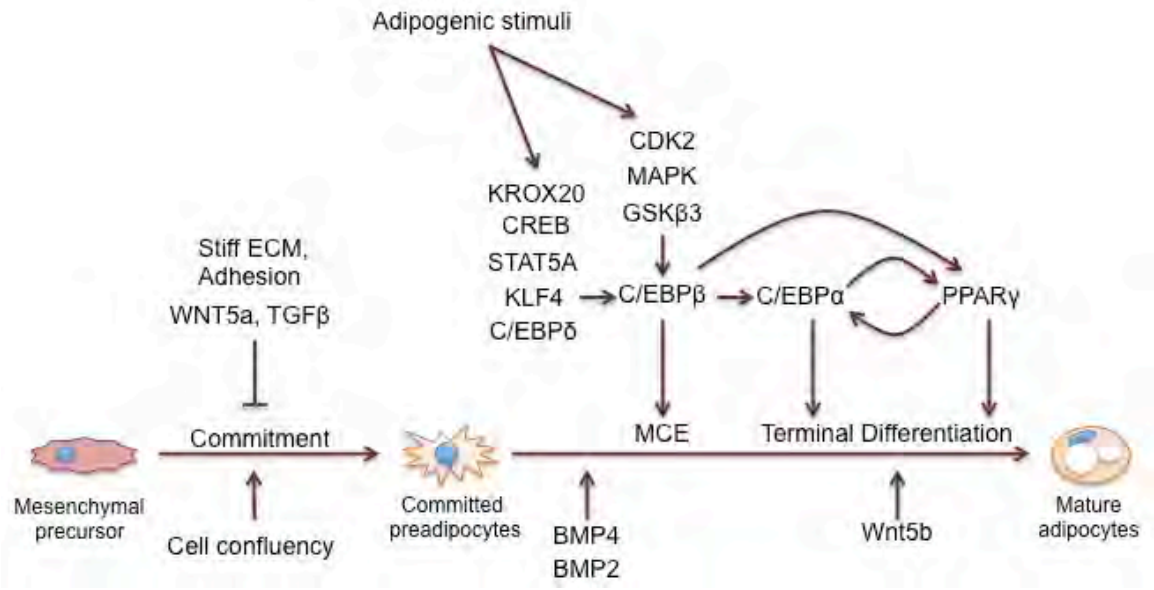


Figure 1.2: Extracellular and intracellular factors involved in adipogenesis
In the first step of adipogenesis, mesenchymal stem cells commit to the adipogenic lineage. Developmental pathways such as WNT and TGFβ are involved in the negative regulation of this step. Dense extracellular matrix and sparse cell confluency inhibits adipogenic commitment. In the second step, so-called terminal differentiation, adipogenic stimuli activate a plethora of transcription factors that promote mitotic clonal expansion (MCE) and stimulate the expression of C/EBPα and PPARγ. BMP2, BMP4 and WNT5b signaling positively regulate adipocyte differentiation. Modified from Cristancho and Lazar, 2011 (114).

Adipose tissue expansion in obesity- adipose tissue remodeling

Adipose tissue is very dynamic and can expand up to 15-fold beyond its original volume when there is a positive energy balance (116). It is also capable of reducing its volume rapidly upon nutrient deprivation by mobilizing lipids to fuel other tissues in times of need (24). Adipose tissue remodeling upon different nutrient and energy expenditure situations is a complex phenomenon in which many factors such as adipocytes, immune cells, vasculature and the extracellular matrix are implicated (12). In this section, I will summarize the events that occur in adipose tissue during obesity and eventually contribute to systemic insulin resistance.

Until recently, it was hypothesized that adipocyte number was predetermined at an early age and was very unlikely to change with nutritional state. However, a study by Spalding et al. showed that approximately 10% of adipocytes renew annually, which generated a new concept in understanding the mechanisms of adipose tissue expansion (212). It is now largely accepted that adipose tissue expands by two mechanisms. In hypertrophy, mature adipocytes enlarge in cell size. In hyperplasia, however, adipocyte number is increased. Some studies suggested that mature adipocytes undergo dedifferentiation and form progenitor cells, which can redifferentiate into adipocytes (213, 214). However, hyperplasia will refer to *de novo* adipocyte differentiation from now on, as mature adipocytes are post mitotic.

It has been debated whether adipose depots have different adipogenic capacities (215, 216). Primary preadipocytes isolated from subcutaneous adipose tissue have higher competence to differentiate in culture, whereas those isolated from visceral depots either do not differentiate or have very low differentiation potential (217, 218). It could be argued that a lack of unknown local factors *ex vivo* is the reason why visceral preadipocytes cannot differentiate into adipocytes. One study addressed the question of whether fat depots have different adipogenic potential *in vivo* and demonstrated that a long term HFD resulted in a significant increase in the number adipocyte progenitor cells in subcutaneous but not visceral adipose tissue. The same study also suggested that hypertrophy is the main expansion mechanism for visceral adipose tissue while subcutaneous depots expand via hyperplasia (219).

Conversely, recent studies using adipocyte-tracing techniques implied that higher levels of adipogenesis/hyperplasia occur in visceral adipose tissue compared to subcutaneous fat depots in mice. A mouse model generated by Scherer and colleagues determined that adipose tissue is expanded by hypertrophy at the early time points after HFD challenge in both depots. However, visceral adipose tissue undergoes hyperplasia during prolonged HFD feeding (133). These results were further supported by a recent study showing that visceral adipose tissue expands via hyperplasia upon HFD feeding. Using BrdU labeling at different time points of HFD feeding, it was also demonstrated that proliferation of adipocyte progenitors was significantly increased in visceral

adipose depots whereas subcutaneous adipose tissue did not show any significant increase in progenitor proliferation during the first week of dietary challenge (220). Regardless of the mechanism, expansion occurs to increase adipose tissue energy storage capacity. Hence, proper adipose tissue expansion could be considered as a beneficial activity for animals (Figure 1.3) (221-224).

Figure 1.3

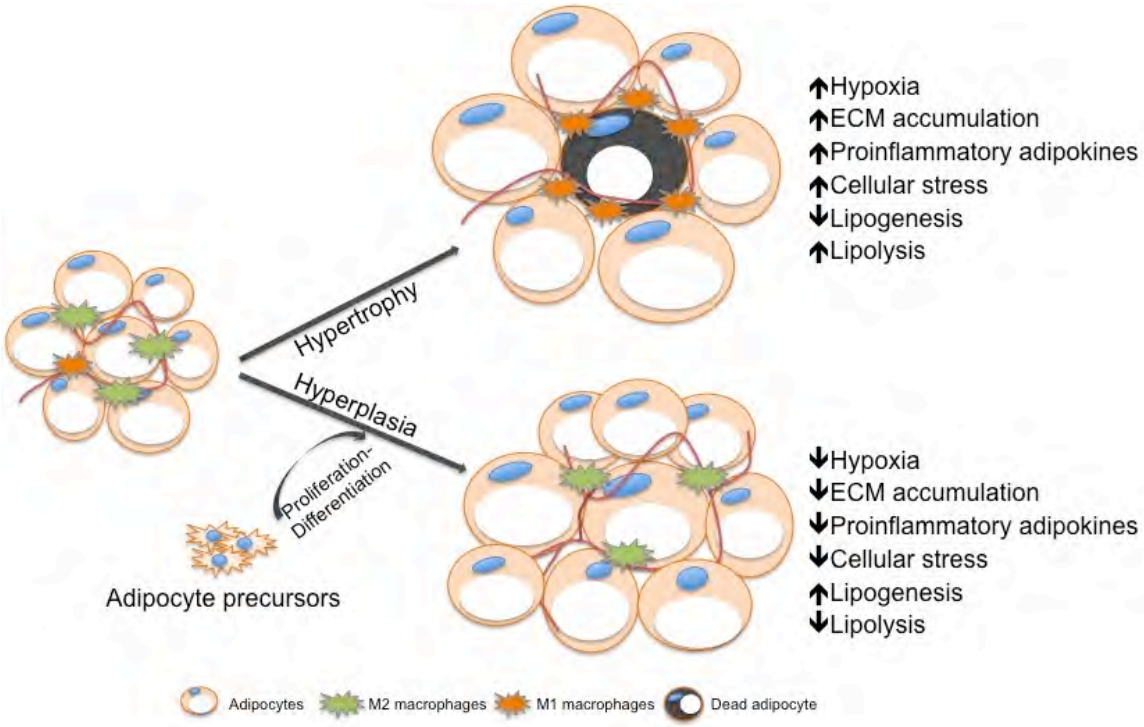


Figure 1.3 Two mechanisms of adipose tissue expansion
In lean adipose tissue, mature adipocytes are surrounded by proper vasculature, loose extracellular matrix and pro-inflammatory (M1) macrophages. Upon excess nutrient intake and decreased energy expenditure, adipose tissue expands via two ways. In hypertrophy, mature adipocytes undergo cell enlargement to meet increased demand for energy storage. A rapid increase in cell size results in cellular stress, inflammation, hypoxia and finally cell death. Proinflammatory macrophages are, in turn, infiltrated into adipose tissue, which stimulate adipose tissue insulin resistance. In hyperplasia, however, adipocyte precursors are recruited to make new functional adipocytes. Such expansion leads to healthier expansion of adipose tissue, which is characterized by adipocytes that are insulin sensitive, the presence of anti-inflammatory (M2) macrophages and efficient vascularization.

Blood vessel formation is vital for tissue expansion but cannot always keep up with the rapid increase in adipose tissue volume. Therefore, some cells become hypoxic in early obesity (223). Hypoxia inducible factor 1 alpha (HIF-1α) is regulated by oxygen levels in the cellular microenvironment (225). Hypoxia and HIF-1α are implicated in adipose tissue dysfunction via several proposed mechanisms such as reducing adiponectin levels as well as stimulating inflammation and fibrosis (226-228). Extracellular matrix (ECM) proteins and proteinases that cleave these proteins are implicated in adipose tissue expansion. As mentioned before, an environment that allows adipocytes to manipulate their differentiation, cell shape and volume is essential for their function. Thus, it is not surprising that ECM accumulation and fibrosis is associated with rapid and unhealthy adipose tissue expansion in addition to stress pathway stimulation and inflammation (229). Many ECM proteins are targets of the TGFβ signaling pathway. Connective tissue growth factor (CTGF) for instance is involved in tissue fibrosis and inhibits adipogenesis (230, 231). Furthermore, various types of collagens have been investigated in the context of adipose tissue expansion and metabolic dysfunction (232). Expressions of many collagens are increased in obese mice compared to wild type controls (233, 234). Collagen VI is mainly expressed in adipose tissue and forms a heterotrimer with three alpha chains. Among them, disruption of alpha 1 results in a knockout phenotype. In both *ob/ob* and high fat diet-fed mice, Col6a1 *null* mice displayed larger adipocytes yet a better metabolic phenotype, which was attributed to

reduced ECM stress during adipose tissue expansion (233). This phenomenon has also been observed in many other genetic models and is termed “healthy adipose tissue expansion” (61, 235, 236). The function of matrix metalloproteinases (MMPs) and tissue inhibitor of MMPs (TIMPs) has also been investigated in adipocytes (237). For example, mice with MMP14 (MT1-MMP) haploinsufficiency demonstrated decreased weight gain, small adipocytes and a lipodystrophic phenotype (167, 238). Male TIMP-2 knockout mice also developed insulin resistance during HFD (239). Taken together, these studies suggested a functional role for MMPs and TIMPs in the regulation of adipose tissue expansion.

There is substantial number of immune cells present in adipose tissue, which increases significantly in obesity (240). Cell death occurs in rapidly enlarged adipocytes, and one hypothesis is that phagocytic macrophage infiltration into adipose tissue serves to eradicate dead cells. Therefore, especially in the visceral adipose depot, these newly immigrated macrophages form ‘crown like structures’ consisting of macrophages surrounding dead adipocytes (241, 242). Several studies have demonstrated that pro-inflammatory cytokines are increased in obese adipose tissue, are secreted by macrophages, and are implicated in adipose tissue remodeling in obesity (65, 243-245). This is not to say all the macrophages recruited into adipose tissue are metabolically harmful. Macrophages have the capacity to take up excess lipids upon fasting and weight loss, which might be an adaptive response to protect adipose tissue

from potentially toxic levels of free fatty acids (246, 247). Moreover, some reports have suggested that M2 (anti-inflammatory) macrophages secrete catecholamines and promote browning of white adipose tissue upon cold exposure (111, 248). Furthermore, the two macrophages types display opposite phenotypes in terms of secreting pro- or anti-inflammatory cytokines. M1 (pro-inflammatory) macrophages are abundant in obese adipose tissue, whereas lean fat has a higher number of M2 (anti-inflammatory) macrophages. Surface markers such as CD11c are utilized to distinguish M1 macrophages from M2 macrophages, as the ratio between them is now used as indicator of the adipose tissue inflammatory state (249-251).

Recently, it was shown that when inflammation is impaired specifically in adipocytes, *in vivo* adipogenesis was attenuated and mice displayed ectopic lipid deposition, systemic inflammation and insulin resistance (252). Adipose tissue inflammation has long been associated with systemic insulin resistance and type II diabetes. Many factors contribute to inflammation-mediated adipose tissue dysfunction. First, adipokine secretion is affected in the obese state (253-256). Monocyte chemoattractant protein-1 (MCP-1) is released by stressed adipocytes that are stimulated by nutrient overload, which attracts pro-inflammatory macrophages into adipose tissue (254, 257). As previously mentioned, pro-inflammatory M1 macrophages secrete tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and interleukin 6 (IL-6), which can have deleterious effects on insulin signaling (258, 259). Free fatty acids that are not properly

stored in obese adipose tissue and lipopolysaccharide (LPS) from the gut microbiota activate the toll like receptor 4 (TLR4) pathway and stimulate adipose tissue inflammation (260, 261). How inflammation causes systemic insulin resistance is not fully understood. One mechanism suggests that TNF-α along with saturated free fatty acids activate c-Jun NH_2-terminal kinase (JNK) and inhibitor of nuclear factor κβ kinase β (IKKβ) to phosphorylate Ser-307 of IRS and suppress insulin signaling (261, 262). Pro-inflammatory cytokines can also stimulate lipolysis and repress triglyceride synthesis by inhibiting PPARγ (263-265).

Insulin resistance

The balance between lipogenesis and lipolysis in adipose tissue is of great importance in maintaining glucose and lipid metabolism. Dysfunctional adipose tissue is characterized by diminished lipogenesis, reduced GLUT4-mediated glucose transport and stimulated lipolysis, all of which result in inefficient storage of lipids (24, 262). Both hyperphagia (increased food intake) and leakage from adipose tissue cause elevated levels of free fatty acids in the circulation, which can then be stored in peripheral tissues such as liver and muscle (62). Lipids released from visceral adipose tissue depots directly circulate into the liver via the portal vein and give rise to hepatosteatosis (266). The liver also also exhibits a high capacity for *de novo* lipogenesis. This process is mainly regulated by

SREBP1c and ChREBP1, which are stimulated by insulin and glucose, respectively. Free fatty acids are then esterified into TAG and stored in liver lipid droplets or driven into circulation in the form of VLDL (267). Moreover, the liver maintains glucose homeostasis by taking up, storing and producing glucose. In lean and healthy conditions, hepatic gluconeogenesis (production of glucose) and glycogen release (glycogenolysis) are inhibited by insulin. Hepatic inflammation and lipotoxicity can contribute to dysregulation of these processes is dysregulated (268). Muscle, on the other hand, is the biggest contributor to insulin-stimulated glucose uptake and can synthesize glycogen after feeding (269, 270). Both liver and muscle are responsive to insulin in the lean state. However, in the obese state, elevated lipid metabolites such as fatty acyl co-A, diacylglycerol and ceramides can inhibit insulin action by modulating different components of the insulin signaling pathway (271). Furthermore, mitochondrial dysfunction, endoplasmic reticulum stress, low-grade inflammation and oxidative stress can all cause insulin resistance in peripheral tissues in obesity (272). Collectively, lack of a peripheral insulin response results in chronic hyperglycemia. Beta cells try to overcome hyperglycemia by producing and secreting more insulin, which in turn causes beta cell exhaustion, failure and eventually type II diabetes, (Figure 1.4). Therefore, optimal lipid synthesis and storage together with restraining controlled lipolysis in adipose tissue is critical. In order to find the novel regulators of these processes and the mechanisms that are implicated in the pathogenesis of metabolic diseases, our laboratory

performed a microarray study and identified a gene called TNMD as a potential candidate.

Figure 1.4

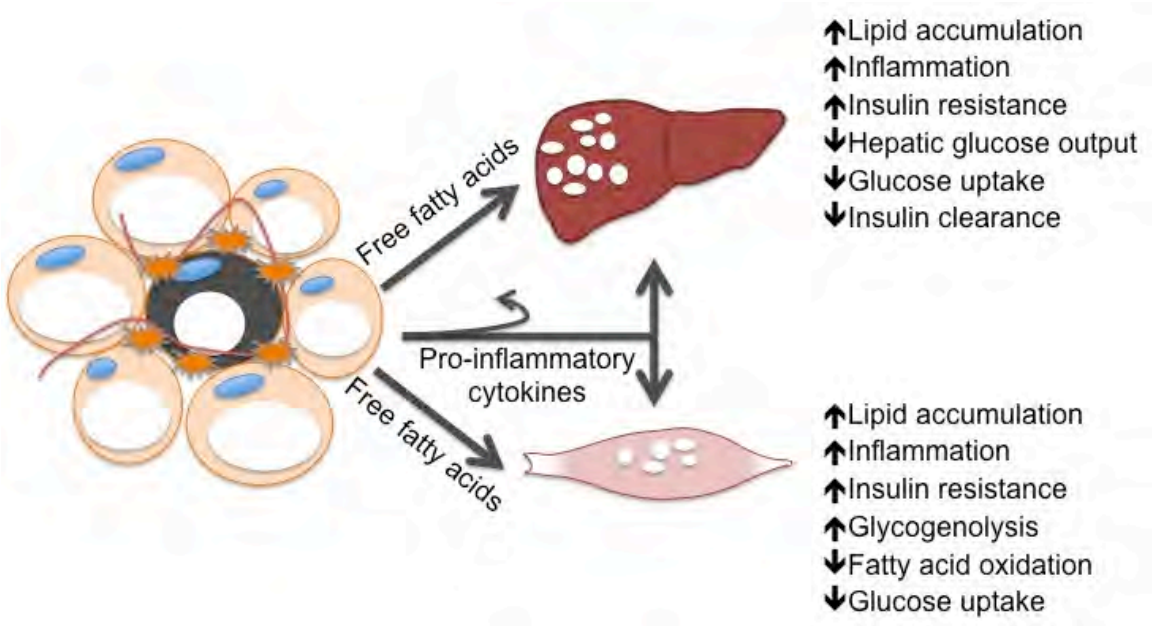


Figure 1.4: Ectopic lipid deposition and insulin resistance
Free fatty acids are released into the circulation from dysfunctional adipose tissue and stored in the peripheral tissues. Lipotoxicity along with proinflammatory cytokines that are secreted from hypertrophic adipocytes and activated adipose tissue macrophages leads to insulin resistance in liver and skeletal muscle. Hepatic glucose production and glycogen break down can no longer be inhibited by insulin in liver and muscle, respectively. Glucose uptake is also diminished in these tissues upon insulin resistance, which eventually results in beta cell failure and type II diabetes.

Tenomodulin (TNMD) and its putative role in metabolism

Tenomodulin (TNMD) is a 317 aa protein that is encoded by the *TNMD* gene, which is located on the X chromosome and spans approximately 15 kb. TNMD was identified by multiple groups as homologous to Chondromodulin-1 (Chm1) and predicted to be a type II transmembrane glycoprotein (273-276). TNMD has similarities to chondromodulin-1 in both exon and domain organization. Moreover, the Cys-rich C-terminal domains of these proteins display approximately 33% sequence homology. High *TNMD* expression was demonstrated in dense connective tissues such as tendons; therefore, the protein encoded by the *TNMD* gene was named as “tenomodulin” (275) and has been widely used as a tendon and tenocyte differentiation marker (277-279). TNMD is composed of three distinct domains. It spans the membrane once; therefore, it has a short transmembrane domain, BRICHOS domain and a Cys rich C-terminal domain. The BRICHOS domain also exists in other proteins such as Chondromodulin-1 and Integral membrane protein 2B (ITM2B or BRI2). The exact function of this domain is not fully understood. However, it has been suggested to serve as an intra-molecular chaperone for secreted members of this family that have an active furin cleavage site (280-282). TNMD has a putative furin cleavage motif (R-X-X-R) between amino acids 233-236 (Figure 1.5) (277).

Figure 1.5

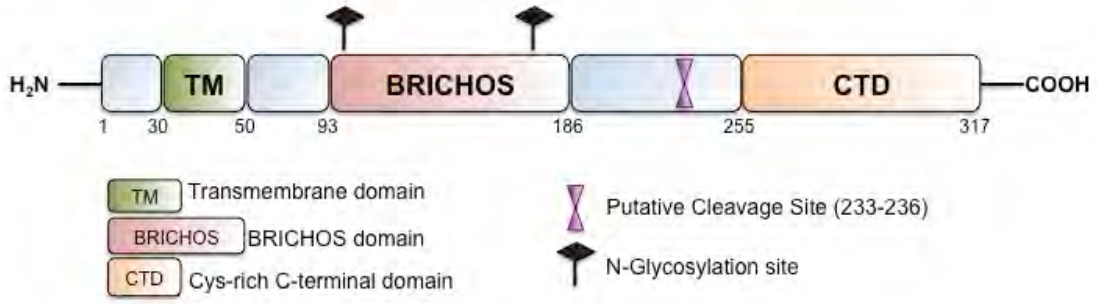


Figure 1.5: Domain structure of tenomodulin
Tenomodulin is predicted to be a type II transmembrane protein with a cytoplasmic N-terminus and extracellular C-terminus. It is composed of a short N-terminal domain (amino acids 1-30), transmembrane domain (amino acids 31-51), BRICHOS domain (amino acids 93-186) and Cys-rich C-terminal domain with 8 Cys residues (amino acids 255-317). The BRICHOS domain consists of two N-glycosylation sites, and a putative furin cleavage site is located between amino acids 233-236.

Chondromodulin-1 (Chm-1) is processed and secreted into the extracellular matrix by chondrocytes (283, 284). Furthermore, when administered into mice, purified chondromodulin-1 inhibited vascularization in cartilage (283). Moreover, the secreted portion of chondromodulin-1 inhibited HUVEC cell proliferation and tube formation *in vitro*. When a signal sequence was introduced into the TNMD sequence, TNMD was also secreted into media and displayed similar effects on HUVEC cells, suggesting that the C-terminal tail of TNMD might be cleaved from the putative furin recognition site and be physiologically functional (285). TNMD knockout mice, however, did not display any reduced vasculature phenotype in tendons.

Three isoforms of TNMD have been proposed due to alternative splicing. The cellular localization of GFP-tagged isoforms has been analyzed by immunofluorescent staining. Isoform I (the most common 7 exon protein) and isoform III were detected in the cytoplasm whereas isoform II was mostly associated with the nuclear envelope (286). In another study, overexpressed TNMD was colocalized with plasma membrane, cytoskeleton and Golgi apparatus markers (287). Collectively, these findings suggest that TNMD does not solely localize in the plasma membrane, and further studies designed to especially detect endogenous levels of TNMD are needed to understand the intracellular location of this protein.

TNMD was previously predominantly detected in dense connective tissues such as tendons, ligaments and cornea (273, 288, 289). However, a

comprehensive study demonstrated that adipose tissue has the highest expression of TNMD among tissues examined (not including tendons in this study) (290). When compared to tendons, however, adipose tissue displayed lower TNMD expression (291). Interestingly, *TNMD* expression in adipose tissue correlates with body mass index and increases during obesity in both subcutaneous and visceral adipose depots (290). Furthermore, TNMD is one of the most responsive genes to weight reduction in human adipose tissue (290, 292, 293). On the contrary, rat adipose tissue either lacks or has non-detectable levels of TNMD expression (291).

In a Finnish Diabetes Prevention Study (DPS), SNPs in the *TNMD* gene were associated with some metabolic parameters such as obesity, BMI, and risk of diabetes. However, these associations could not be confirmed in a Metabolic Syndrome in Men Study (METSIM) study, which had a larger cohort (294-296). On the other hand, a BMI-dependent association was found between some SNPs in *TNMD* and serum levels of HDL and LDL (297). Moreover, serum levels of some immune mediators such as C-reactive protein, chemokine (C-C motif) ligand-5 (CCL-5), CCL-3 and serum amyloid A were associated with SNPs in *TNMD* (298).

Scope of this thesis

The mechanisms by which obesity and adipose tissue dysfunction leads to insulin resistance are not fully revealed. Identification of adipocyte specific molecules that may protect obese subjects from metabolic syndrome is an important approach to understanding these mechanisms as well as developing therapeutic approaches for a major health care problem. The aim of the research that presented in this thesis is to characterize TNMD as such a molecule. Two main questions were addressed here:

1) Does TNMD play a role in adipocyte differentiation? If so what is the mechanism by which TNMD regulate adipogenesis?
2) Does TNMD affect adipose tissue function? If so, what is its role of TNMD in regulating adipocyte specific processes?

In Chapter II, I present the approach that we took to address these questions. Using *in vitro* cell culture models, I demonstrated that TNMD is required for adipocyte differentiation and adipogenic gene expression. Furthermore, in a transgenic mouse model, I found that overexpressing TNMD selectively in the adipose tissue increased hyperplastic expansion and lipogenic gene expression upon HFD feeding. Such healthy adipose tissue expansion leads to attenuated liver steatosis and improved systemic insulin resistance. Therefore, TNMD may possess therapeutic potential for insulin resistance and type II diabetes.

CHAPTER II

TENOMODULIN PROMOTES HUMAN ADIPOCYTE DIFFERENTIATION AND BENEFICIAL VISCERAL ADIPOSE TISSUE EXPANSION

This chapter is derived from the article with the same name published in *Nature Communications*:

Senol-Cosar O., Roth Flach R.J., DiStefano M., Chawla A., Nicoloro S., Straubhaar J., Hardy O.T., Noh H.L., Kim J.K., Wabitsch M., Scherer P.E., Czech M.P. (2016*)*

Author Contributions

Figure 2.1, a, b. The Gene Chip microarray studies were performed by **Sarah M. Nicoloro**. **Juerg Straubhaar** analyzed the data from these microarrays.

Figure 2.2. SVF and primary adipocytes fractionation and RNA isolation was performed by **Sarah M. Nicoloro.**

Figure 2.7. Juerg Straubhaar analyzed the data from these microarrays

Figure 2.11. Transgenic and adenoviral expression constructs were generated by **Anil Chawla**.

Figure 2.14,d. Marina T. DiStefano performed the glucose incorporation experiment and provided help in animal studies.

Figure 2.15, a. Rachel Roth Flach performed the isolectin staining and vessel density quantification, provided help in animal studies.

Figure 2.16; Figure 2.19. Hye-Lim Noh performed metabolic cage and clamp studies and **Jason K. Kim** analyzed the data.

SGBS cells were obtained from **Martin Wabitsch**'s laboratory.

Philipp E. Scherer provided the adiponectin 5.4kb promoter plasmid.

The rest of the experiments presented in this chapter were performed by **Ozlem Senol-Cosar**.

This manuscript was written by **Ozlem Senol-Cosar** with helpful suggestions from **Rachel Roth Flach, Joseph V. Virbasius,** and **Michael P. Czech**.

Summary

Proper regulation of energy storage in adipose tissue is crucial for maintaining insulin sensitivity, and molecules contributing to this process have not been fully revealed. Here, we show that type II transmembrane protein tenomodulin (*TNMD*) is upregulated in adipose tissue of insulin resistant versus insulin sensitive individuals who were matched for BMI. *TNMD* expression increases in human preadipocytes during differentiation, whereas silencing *TNMD* blocks adipogenesis. Upon high fat diet feeding, transgenic mice overexpressing *Tnmd* develop increased epididymal adipose tissue (eWAT) mass, and preadipocytes derived from *Tnmd* transgenic mice displayed greater proliferation, consistent with elevated adipogenesis. In *Tnmd* transgenic mice, lipogenic genes are were upregulated in eWAT and *Ucp1* was upregulated in brown fat, while liver triglyceride accumulation was attenuated. Despite expanded eWAT, *Tnmd* transgenic animals display improved systemic insulin sensitivity, decreased collagen deposition and inflammation in eWAT as well and increased insulin- stimulation stimulated of Akt phosphorylation. Our data suggest that TNMD acts as a protective factor in visceral adipose tissue to alleviate insulin resistance in obesity.

Introduction

A large body of work has suggested that adipose tissue plays a key role in determining metabolic health as a major regulator of carbohydrate and lipid homeostasis. Expansion of adipose tissue in overweight or obese humans can lead to a spectrum of dysfunctions collectively referred to as metabolic syndrome. However, a significant number of metabolically healthy obese human subjects demonstrate a situation of benign adipose tissue expansion whose differences from pathological obesity are poorly understood (61, 236, 299-301). Some studies have suggested that specific physiological mechanisms and anatomical locations of adipose expansion may differentially affect metabolic homeostasis (302-305). Major white adipose depots located in subcutaneous regions and the visceral cavity can dynamically expand during obesity (77). In humans, adipose tissue expands via adipocyte hypertrophy during early obesity, whereas an increase in adipocyte number, denoted hyperplasia, also occurs in prolonged obesity (306, 307). Animal models have demonstrated that subcutaneous adipose tissue enlargement is mostly due to hypertrophy while the visceral depot expands by increasing both cell size and number upon long term high fat diet (HFD) feeding (133, 308). This increase in cell number derives from the differentiation of adipocyte precursors into differentiated adipocytes, a well-defined process that has been extensively modeled in the 3T3-L1 mouse cell line (114, 309). Though mouse adipocyte lines such as 3T3-L1 cells have greatly

contributed to identifying the molecular mechanisms involved in differentiation and maintaining mature adipocyte function (139), interspecies differences in gene expression and regulation between mouse and human adipocytes are important to consider and further investigate (158, 310).

Central obesity is linked to several metabolic morbidities such as type 2 diabetes and cardiovascular disease (311). Visceral adipose tissue is more prone to inflammation than subcutaneous fat in obesity through mechanisms that enhance immune cell content (312) and increase pro-inflammatory cytokine expression (74, 244, 245, 313). A leading hypothesis suggests that low-grade inflammation in fat depots is involved in metabolic syndrome (65, 314). Moreover, visceral adipose tissue may be more lipolytic than subcutaneous adipose tissue due to dampened insulin suppression of lipolysis and a higher response to catecholamines. This, in turn, increases both non-esterified fatty acid release into the circulation and hepatic lipid deposition due to the close proximity of visceral adipose tissue to the hepatic portal vein (315, 316). Ectopic lipid storage in liver and muscle is thought to trigger insulin resistance in these tissues, although not under all conditions (317). Therefore, promoting healthy expansion and better lipid storage in visceral adipose tissue is crucial to maintain glucose homeostasis and insulin sensitivity.

In order to identify and explore mechanisms in adipose tissues that either cause insulin resistance or preserve insulin sensitivity in obese individuals, we compared gene expression in subcutaneous and omental adipose tissues from

obese human subjects matched for body mass index (BMI) but differing in insulin resistance. Among several differentially expressed genes identified, we focused on tenomodulin (*TNMD*), a type II transmembrane protein, due to its high and predominant expression in human adipose tissue, also noted by others (275, 290). Comparative analysis of adipose tissue *TNMD* expression in obese and lean individuals also previously indicated that TNMD is strongly correlated with BMI (290, 292, 318). Moreover, many genome-wide association studies revealed that single nucleotide polymorphisms (SNPs) in the *TNMD* gene are associated with various metabolic characteristics such as BMI, serum low-density lipoprotein levels and inflammatory factors (296, 297, 319). Though these studies indicate a potential role for TNMD in human adipose tissue, the function of TNMD has not been evaluated.

Here, by gene silencing and generating a transgenic overexpression mouse model, we demonstrate that TNMD is required for adipocyte differentiation, and overexpression of *Tnmd* in adipose tissue protects mice from obesity-induced systemic insulin resistance. These data suggest that adipocyte TNMD is a protective factor that enhances insulin sensitivity in obesity, potentially via promoting hyperplasia and beneficial lipid storage in the visceral adipose tissue.

Experimental Procedures

Animals

All of the studies were approved by The University of Massachusetts Medical School Institutional Animal Care and Use Committee. Mice were housed in an animal facility with a 12 hour light/dark cycle and had access to water, chow or high fat diet (12492i Harlan) *ad libitum* during the indicated periods. For *in vivo* preadipocyte proliferation studies, mice were treated with 0.8 mg ml^{-1} BrdU in water with 1% sucrose. Water was changed every 72 hours and kept in the dark. Mice were euthanized by CO_2 and bilateral pneumothorax.

Human samples

Human adipose tissue samples collected from morbidly obese patients who underwent gastric bypass surgery between 2005 and 2009 at the University of Massachusetts Medical School were selected for this study (300). Samples used for microarray analysis were from BMI-matched female patients, whereas qRT-PCR and Western Blot validations were performed in samples from both males and females. Adipose tissue samples were obtained from the lower abdominal wall (for subcutaneous) and omentum (for visceral) during the surgery. Informed consent was given by the patients, and the study was approved by the University of Massachusetts Medical School Institutional Review Board.

Generation of Adiponectin-*Tnmd*-Flag transgenic mice

Full-length mouse *Tnmd* with a C-terminal Flag tag was inserted 3' to 5.4 kb adiponectin promoter at the Cla I site (320). After verifying both ends by sequencing, the transgenic cassette was linearized by Kpn1 and Xho1 digestion, purified and submitted for pro-nuclear injection. The transgene was introduced into embryos from C57BL/6J mice (000058) (Jackson Laboratories). Embryos were then implanted into pseudopregnant C57BL6/J females by the UMASS Transgenic Animal Facility. Male transgenic animals were crossed with C57BL6/J females. Genotyping was performed by PCR from genomic DNA with the following primers: 5'-GACCAGAATGAGCAATGGGTG, 3'-ATCGTCGTCATCCTTGTAGTCG. 6-8 weeks old Adiponectin-*Tnmd*-Flag transgenic mice and age matched wild-type littermates were used in the experiments. Male animals were used unless otherwise was stated.

Western Blotting

Cell lysates were prepared using RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% sodium deoxycholate, 1% NP-40, 0.2 % SDS, 50 mM EDTA) containing 1X HALT protease and phosphatase inhibitors (Thermo Scientific). Total protein was separated on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk solution in TBS-T and immunoblotted with an antibody generated against NGIEFDPMLDERGYC peptide from C-terminus of TNMD (Rockland, 1:5000) and antibodies against

C/EBPα (8178), C/EBPβ (3082), PPARγ (2443), phospho AKT(S308) (9275), phospho AKT(T473) (4060), total AKT (4691 or 2920), ACLY (4332), ACC (3662), PLIN (3470), (Cell Signaling, 1:1000), FASN (BD Biosciences, 610963, 1:2000), FLAG (F7425, 1:2000) tubulin and actin (Sigma, 1:5000).

qRT-PCR analysis

Total RNA was isolated using TriPure (Roche) according to the manufacturer's protocol. DNAse (DNA-free, Life Technologies)-treated RNA was reverse transcribed into cDNA using iScript (BioRad). Quantitative PCR analyses were performed using SYBR green (iQ SYBR Green Supermix, BioRad) on BioRad CFX97. Primer sequences used for qRT-PCR analyses were listed in Table 2.1. HUGO Gene Nomenclature Committee's Guideline was used for gene names written in this thesis.

Accession Codes

Microarray data have been deposited in GEO database under accession code GSE76319 and GSE20950.

Table 2.1

Human Primers		
Gene	**Forward**	**Reverse**
TNMD	ATTCAGAAGCGGAAATGGCACTGA	TAGGCTTTTCTGCTGGGACCCAA
C/EBPA	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAGCTCCAGCAC
PPARG2	CTCCTATTGACCCAGAAAGCGA	TGCCATGAGGGAGTTGGAAG
PLIN1	GACCTCCCTGAGCAGGAGAAT	GTGGGCTTCCTTAGTGCTGG
KLF4	GATGAACTGACCAGGCACTA	TCGGGAAGACAGTGTGAAAA
SPP1	CTCCATTGACTCGAACGACTC	CAGGTCTGCGAAACTTCTTAGAT
ADIPOQ	TCTGCCTTCCGCAGTGTAGG	GGTGTGGCTTGGGGATACGA
RPLP0	CAGATTGGCTACCCAACTGTT	GGGAAGGTGTAATCCGTCTCC

Mouse Primers		
Gene	**Forward**	**Reverse**
Tnmd	AATGGGTGGTCCCGCAAGTG	ACAGACACGGCGGCAGTAAC
C/ebpa	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAGCTCCAGCAC
C/ebpb	GCAAGAGCCGCGACAAG	GGCTCGGGCAGCTGCTT
Pparg2	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT
Cidec	ATCAGAACAGCGCAAGAAGA	CAGCTTGTACAGGTCGAAGG
Lept	GAGACCCCTGTGTCGGTTC	CTGCGTGTGTGAAATGTCATTG
Adipoq	TGTTCCTCTTAATCCTGCCCA	CCAACCTGCACAAGTTCCCTT
Glut4	GTGACTGGAACACTGGTCCTA	CCAGCCACGTTGCATTGTAG
Plin1	CTGTGTGCAATGCCTATGAGA	CTGGAGGGTATTGAAGAGCCG
Srebp1c	GGCCCGGGAAGTCACTGT	GGAGCCATGGATTGCACATT
Fasn	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
Acly	ACCCTTTCACTGGGGATCACA	GACAGGGATCAGGATTTCCTTG
Acc1	TGTACAAGCAGTGTGGGCTGGCT	CCACATGGCCTGGCTTGGAGGG
Cd68	CCATCCTTCACGATGACACCT	GGCAGGGTTATGAGTGACAGTT
Ccl2	GCTGGAGAGCTACAAGAGGATCACC	TCCTTCTTGGGGTCAGCACAGAC
Il-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Tnfa	CCCTCACACTCAGATCATCTTCT	GTCACGACGTGGGCTACAG
Ucp1	ACTGCCACACCTCCAGTCATT	CTTTGCCTCACTCAGGATTGG
Prdm16	CCAAGGCAAGGGCGAAGA	AGTCTGGTGGGATTGGAATGT
Plin2	GACCTTGTGTCCTCCGCTTAT	CAACCGCAATTTGTGGCTC
Mmp2	AACACTGAAGATCTTGCTCTGAGAT	TTGAGAGACTGAGACAGGGAGTC
Mmp3	TGATGAACGATGGACAGAGG	GAGAGATGGAAACGGGACAA
Mmp9	ACCACATCGAACTTCGA	CGACCATACAGATACTG
Mmp12	CTGGACAACTCAACTCT	AGAGGAGTCACATCACT
Mmp14	CAGTATGGCTACCTACCTCCAG	GCCTTGCCTGTCACTTGTAAA
Fn1	ATGTGGACCCCTCCTGATAGT	GCCCAGTGATTTCAGCAAAGG
Smad2	ATGTCGTCCATCTTGCCATTC	AACCGTCCTGTTTTCTTTAGCTT
Smad3	CACGCAGAACGTGAACACC	GGCAGTAGATAACGTGAGGGA
Cola1a1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
Col5a1	CTTCGCCGCTACTCCTGTTC	CCCTGAGGGCAAATTGTGAAAA
Col6a1	AACAGGAATAGGAAATGTGACCC	ACACCACGGATAGGTTAGGGG
Col6a3	CAGAACCATTGTTTCTCACT	AGGACTACACATCTTTTCAC
Tgfb1	ATTCCTGGCGTTACCTTGG	AGCCCTGTATTCCGTCTCCT
Vwf	CTTCTGTACGCCTCAGCTATG	GCCGTTGTAATTCCCACACAAG
Cd31	ACGCTGGTGCTCTATGCAAG	TCAGTTGCTGCCCATTCATCA
Cdh5	CACTGCTTTGGGAGCCTTC	GGGGCAGCGATTCATTTTTCT
Thbs1	GGGGAGATAACGGTGTGTTTG	CGGGGATCAGGTTGGCATT
36b4	TCCAGGCTTTGGGCATCA	CTTTATCAGCTGCACATCACTCAGA

Table 2.1: Primer sequences used in the qRT-PCR analyses

Cell culture

Simpson Golabi Behmel Syndrome (SGBS) cells were obtained from Dr. Martin Wabitsch's laboratory and cultured in DMEM/F12 media supplemented with 10% fetal bovine serum, 33 uM biotin, 17 uM pantothenic acid, 100 units ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin until full confluence. Cells were washed with PBS before differentiation, and differentiation was stimulated with serum-free media containing rosiglitazone, dexamethasone, 3-isobutyl-1-methylxanthine, cortisol, transferrin, triiodothyronine, and human insulin. 4 days later, the differentiation cocktail was replaced with adipocyte maintenance media (DMEM/F12, biotin, pantothenic acid, transferrin, insulin and cortisol). Cells were maintained until they are fully differentiated (day 14). 3T3-L1 preadipocytes were obtained from AATC (CL-173). Cells were cultured in high glucose DMEM media supplemented with 10% FBS, 50 µg ml^{-1} streptomycin, and 50 units ml^{-1} penicillin and differentiated into adipocytes with high glucose DMEM media with 10% FBS, 50 µg ml^{-1} streptomycin, 50 units ml^{-1} penicillin, 5 µg ml^{-1} insulin, 0.25 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine.

Oil Red O Staining

Differentiated adipocytes were fixed with 10% formalin, washed with 60% isopropanol and air dried. Oil Red O working solution was added on cells and washed with distilled water to remove excess dye. Oil Red O was extracted using

100% isopropanol and absorbance was measured in 520 nm in spectrometer for quantification.

siRNA transfection of SGBS and 3T3-L1 preadipocytes

SGBS or 3T3-L1 preadipocytes were plated into 12-well plates (10^5 cells per well) and transfected with 100 nM (SGBS) or 50 nM (3T3-L1) scrambled siRNA or TNMD siRNA (Dharmacon, siGENOME, Smartpool) using Lipofectamine RNAiMax (Life Technologies) according to the manufacturer's protocol. 48 hours after transfection, cells were harvested or stimulated for adipogenesis.

MTT assay

SGBS preadipocytes (10^4 cells per well) were plated in 96 well plates. 24 hours later, they were transfected with either scrambled or TNMD siRNA. Cell viability was measured 0, 24 and 48 hours after transfection using an MTT assay kit (Biotium Inc.) according to the manufacturer's instructions.

Immunofluorescence staining

Differentiated adipocytes were fixed with 10% formalin and permeabilized in 0.1% Triton X in PBS. 3% BSA in PBS was used for blocking. Cells were incubated overnight with PLIN antibody (Cell Signaling) diluted in 1% BSA solution and 45 minutes at 37 ^{0}C with secondary antibody (Alexa Fluor 488, Life

Technologies). Nuclei were stained with DAPI. Preadipocytes were fixed in 70% cold methanol and treated with 1.5 M HCl for 30 min. After washing, they were washed and blocked with 5% normal goat serum and 0.3% Triton-X100 in PBS. Primary antibodies for BrdU and Pref1 (Cell Signaling 5292, 1:1000 and EMD Millipore, AB3511, 1:100) were applied overnight. Cells were washed and incubated with secondary antibodies for 90 min at room temperature and mounted with Prolong® Gold Antifade Reagent with DAPI. Images from at least 3 different areas were taken, and nuclei were counted by Image J Analysis Software. For vessel density in adipose tissue, whole-mount staining was performed after 10% formalin fixation. Tissues were blocked overnight in 10% BSA and 0.3% triton X-100 in PBS at 4 ^{0}C, stained overnight with Isolectin B4 (Life Technologies I21411; 1:40) in 100 mM MgCl2, 100 mM CaCl2, 10 mM MnCl2, and 1%Triton X-100 in PBS at 4 ^{0}C, and washed 3x 20 minutes in 5% BSA, 0.15% triton X-100 in PBS at room temperature. Approximately 1 mm cubes were whole-mounted in ProLong Gold (Life Technologies). Images were visualized in flattened 25 um z-stacks with confocal microscopy at 10x. Images were acquired with MetaMorph Software, version 6.1 (Universal Imaging, Downingtown, PA). At least 3 technical replicates of adipose tissue images were quantified per mouse and averaged for average vascular density. Images were quantified using Image J Analysis Software.

Histology

Tissue samples were fixed in 10% formalin and embedded in paraffin. Sectioned slides were then stained with hematoxylin and eosin by the UMass Medical School Morphology Core. Adipocyte size was assessed using Adiposoft software (321).

Insulin and Glucose tolerance tests

Mice fed with the indicated diets were fasted 16 hours for glucose tolerance tests and or 4 hours for insulin tolerance tests. Basal blood glucose levels were measured with a Breeze-2-glucose meter (Bayer) before and after they were intraperitoneally injected with glucose (1 g kg^{-1}) or insulin (1IU kg^{-1}).

Hyperinsulinemic-euglycemic clamp and metabolic cage studies

The clamp and metabolic cage studies were performed at the UMass Mouse Metabolic Phenotyping Center. Mice fed with HFD for 12 weeks were subjected to a 4-hour fast, and a 2-h hyperinsulinemic-euglycemic clamp was performed with a primed and continuous infusion of human insulin (150 mU kg^{-1} body weight priming followed by 2.5 mU kg^{-1} min^{-1}; Humulin, Eli Lilly). During the clamp, 20% glucose was infused at variable rates to maintain euglycemia (322). Whole body glucose turnover was assessed with a continuous infusion of [3-^{3}H] glucose, and 2-deoxy-D-[1-^{14}C]glucose (PerkinElmer, Waltham, MA) was administered (10 μCi) at 75 min after the start of clamps to measure insulin-

stimulated glucose uptake in individual organs. At the end of the study, mice were anesthetized, and tissues were taken for biochemical analysis. The metabolic cages were used to measure food intake, respiratory exchange ratio, VO_2 consumption and physical activity over a 3-day period, and average for each parameter was calculated (TSE Systems).

Plasma analysis

Blood samples collected from animals after a 16-hour fast via cardiac puncture. Serum triglyceride levels were measured using a Serum Triglyceride Determination Kit (Sigma). Free fatty acid and total cholesterol in plasma samples were analyzed using NEFA (Free Fatty Acid) Kit (Wako Diagnostics) and Cholesterol/Cholesteryl Ester Quantitation Kit (Abcam) respectively.

***Ex vivo* lipogenesis assay**

Adipose tissue explants were incubated with labeling media containing 2.50% FA free BSA, 1% (v/v) Pen/Strep, 0.5 mM d-Glucose, 2 mM sodium pyruvate, 2 mM glutamine, 2 µCi mL^{-1} [^{14}C]-U-glucose. Insulin (1 µM) was added to insulin-stimulated conditions and incubated for 4.5 h before lipid extraction at 37 °C. The reaction was stopped by adding modified Dole's extraction mixture (80 mL isopropanol, 20 mL hexane, 2 mL of 1N H_2SO_4). Total triglyceride was extracted with hexane, washed and evaporated and counted by liquid scintillation

Ex vivo lipolysis assay

Adipose tissue explants were isolated from iWAT and eWAT of mice that had been fed chow diet for 12 weeks. Fat pads were measured, and a 30 mg piece was placed in freshly prepared KRH buffer (125 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 2.6 mM MgSO4, 5 mM HEPES, pH 7.2) containing 2.5% BSA (fatty acid free) and 1 mM sodium pyruvate until stimulation. Subsequently, extracts were treated with PBS or isoproterenol (10 μM) for 2 h at 37°C. Free glycerol content in the buffer was quantified for each sample using the Free Glycerol Determination Kit (Sigma). Glycerol release from each sample was normalized to the weight of each fat pad.

Statistical Analysis

A two-tailed Student's t-test with Welch's correction was performed to analyze the difference between two groups using Microsoft Excel or Graph Pad Prism 6.0. The Grubb's test was used to determine the statistical outliers. In case of an outlier was determined, it was removed from the statistical analysis. Experimental data were represented as the mean of at least three biological replicates. *P* values < 0.05 were considered to be statistically significant. Variance was estimated using the standard error of the mean for both groups that were statistically compared. No statistical methods were used to predict sample size. No randomization or blinding was performed to allocate the samples for animal experiments.

Results

Higher expression of human *TNMD* in insulin resistance

To assess gene expression differences in insulin resistant vs. insulin sensitive obese individuals, total RNA was isolated from snap frozen adipose tissue biopsies from omental and subcutaneous fat depots of human subjects undergoing bariatric surgery, and analyzed for genome-wide gene expressions. Clinical data of the subjects used in gene expression analysis are shown in Table 2.2. DNA microarray data identified *TNMD* as a gene that is significantly upregulated in omental fat from insulin-resistant individuals compared with BMI-matched, insulin sensitive subjects (Figure 2.1, a). However, no significant difference in *TNMD* gene expression was observed between the two groups in subcutaneous adipose tissue (Figure 2.1, b). These results were validated by assessing mRNA and protein levels using qRT-PCR and Western blotting respectively (Figure 2.1, c-d).

Table 2.2

	Insulin Sensitive (n=8) (Mean±SE)	**Insulin Resistant (n=6) (Mean±SE)**	***P***
Age	37 (±1.57)	44 (±3.72)	0.1060
Weight	295.875 (±14.89)	317.17 (±22.38)	0.4257
BMI	48.25 (±1.04)	50 (±3.34)	0.5829
EBW	167.25 (±11.15)	182.67 (±21.05)	0.5006
Fasting glucose (mg/dl)	88.125 (±3.28)	104.83(±5.20)	0.0145
Fasting insulin (mIU/l)	9.375 (±1.54)	24 (±3.72)	0.0017
HOMA-IR (mmol/l)	1.375 (±0.22)	3.57 (±0.53)	0.0013
%B Beta cell sensitivity	125.4 (±18.47)	174.12 (±25.89)	0.1405

Table 2.2: Clinical characteristics of subjects selected for human Affymetrix GeneChip analysis.
BMI: Body-Mass Index
EBW: Excess body weight
HOMA-IR: homeostasis model assessment of insulin resistance

Figure 2.1

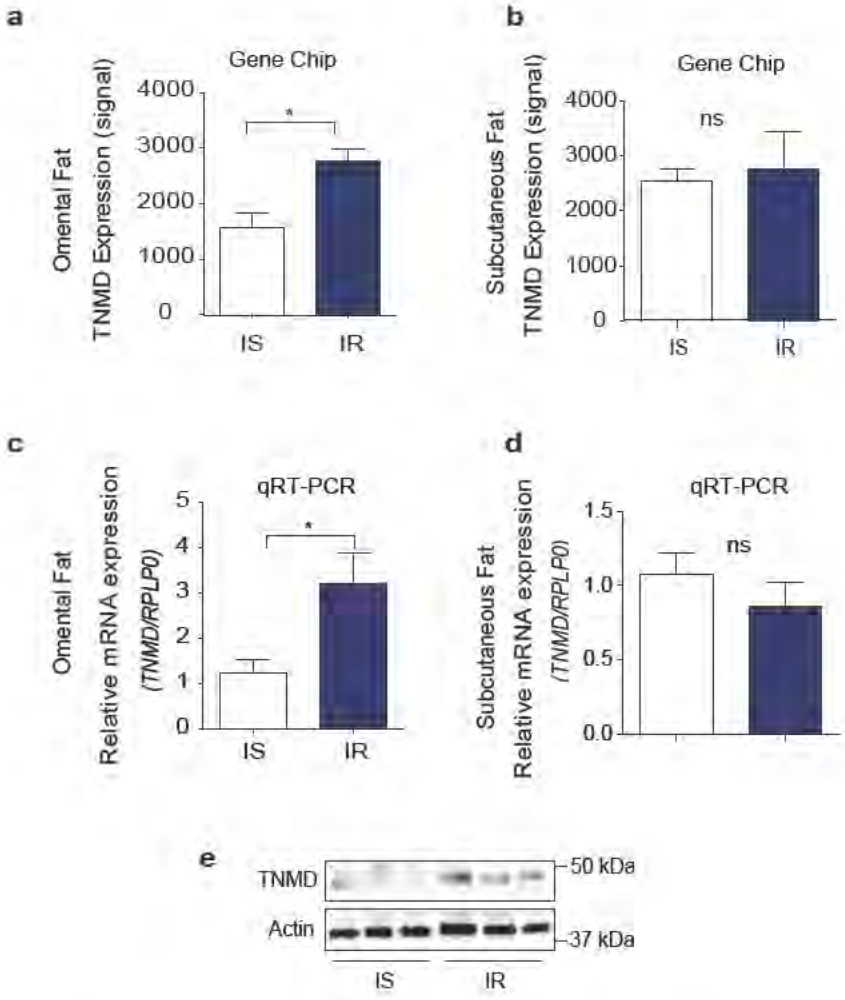


Figure 2.1: Higher expression of human *TNMD* in insulin resistance
Omental and subcutaneous adipose tissue was isolated from obese patients undergoing bariatric surgery. **(a,b)** RNA was isolated, and a microarray was performed (mean±SEM; n=6-7, insulin sensitive, n=8, insulin resistant, *p<0.05, by Student's t-test). **(c,d)** Quantitative PCR validation of *TNMD* expression in obese individuals (mean±SEM; n=6, insulin sensitive, n=8, insulin resistant, *p<0.05, by Student's t-test). **(e)** Western blot analysis of TNMD and actin in omental adipose tissue lysates from insulin sensitive and insulin resistant patients.

It was previously demonstrated that white adipose tissue and primary adipocytes have the highest expression of *TNMD* among human tissues (290). Although this original study claimed similar *TNMD* expression in the stromal vascular fraction (SVF) and whole adipose tissue, we observed that *TNMD* is predominantly expressed in adipocytes compared with cells from the SVF in both visceral and subcutaneous white adipose tissues (Figure 2.2, a,b). *SPP1* and *PLIN1* were used as specific markers for SVF and primary adipocytes, respectively (Figure 2.2, c,d).

Figure 2.2

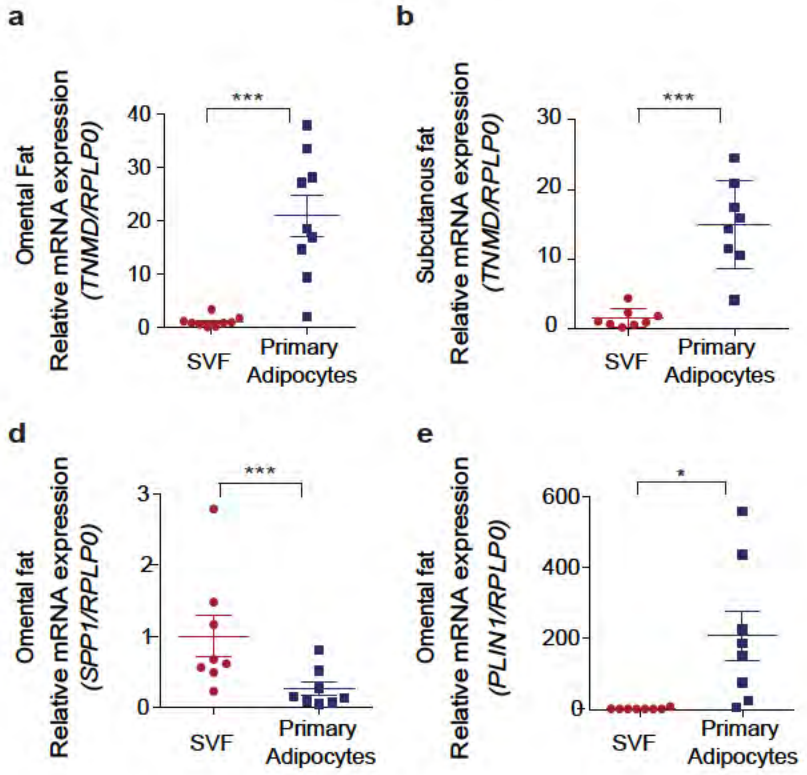


Figure 2.2: *TNMD* is predominantly expressed in adipocytes in human adipose tissue

Primary adipocytes were separated from the SVF by centrifugation. Quantitative PCR analysis of *TNMD* **(a)**, *PLIN1* **(c)** and *SPP1* **(d)** expression in the stromal vascular fraction (SVF) and in primary adipocytes isolated from omental adipose tissue of obese individuals (mean ± SEM; n=9 for both SVF and primary adipocytes *p<0.05, **p<0.01, ***p<0.001, by Student's t-test). **(b)** TNMD expression in the SVF or primary adipocytes isolated from human subcutaneous fat depot (mean ± SEM; n=8 for both SVF and primary adipocytes, *p<0.05, **p<0.01, ***p<0.001, Student's t-test).

To assess the role of TNMD in human adipocyte function, we utilized human preadipocytes obtained from a subject with Simpson-Golabi-Behmel Syndrome (SGBS cells) (148) and discovered that *TNMD* expression increased by several hundred fold during adipocyte differentiation in culture (Figure 2.3, a). Expression of mature adipocyte markers such as *PPARG2* and *ADIPOQ* certified that these cells had undergone adipocyte differentiation after induction with the adipogenic cocktail. *TNMD* expression was increased even 18 hours after stimulation of the preadipocytes. Moreover, *TNMD* exhibited a time-dependent expression profile similar to peroxisome proliferator-activated receptor gamma 2 (*PPARG2)* and CCAAT/enhancer-binding protein alpha (*C/EBPA),* but not Kruppel-like factor 4 (*KLF4)*, which is an early transcription factor in the adipogenic cascade (Figure 2.3, b).

Figure 2.3

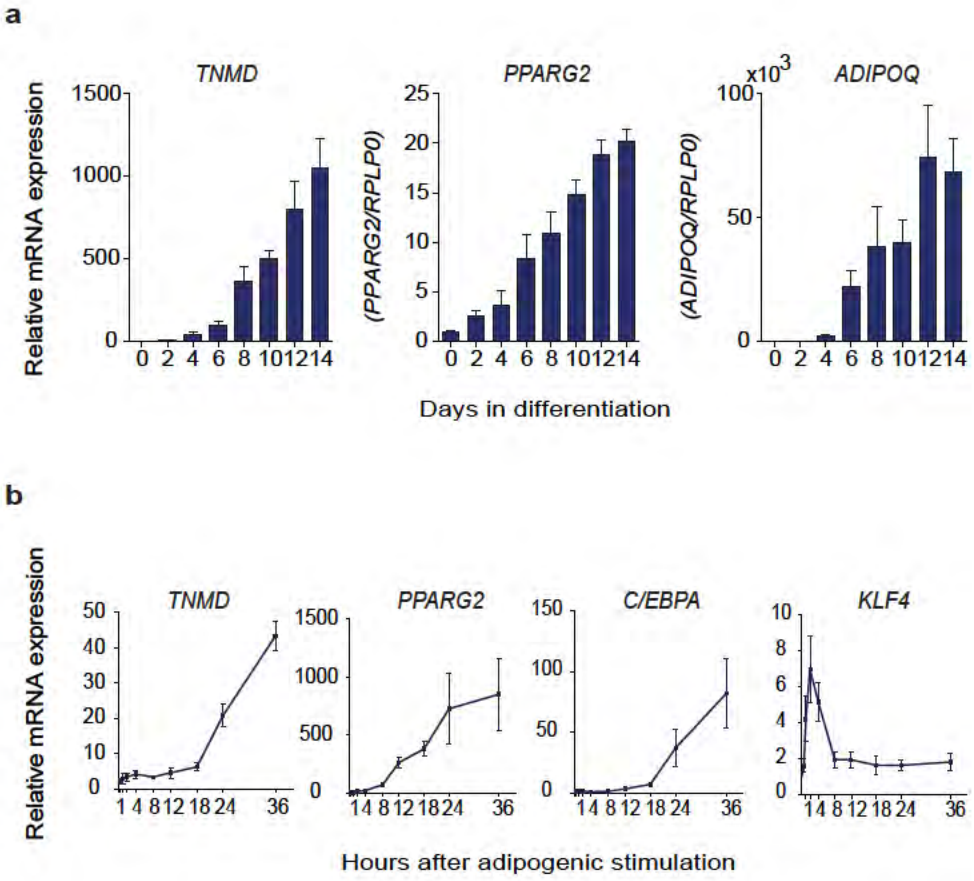


Figure 2.3: *TNMD* expression increases during human adipocyte differentiation

SGBS cells were induced to differentiate, and; RNA was isolated at the indicated time points of differentiation. **(a)** Quantitative PCR analysis for *TNMD, PPARG2, ADIPOQ* expression at various time points during differentiation of SGBS human adipocytes (mean±SEM; n=3-4, *p<0.05, **p<0.01, ***p<0.001, by Student's t-test). **(b)** *TNMD, PPARG2, C/EBPA* and *KLF4* expression at the indicated early time points (mean±SEM; n=3, Student's t-test).

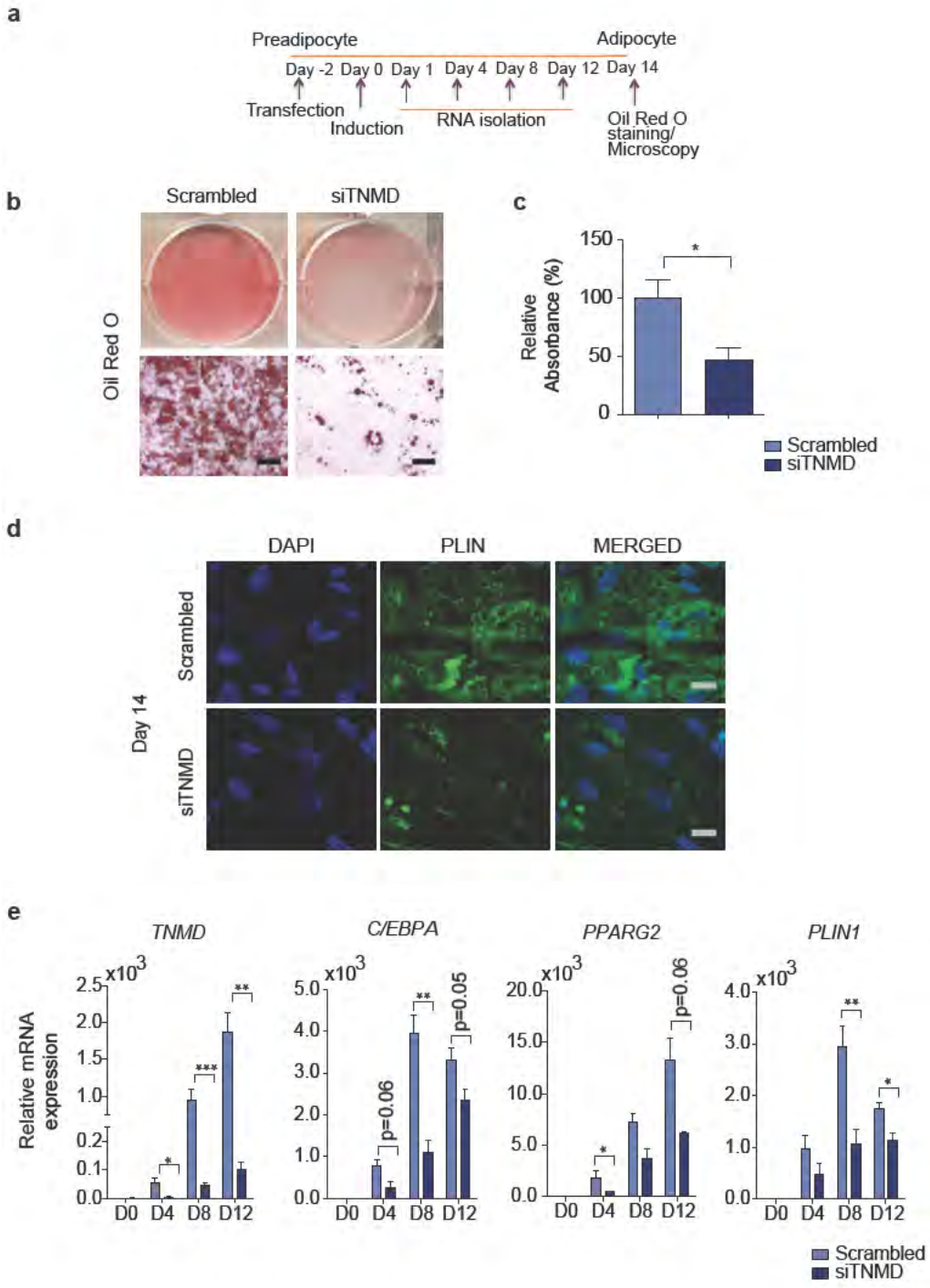
TNMD is required for human adipogenesis

Several studies have demonstrated that adipose tissue expansion in obesity is promoted by adipogenesis in addition to adipocyte hypertrophy in both mice and humans (133, 220, 323-325). Because *TNMD* had an expression profile that was similar to genes that are critically involved in adipocyte differentiation and its expression is increased in obese adipose tissue, we hypothesized that TNMD might be involved in human adipocyte differentiation. To test this hypothesis, *TNMD* was silenced in SGBS preadipocytes two days prior to adipogenic stimulation (Figure 2.4, a). Remarkably, silencing of *TNMD* significantly attenuated differentiation as demonstrated by substantially decreased accumulation of neutral lipids measured by Oil Red O staining at day 14 (Figure 2.4, b,c). Analysis of mature SGBS adipocytes also revealed that preadipocytes lacking TNMD had fewer lipid droplets and lacked a differentiated adipocyte morphology as assessed by perilipin (PLIN) staining (Figure 2.4, d). Thus, TNMD is required for human adipocyte differentiation. To further investigate whether TNMD is required for adipogenic gene expression during differentiation, total mRNA was isolated at different time points of differentiation (Fig. 2a), and expression of adipogenic markers such as *PLIN1*, *C/EBPA*, and *PPARG2* were analyzed by qRT-PCR. Indeed, expression of these genes was significantly blunted at most of the majority of time points in *TNMD* silenced cells compared with cells that had been transfected with scrambled siRNA (Figure 2.4, e).

Figure 2.4: siRNA mediated silencing of *TNMD* attenuates differentiation in human adipocytes

SGBS preadipocytes were transfected with siRNA two days prior to adipogenesis induction. Total RNA isolation and Oil Red O staining were performed on the stated days of differentiation. **(a)** Schematic demonstration of experimental method. **(b,c)** Adipocytes were differentiated for 14 days in culture and stained with Oil Red O. **(b)** Representative images (Scale bars represent 100 µm). **(c)** Quantification of Oil Red O staining (mean±SEM; n=4 *p<0.05, Student's t-test). **(d)** Immunofluorescent staining of mature adipocytes with PLIN (green) and DAPI (blue) (Scale bars represent 20 µm). **(e)** Quantitative PCR analysis of *TNMD, C/EBPA, PPARG2 and PLIN1* expression at days 0, 4, 8 and 12 of adipocyte differentiation in SGBS cells (mean±SEM; n=3 *p<0.05, **p<0.01, ***p<0.001).

Figure 2.4


a
Preadipocyte
Adipocyte
Day -2 Day 0 Day 1 Day 4 Day 8 Day 12 Day 14
Transfection
Induction
RNA isolation
Oil Red O staining/ Microscopy
b
Scrambled
siTNMD
Oil Red O
c
Relative Absorbance (%)
150
100
50
0
*
Scrambled
siTNMD
d
DAPI
PLIN
MERGED
Day 14
Scrambled
siTNMD
e
TNMD
C/EBPA
PPARG2
PLIN1
Relative mRNA expression
x10^3
D0 D4 D8 D12
p=0.06
p=0.05
p=0.06
Scrambled
siTNMD

Although *TNMD* expression is low in murine adipocytes, TNMD depletion in 3T3-L1 preadipocytes revealed that TNMD is also required for mouse adipocyte differentiation and plays a similar role to regulate adipogenic gene expression as in SGBS cells (Figure 2.5, a,b). These data suggest that even though expression profiles are different between mature human and mouse adipocytes, the requirement of TNMD for differentiation is conserved between the two species.

Figure 2.5

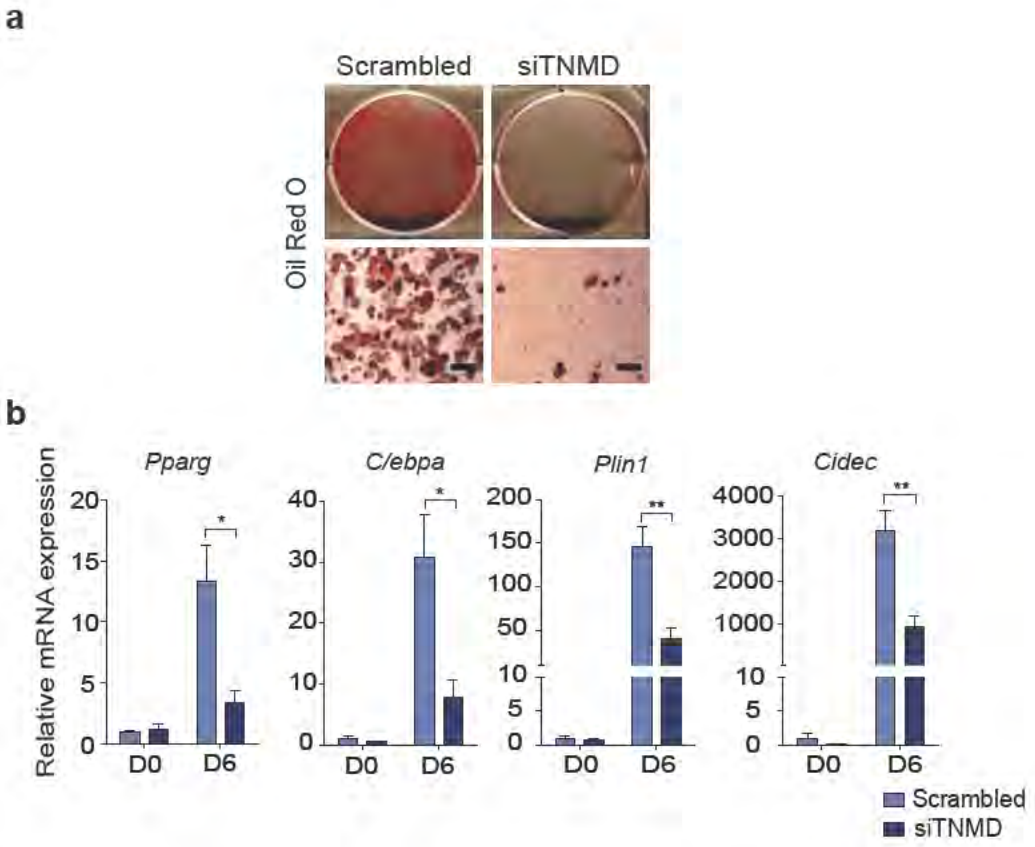


Figure 2.5: Knockdown of *TNMD* diminished differentiation in 3T3-L1 preadipocytes

3T3-L1 adipocytes were treated with scrambled or TNMD siRNA and differentiated for 6 days **(a)** Representative Oil -Rred O staining in 3T3-L1 cells on day 6 of differentiation. Whole well pictures (upper panel) and light microscopy image. Scale bar represents 100um **(b)** Quantitative PCR analysis for *Pparg*, *C/ebpa, Plin1* and *Cidec* on day 0 and day 6 of differentiation (n=3, *p<0.05, **p<0.01, ***p<0.001, Student's t-test).

Unlike the mouse cell line 3T3-L1, human primary adipocytes do not undergo clonal expansion (326, 327). SGBS cells also did not display any increase in cell number during the first five days of differentiation (Figure 2.6, a), suggesting that clonal expansion also does not occur in these cells. Therefore, full confluence is important for SGBS cells to differentiate. To assess whether TNMD affected cell death in SGBS preadipocytes, an MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cell viability assay was performed to assess viability in *TNMD*-silenced SGBS cells. The results show that siRNA-mediated silencing of *TNMD* did not affect the viability in these cells (Figure 2.6, b).

Figure 2.6

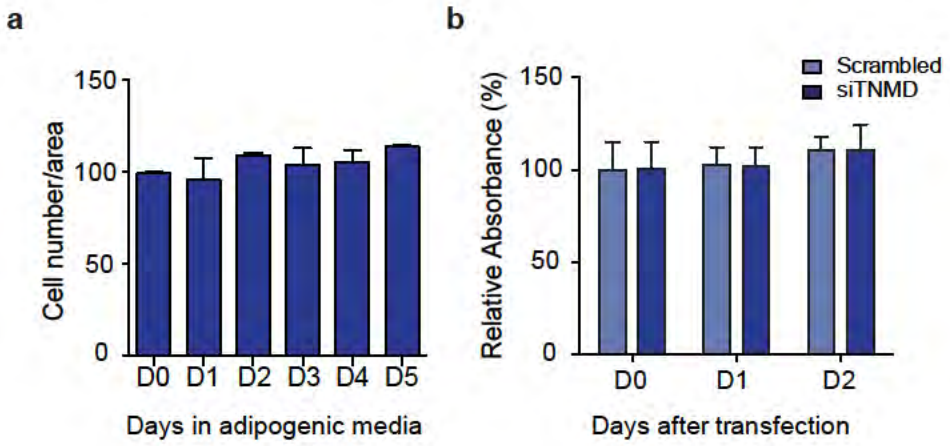


Figure 2.6: siRNA knockdown does not affect viability in SGBS cells viability

(a) Equal numbers of SGBS preadipocytes were seeded in wells three days before adipogenic media was added to the cells. Cells were fixed and stained with Hoechst. The number of nuclei per area was calculated at the indicated days using Image J. cells (mean cell number ± SEM; n=3). **(b)** MTT cell viability assay (mean± SEM; n=3)

Because the expression of adipogenic transcription factors increases dramatically even during the first day of differentiation, we asked whether *TNMD* silencing diminishes the early induction of these transcription factors as well as their target genes. Thus, a DNA microarray analysis was performed in SGBS cells that had been transfected with either scrambled *or* TNMD siRNA at day 1 of differentiation. The induction of many adipogenic genes was significantly diminished after TNMD depletion (Figure 2.7, a, b). Importantly, in the absence of TNMD, stimulation of both the early transcription factor CCAAT/enhancer-binding protein beta (C/EBPβ) as well as late transcription factors C/EBPα and PPARγ were diminished (Figure 2.8, a, b).

Figure 2.7

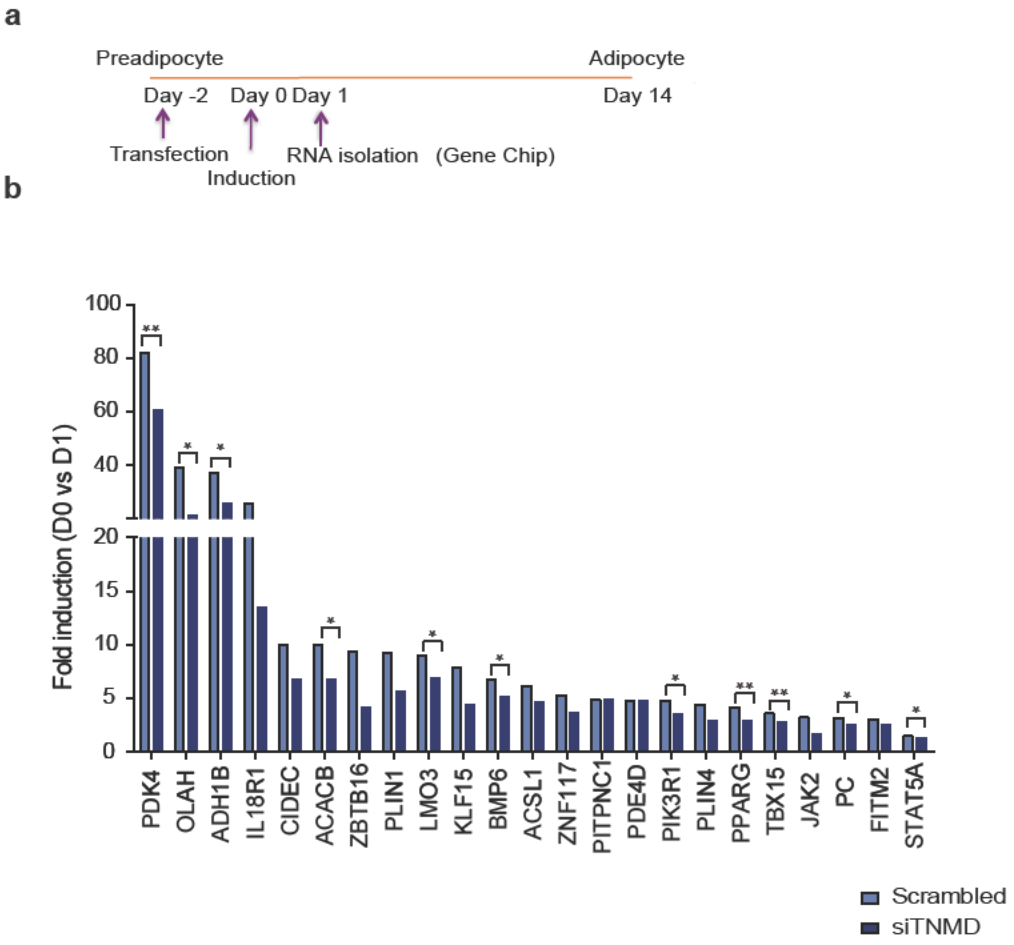


Figure 2.7: Microarray analysis in SGBS cells transfected with scrambled and siTNMD

(a) Experimental design of microarray analysis. SGBS cells were transfected with either scrambled or siTNMD. Total RNA was isolated before and one day after adipogenic induction **(b)** Fold change in various genes upon adipogenic stimuli and comparison among cells transfected with scrambled siRNA and siTNMD (n=3, *p<0.05, **p<0.01, ***p<0.001, Student's t-test).

Figure 2.8

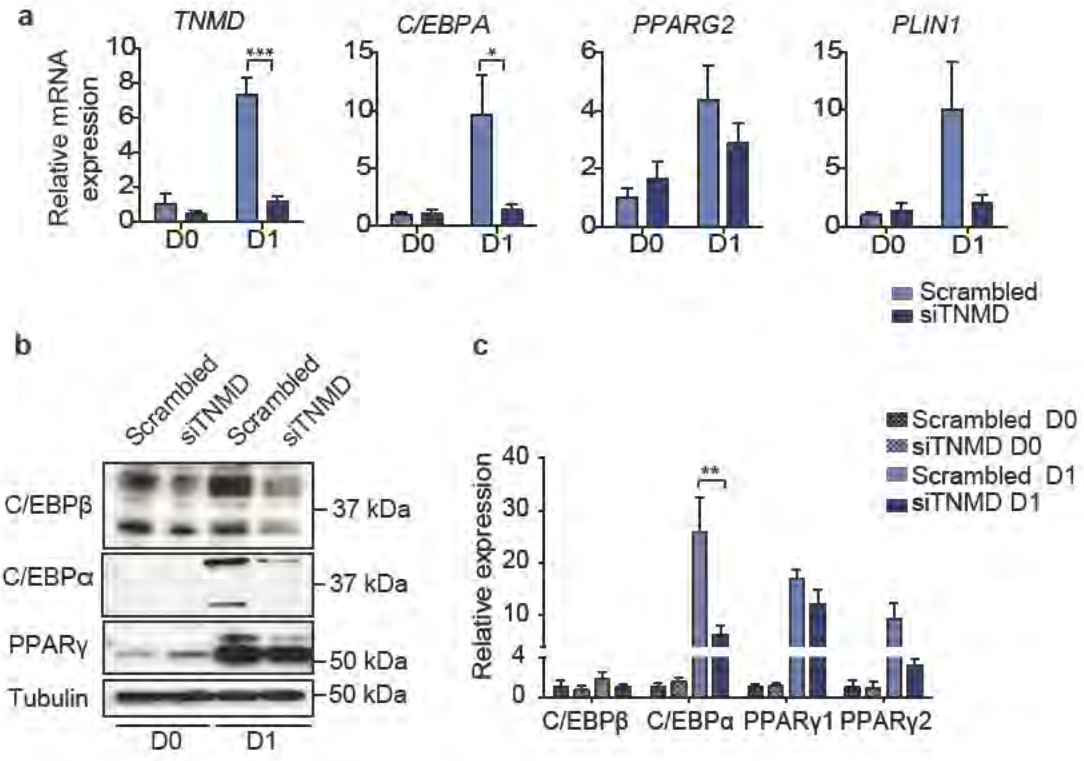


Figure 2.8: Induction of early adipogenic factors was diminished by TNMD silencing

SGBS preadipocytes were transfected with siRNA two days prior to adipogenesis induction. Total RNA and protein isolation were performed on day 0 and day 1 of differentiation. **(a)** Quantitative PCR analysis of *TNMD, C/EBPA, PPARG2 and PLIN1* expression at day 0 and day 1 after stimulation (mean±SEM; n=3 *p<0.05, **p<0.01, ***p<0.001, by Student's t-test). **(b)** Immunoblots for C/EBPβ, C/EBPα, PPARγ and tubulin at day 0 and day 1 of adipogenic differentiation (n=3 for C/EBPβ and PPARγ, n=6 for C/EBPα) **(c)** Quantification of Western blot analysis (n=3-6, *p<0.05, **p<0.01, ***p<0.001, Student's t-test).

The C-terminal domain of TNMD is similar to the secreted and functional portion of its homologous protein Chondromodulin 1 (CHM1) (275), and studies have suggested that the C-terminal domain of TNMD is functionally active (285). We sought to understand whether the effect of TNMD to regulate adipogenesis is cell autonomous. Thus, SGBS cells treated with siTNMD were mixed with non–transfected cells in equal numbers to determine whether wild-type TNMD rescues the adipogenic defect in TNMD-silenced cells via a paracrine mechanism. After adipogenic stimulation, we observed that Oil Red -O staining was diminished by approximately 50% when control cells were co-cultured with transfected cells, suggesting that the adipogenic effect of TNMD is cell autonomous *in vitro* (Figure 2.9, a).

Figure 2.9

a

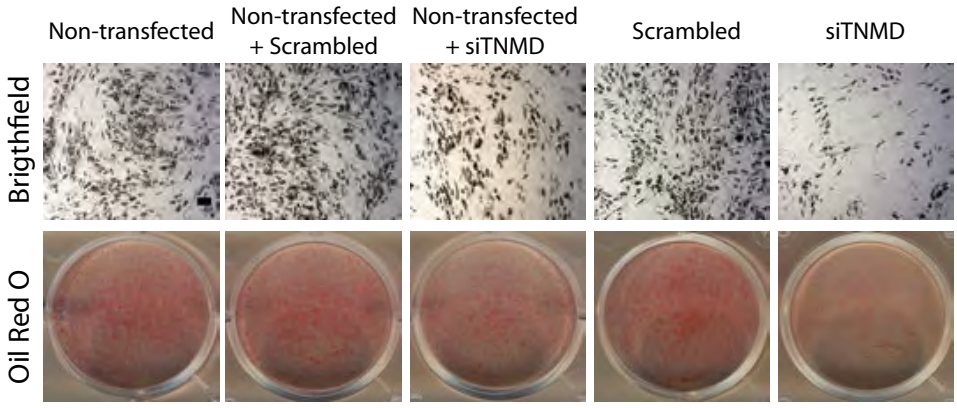


Figure 2.9: Co-culture of non-transfected SGBS preadipocytes with TNMD transfected cells. (a) SGBS preadipocytes were transfected with either Scrambled or siTNMD. 24 hours after transfection, cells were trypsinized and seeded in equal numbers from stated groups in a different well. 2 days after transfection, cells were stimulated with adipogenic cocktail. Adipogenesis was analyzed by Oil Red O staining and microscopy. The data are representative of two independent experiments. The scale bar represents 100um.

Increased eWAT expansion in *Tnmd* overexpressing mice

To gain a better understanding of the role of *Tnmd* in adipose tissue, we generated transgenic mice that overexpress mouse *Tnmd* under the adipose tissue-specific Adiponectin promoter (Figure 2.10, a). A transgenic line that exhibited significant overexpression of *Tnmd* in inguinal (iWAT), epididymal (eWAT) and brown adipose tissue (BAT) were utilized in these experiments. qRT-PCR and Western Blot analysis demonstrated that *Tnmd* expression was specific to adipose tissue in these mice (Figure 2.10, b,c).

Given that *TNMD* expression is increased during obesity in human adipose tissue (290), weight gain was assessed in the *Tnmd* transgenic mice, and no weight difference was observed compared with littermate controls after both chow and high fat diet (HFD) fed conditions (Figure 2.11, a). However, a significant increase in eWAT weight was observed in HFD-fed but not in chow-fed transgenic animals (Figure 2.11, b). The transgenic mice also displayed a concomitant decrease in liver weight after HFD, suggesting that the *Tnmd* transgenic mice may have enhanced adipose tissue storage capacity, which may attenuate lipid deposition in non-adipose tissues. Transgenic mice also had significantly smaller brown adipose tissue when compared with controls. However, no significant difference was detected in inguinal and axillary white adipose tissue weights after either feeding regimens (Figure 2.11, b).

Figure 2.10

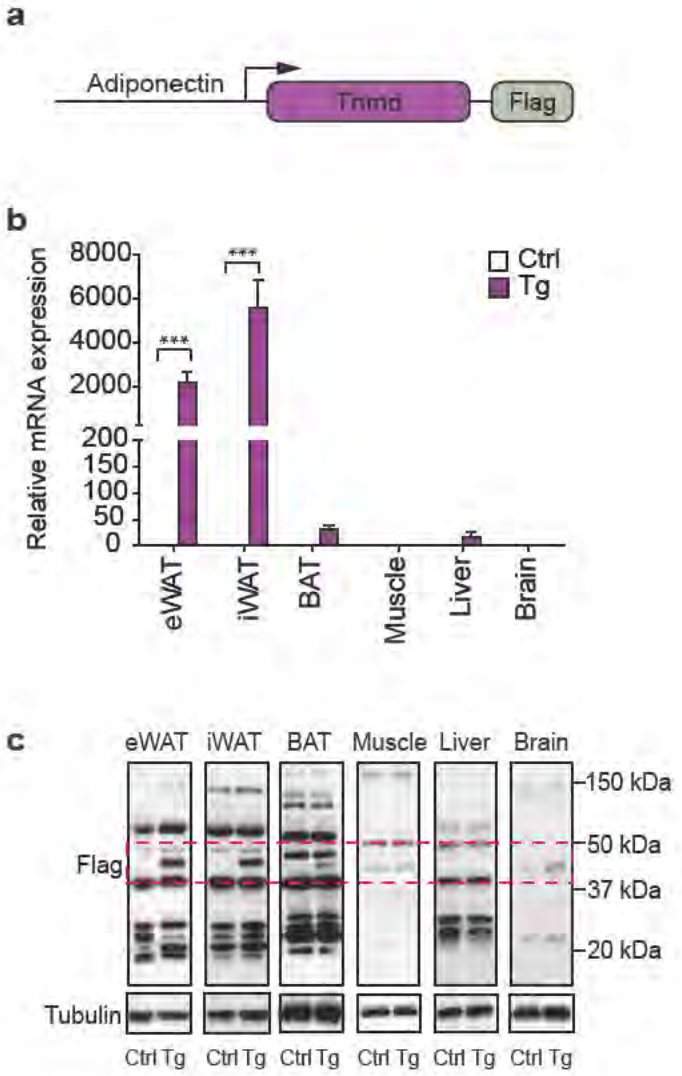


Figure 2.10: Specific *Tnmd* expression in adipose tissue
(a) Schematic of transgene construct used to generate adipose tissue specific *Tnmd* transgenic mice. **(b)** Quantitative PCR analysis of Tnmd in eWAT, iWAT, BAT, Liver, Muscle and Brain isolated after 16 weeks of HFD in male control (Ctrl) and transgenic (Tg) mice (mean± SEM; n=5-11; *p<0.05, **p<0.01, ***p<0.001, Student's t-test). **(c)** Representative Flag and Tubulin immunoblots in iWAT, eWAT, BAT, Muscle, Liver and Brain lysates that were isolated from male control (Ctrl) and transgenic mice (Tg) fed a HFD for 16 weeks.

Figure 2.11

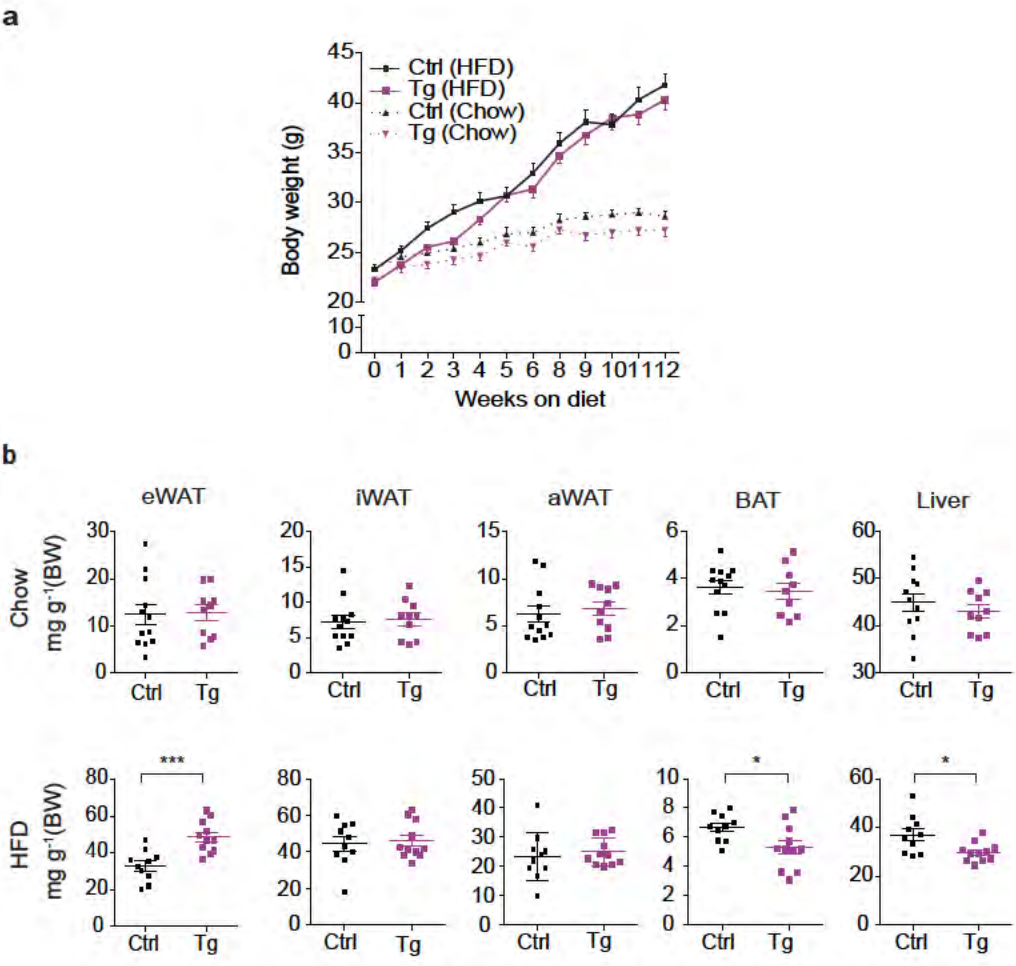


Figure 2.11 Specific *Tnmd* expression in adipose tissue enhances HFD-induced epididymal fat expansion.
(a) 6-week-old male control (Ctrl) and transgenic (Tg) mice were fed chow or HFD for 12 weeks. Body weights were measured at the indicated time points (HFD, n=11 (control), n=11 (transgenic), Chow n=11 (control) and n=7 (transgenic)). **(b)** Epididymal, inguinal and axillary white adipose tissue, brown adipose tissue and liver weights were measured in control (Ctrl) or transgenic (Tg) male mice after 16 weeks of chow or HFD and normalized to overall body weight (mean±SEM; Chow n=12 (control), n=9 (transgenic), HFD n=10 (control), n=11 (transgenic), *p<0.05, **p<0.01, ***p<0.001, by Student's t-test).

TNMD promotes healthy visceral adipose tissue expansion

Because *Tnmd* transgenic mice had larger eWAT (Figure 2.11, b) and because TNMD is required for adipogenesis (Figure 2.4), white adipose tissue cell size was assessed to understand whether the significant increase in eWAT weight in *Tnmd* transgenic mice was due to hypertrophy or hyperplasia. Though the eWAT pads were larger, no significant difference in adipocyte size was observed in eWAT or iWAT depots of HFD-fed *Tnmd* transgenic mice (Figure 2.12, a,b), and this was also the case after a short term (4-week) HFD (Figure 2.12, c,d,e). These results suggest that the increased eWAT weight (Figure 2.11, b) was caused by an increase in adipocyte number rather than hypertrophy.

We thus investigated whether preadipocyte proliferation was affected in TNMD transgenic mice. Recently, Jeffery et al. demonstrated that there is a significant increase in preadipocyte proliferation during first week of HFD in visceral adipose tissue (220). To assess preadipocyte proliferation *in vivo*, we treated control and *Tnmd* transgenic mice with BrdU for one week in their drinking water and concurrently fed them HFD for 6 days (Figure 2.13, a). Preadipocytes were then isolated, seeded on coverslips and media selected for 24 hours followed by immunostaining for BrdU and Pref1 as a preadipocyte marker. Remarkably, a significant increase in BrdU incorporation was observed in preadipocytes that were isolated from the eWAT of *Tnmd* transgenic mice (Figure 2.13, b,c). These data suggest that TNMD promotes eWAT expansion by enhancing preadipocyte proliferation in response to HFD.

Figure 2.12: Assesment of iWAT and eWAT depots at 4 and 16 weeks of HFD in control and transgenic mice

6 week-s old male control (Ctrl) and *Tnmd* transgenic (Tg) animals were fed HFD for either 4 or 6 weeks **(a)** Representative H&E images of eWAT and iWAT (n=5 (control), n=9 (transgenic), Scale bars represent 100 µm). **(b)** Adipocyte size was analyzed using Adiposoft software. At least 4 different areas per mouse were analyzed, and the average adipocyte size in each group was calculated (mean±SEM; n=5 (control), n=9 (transgenic), Student's t-test). **(c)** Epididymal and inguinal white adipose tissue weights measured after 4 weeks of HFD in control (Ctrl) and transgenic (Tg) animals and normalized to total body weights (mean±SEM; n=8 (control), n=5 (transgenic), Student's t-test) **(d)** Histologic analysis of eWAT and liver of from transgenic (Tg) and control (Ctrl) mice after 4 weeks. Scale bars represent 100 µm **(e)** Adipocyte size was analyzed and avarage average adipocyte size was calculated for each group (mean±SEM; n=8 (control), n=5 (transgenic), Student's t-test).

Figure 2.12

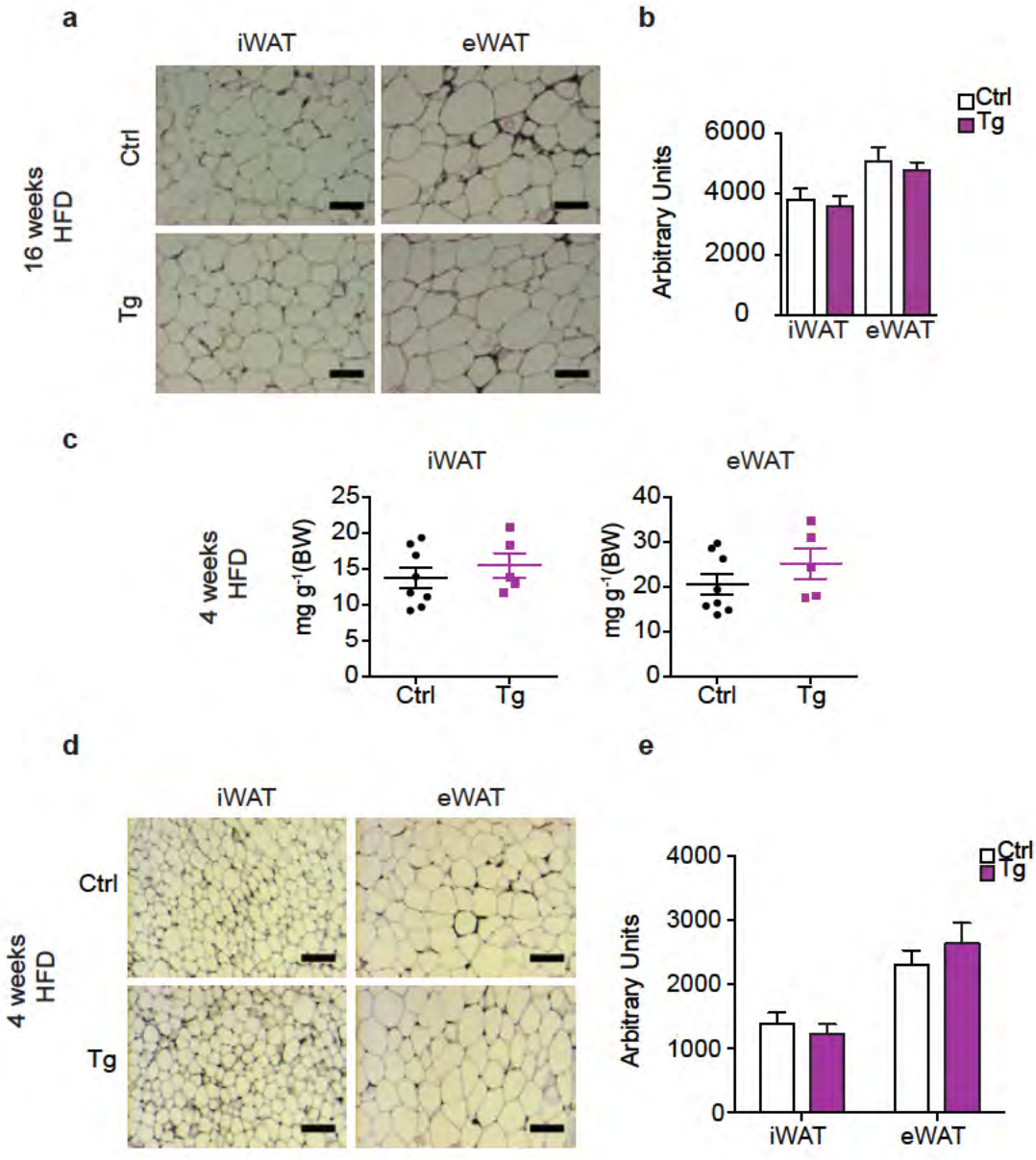

a
iWAT
eWAT
16 weeks HFD
Ctrl
Tg
b
Ctrl
Tg
Arbitrary Units
6000
4000
2000
0
iWAT
eWAT
c
iWAT
eWAT
4 weeks HFD
mg g-1(BW)
25
20
15
10
5
0
40
30
20
10
0
Ctrl
Tg
d
iWAT
eWAT
4 weeks HFD
Ctrl
Tg
e
Ctrl
Tg
Arbitrary Units
4000
3000
2000
1000
0
iWAT
eWAT

Figure 2.13

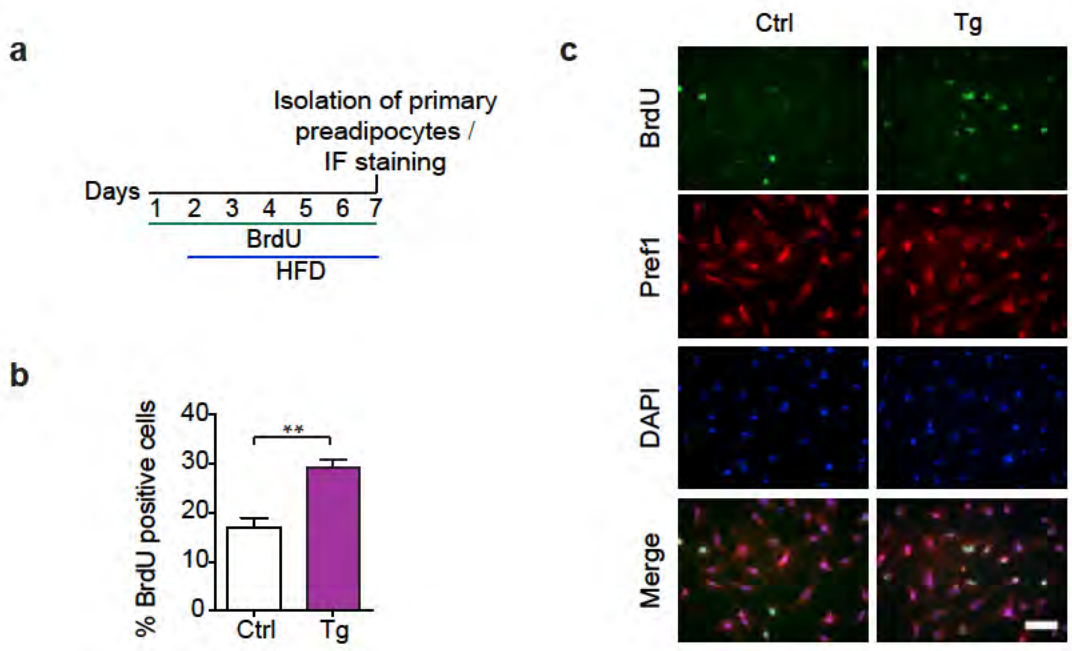
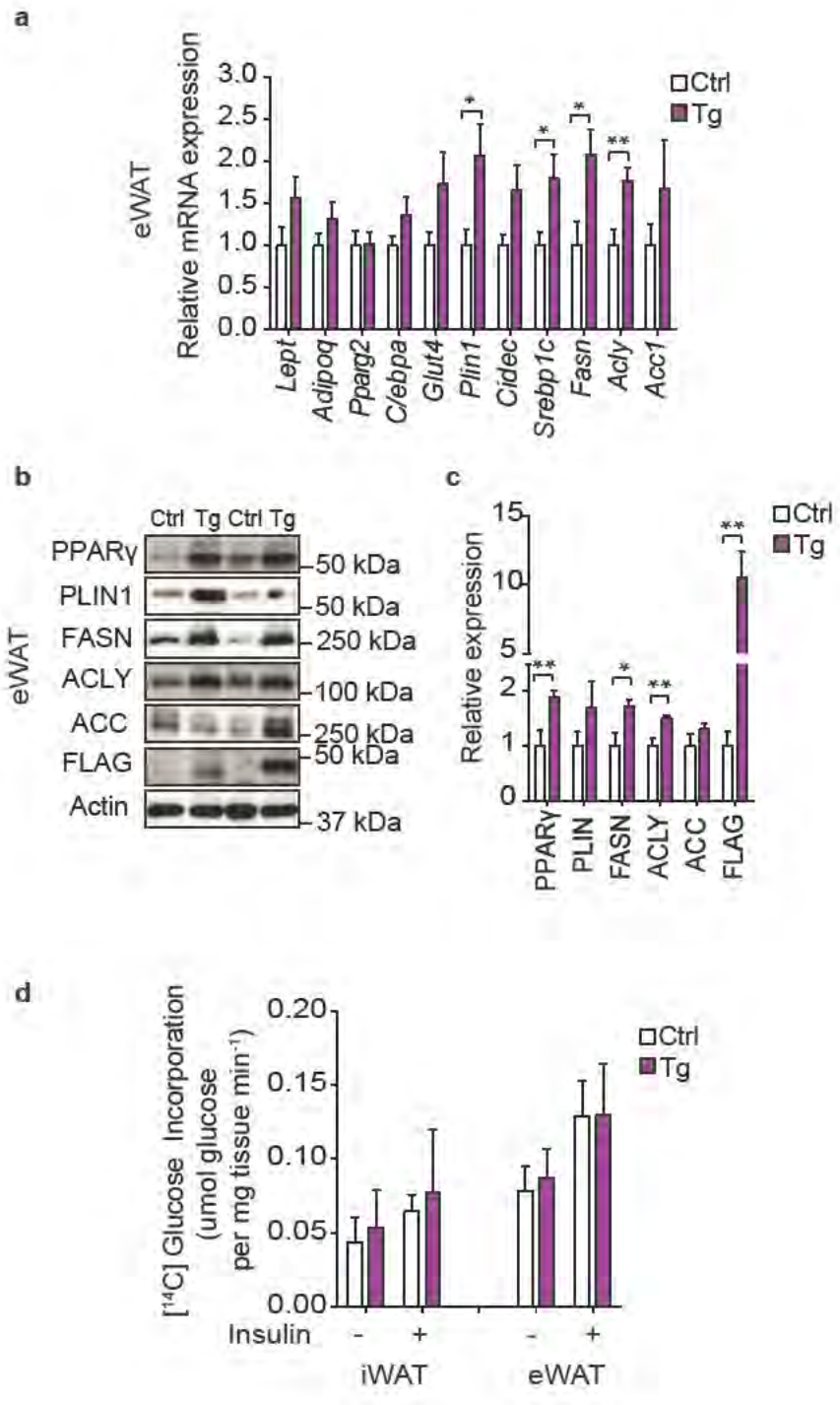


Figure 2.13: TNMD elevated preadipocyte proliferation upon high fat diet feeding.
(a) Experimental set-up for *in vivo* BrdU labeling in primary preadipocytes. 6 week-old female mice were treated with 0.8 mg ml^{-1} BrdU in water containing 1% sucrose. **(b)** Preadipocytes were isolated, seeded on coverslips, and stained with BrdU and Pref1 antibodies. At least three different areas were quantified in each slide, and the percentage of BrdU-positive cells was calculated (mean±SEM; n=5 for both group, *p<0.05, **p<0.01 by Student's t-test). **(c)** Representative immunofluorescence pictures. Scale bars represent 50 µm.

Consistent with these results, mRNA levels encoding adipogenic and lipogenic genes *Plin1*, sterol regulatory element-binding protein 1c (*Srebp1c*), fatty acid synthase (*Fasn*) and ATP-citrate lyase (*Acly*) were significantly increased, and the protein levels of PPARγ, PLIN, FASN, ACLY were 1.5-1.9 fold increased in *Tnmd* transgenic mouse visceral adipose tissue (Figure 2.14, a-c). These observations suggest that overexpressing *Tnmd* in visceral adipose tissue increases its storage capacity by both increasing adipocyte number and upregulating lipogenesis. Although no difference in [^{14}C]-Glucose incorporation into triglyceride was observed in adipose tissue explants that had been isolated from chow-fed control and TNMD transgenic animals (Figure 2.14, d), such assays performed *in vitro* are quite artificial and may not represent physiological conditions.

Figure 2.14: Increased lipogenic gene expression in *Tnmd* transgenic mice **(a)** Quantitative PCR (mean±SEM; n=9 (control), n=11 (transgenic) and **(b,c)** Western blot analysis of adipogenic and lipogenic genes in eWAT of HFD-fed male animals and quantification of blots (mean±SEM; n=5 (control), n=9 (transgenic) *p<0.05, **p<0.01 by Student's t-test) **(d)** *Ex vivo* lipogenesis analysis in adipose depots in transgenic and control mice. Insulin stimulated [14C]-Glucose incorporation into triglyceride was measured in adipose tissue explants that had been isolated from 12 weeks chow fed mice (mean±SEM; n=4, Student's t-test).

Figure 2.14


a
eWAT
Relative mRNA expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
Tg
*
*
*
**
Lept
Adipoq
Pparg2
C/ebpa
Glut4
Plin1
Cidec
Srebp1c
Fasn
Acly
Acc1
b
Ctrl Tg Ctrl Tg
PPARγ
PLIN1
FASN
ACLY
ACC
FLAG
Actin
50 kDa
50 kDa
250 kDa
100 kDa
250 kDa
50 kDa
37 kDa
eWAT
c
Relative expression
15
10
5
2
1
0
Ctrl
Tg
**
*
**
**
PPARγ
PLIN
FASN
ACLY
ACC
FLAG
d
[14C] Glucose Incorporation (umol glucose per mg tissue min-1)
0.20
0.15
0.10
0.05
0.00
Ctrl
Tg
Insulin
-
+
-
+
iWAT
eWAT

Tnmd mice displayed reduced inflammation and tissue fibrosis in eWAT

A previous report suggested that TNMD has antiangiogenic properties in cultured endothelial cells (285). However, *TNMD* knockout mice did not display any obvious vascular abnormalities (277). These previous studies suggested that TNMD might have effects on blood vessel density or extracellular matrix composition. Blood vessel morphology and density was assessed in HFD-fed control and TNMD transgenic mice; however, no differences were observed (Figure 2.15, a). Furthermore, endothelial cell marker gene expression was unaltered in TNMD transgenic adipose tissue as assessed by qRT-PCR (Figure 2.15, b).

Interestingly, a previous report characterizing TNMD-null mice noted disorganized collagen fibrils (277); thus, we hypothesized that TNMD might be involved in ECM processing. Trichrome staining was performed in control and TNMD transgenic mice to investigate whether TNMD is involved in regulating extracellular matrix and tissue fibrosis in eWAT. Remarkably, whereas abundant blue collagen staining was observed in eWAT of control mice, collagen accumulation was clearly decreased in transgenic mice, even in the inflamed areas (Figure 2.15, c). Moreover, gene expression analysis of whole adipose tissue revealed that genes encoding ECM proteins such as *Col1a1, Mmp12, Mmp14* as well as genes involved in TGFβ signaling were significantly decreased in transgenic eWAT compared to control animals suggesting that TNMD might be involved in regulating extracellular matrix composition (Figure 2.15, d). Therefore,

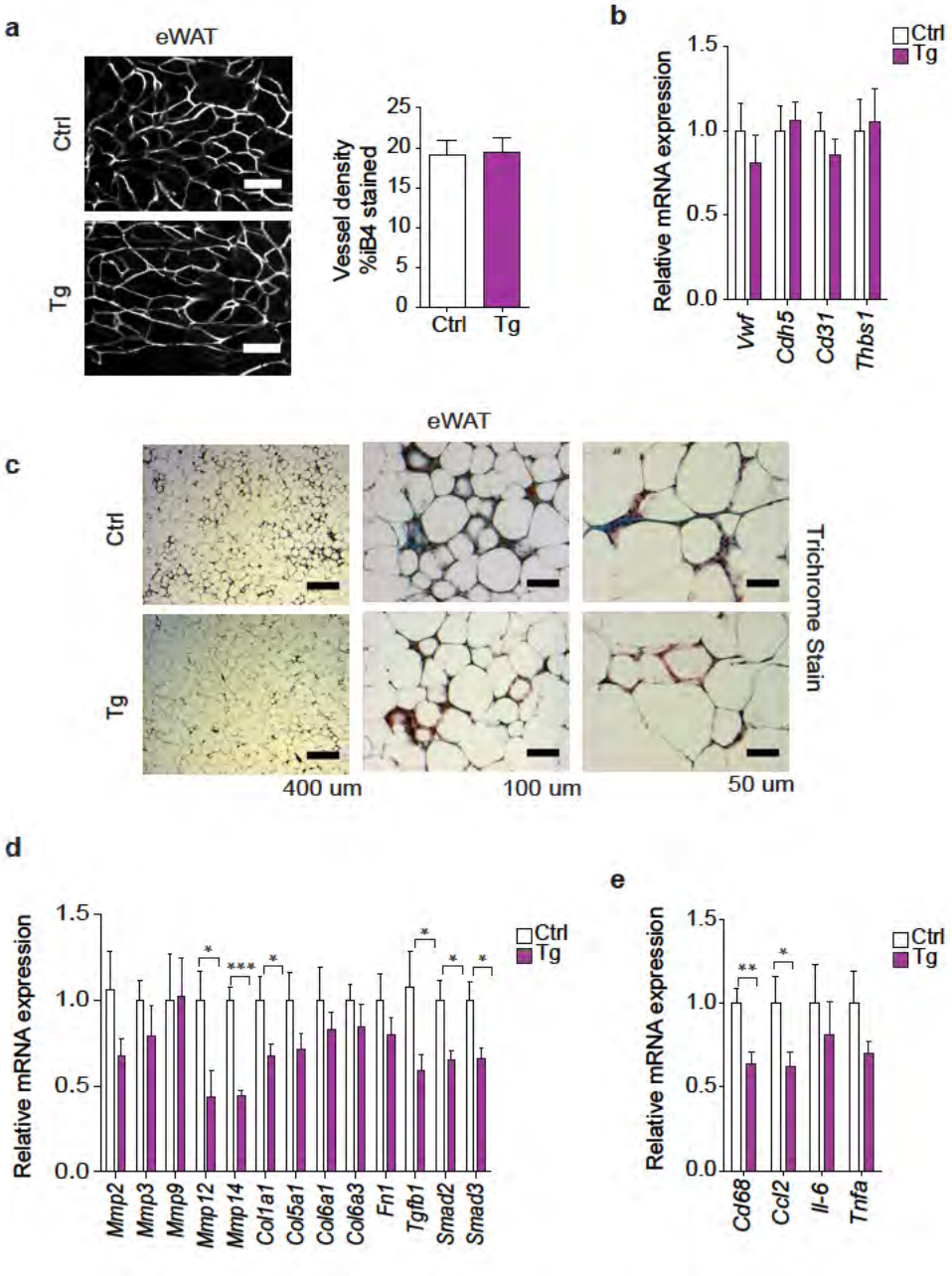
TNMD may promote healthy visceral adipose tissue expansion through a direct interaction with ECM proteins and regulation of ECM remodeling.

Many studies have demonstrated that inflammation, immune cell infiltration and expansion occur in visceral adipose tissue during obesity, which is associated with metabolic dysfunction and insulin resistance (64, 244, 245, 300, 312, 328, 329). After prolonged HFD, immune cell infiltration was increased in eWAT of control mice, whereas transgenic mice displayed fewer crown-like structures by histological analysis (Figure 2.12, a; Figure 2.15, c). Furthermore, qRT-PCR results demonstrated that macrophage marker *Cd68* and macrophage-derived cytokines such as monocyte chemotactic protein 1 (*Ccl2)* were downregulated by 40% in transgenic mice (Figure 2.15, e). Thus, *Tnmd* overexpression also promotes adipose tissue integrity by preventing adipose tissue inflammation in obesity.

Figure 2.15: Decreased inflammation and collagen expression in eWAT of *Tnmd* transgenic mice

(a) Isolectin staining and vessel density quantification in eWAT from control and transgenic animals fed with HFD for 8 weeks of HFD (mean±SEM; n=5 for both control and transgenics, *p<0.05, **p<0.01, by Student's t-test.) Scale bars represent 100 µm. **(b)** Quantitative PCR for angiogenesis markers in eWAT from 16 weeks HFD fed control and transgenic mice (mean±SEM; n=9 (control), n=11 (transgenic), *p<0.05, **p<0.01, by Student's t-test). **(c)** Representative images of Trichrome staining in eWAT from control (Ctrl) and transgenic (Tg) mice that had been fed HFD for 16 weeks. Scale bars represent 400 µm (left panel), 100 µm (middle panel), 50 µm (right panel). **(d)** Quantitative PCR of ECM and TGFβ signaling genes in eWAT from HFD-fed animals (mean±SEM; n=9 (control), n=10 (transgenic), *p<0.05, **p<0.01, by Student's t-test). **(e)** qRT-PCR analysis of inflammatory genes in eWAT from HFD-fed animals (mean±SEM; n=9 (control), n=11 (transgenic), *p<0.05, **p<0.01, by Student's t-test).

Figure 2.15

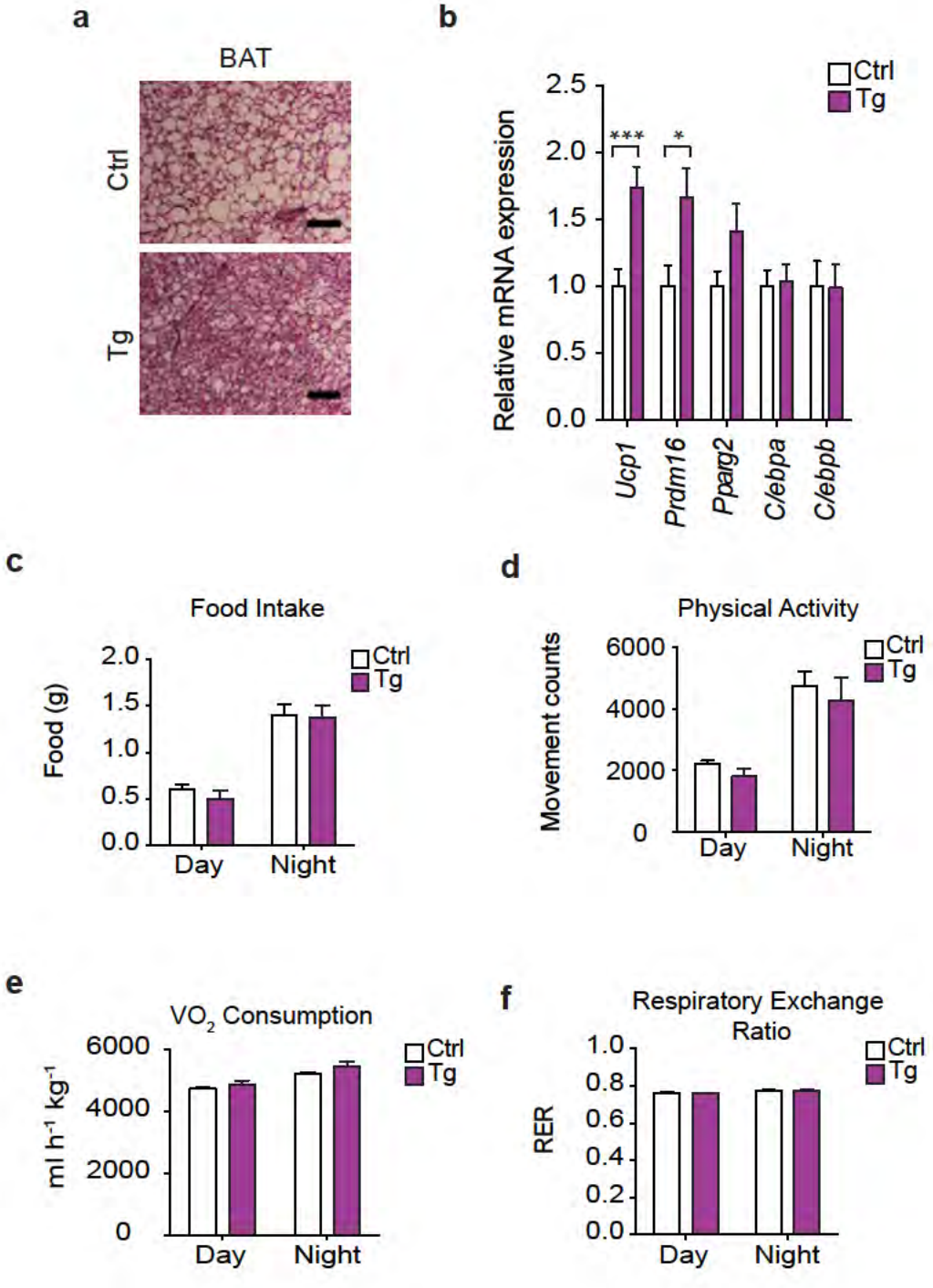

a
eWAT
Ctrl
Tg
Vessel density
%iB4 stained
25
20
15
10
5
0
Ctrl
Tg
b
Ctrl
Tg
Relative mRNA expression
1.5
1.0
0.5
0.0
Vwf
Cdh5
Cd31
Thbs1
c
eWAT
Ctrl
Tg
Trichrome Stain
400 um
100 um
50 um
d
Relative mRNA expression
1.5
1.0
0.5
0.0
Ctrl
Tg
*
***
*
*
*
*
Mmp2
Mmp3
Mmp9
Mmp12
Mmp14
Col1a1
Col5a1
Col6a1
Col6a3
Fn1
Tgfb1
Smad2
Smad3
e
Relative mRNA expression
1.5
1.0
0.5
0.0
Ctrl
Tg
**
*
Cd68
Ccl2
Il-6
Tnfa

TNMD inhibited lipid deposition in liver and BAT

Consistent with our observation that the *Tnmd* transgenic mouse BAT depot was smaller, we observed fewer lipid droplets in histological samples of BAT histological sections (Figure 2.16, a). Importantly, brown adipocyte markers such as uncoupling protein 1 (*Ucp1*) and PR domain containing 16 (*Prdm16*) were significantly increased by nearly 2-fold in transgenic mouse BAT compared with that from controls upon HFD challenge (Figure 2.16, b). These results suggested that *Tnmd* overexpression in BAT may also promote BAT maintenance of mitochondrial fatty acid oxidation during HFD and contribute to overall beneficial metabolism. However, metabolic cage analysis in HFD-fed TNMD transgenic and control mice with similar food intake and physical activity revealed no significant differences in respiratory exchange ratio (RER) or VO_2 consumption (Figure 2.16, c-f).

Figure 2.16: Lipid deposition in BAT was decreased in transgenic mice
a) Representative histological analysis of BAT from HFD-fed animals. Scale bars represent 100 µm. **(b)** Quantitative PCR analysis of *Ucp1, Prdm16, Pparg2, C/ebpa* and *C/ebpb* in BAT of HFD-fed animals (mean±SEM; n=9 (control), n=11 (transgenic), *p<0.05, **p<0.01, ***p<0.001, by Student's t-test). **(c-f)** Metabolic cage analysis was performed in male mice that had been fed with HFD for 12 weeks. **(c)** Average food intake per mouse during the day (7am-7pm) and night (7pm-7am) over 3 days (mean±SEM; n=8 (control), n=5 (transgenic)). **(d)** Physical activity was calculated as average of total movements per mouse during the day (7am-7pm) and night (7pm-7am) over 3 days (mean±SEM; n=8 (control), n=5 (transgenic)). Average **(e)** VO_2 consumption and **(f)** Respiratory Exchange Ratio during the day (7am-7pm) and night (7pm-7am) over 3 days (mean±SEM; n=8 (control), n=5 (transgenic)).

Figure 2.16

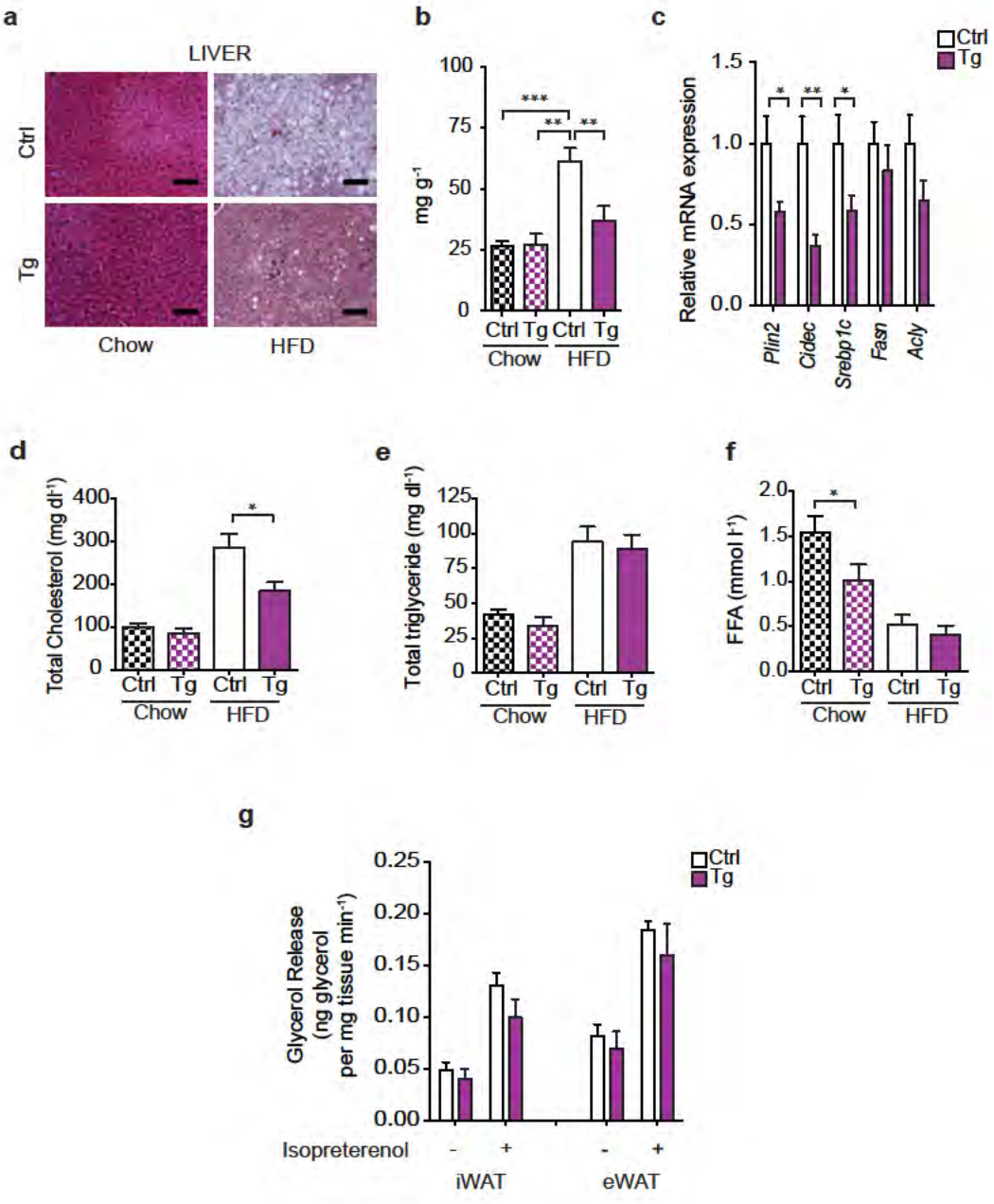

a
BAT
Ctrl
Tg
b
Relative mRNA expression
Ctrl
Tg
***
*
2.5
2.0
1.5
1.0
0.5
0.0
Ucp1
Prdm16
Pparg2
C/ebpa
C/ebpb
c
Food Intake
Food (g)
Ctrl
Tg
2.0
1.5
1.0
0.5
0.0
Day
Night
d
Physical Activity
Movement counts
Ctrl
Tg
6000
4000
2000
0
Day
Night
e
VO2 Consumption
ml h-1 kg-1
Ctrl
Tg
6000
4000
2000
0
Day
Night
f
Respiratory Exchange Ratio
RER
Ctrl
Tg
1.0
0.8
0.6
0.4
0.2
0.0
Day
Night

A significant decrease in liver weight was also observed in *Tnmd* transgenic mice compared with their control littermates (Figure 2.11, b). Therefore, we assessed hepatic lipid content in HFD-fed mice by both histological and triglyceride (TG) analysis. Although hepatic TG content increased by 2-fold in control animals fed HFD, strikingly, *Tnmd* transgenic animals displayed no HFD-induced increase in hepatic TG content and instead displayed a 60% reduction compared with control HFD-fed littermates (Figure 2.17, a,b). Consistent with the decreased lipid content of livers in the HFD-challenged transgenic animals, hepatic genes involved in lipid droplet formation that are associated with fatty liver such as perilipin 2 (*Plin2)* and cell death-inducing DFFA-like effector c (*Cidec)* were significantly downregulated in HFD-fed *Tnmd* transgenic mice (Figure 2.17, c).

Assessment of serum metabolic parameters demonstrated that HFD-fed *Tnmd* transgenic animals had significantly less total plasma cholesterol levels. However, no differences were detected in triglyceride levels on chow or HFD (Figure 2.17, d, e). Furthermore, serum free fatty acid levels were significantly reduced in chow-fed *Tnmd* transgenic animals (Figure 2.17, f). However, though serum fatty acid levels can be a reflection of adipose tissue lipolysis, *ex vivo* lipolysis was not affected basally or after isoproterenol stimulation in *Tnmd* transgenic mice compared with littermate controls (Figure 2.17, g). These data suggest that adipose tissue *Tnmd* overexpression may have a paracrine effect to regulate serum lipid concentrations.

Figure 2.17: Peripheral lipid deposition was reduced in *Tnmd* transgenic mice. (a,b) Livers were isolated from animals fed chow or HFD for 16 weeks. **(a)** H&E staining of liver tissue. Scale bars represent 100 µm. **(b)** Triglyceride content was measured (n=6-8) **(c)** Gene expression in livers of HFD-fed animals (n=9-11) **(d)** Total cholesterol (n=9-12) **(e)** Total triglyceride (n=7-12) and **(f)** Free fatty acid levels (n=7-11, mean±SEM; *p<0.05, **p<0.01, ***p<0.001, by Student's t-test) were assessed in plasma samples from control (Ctrl) and transgenic (Tg) animals after 16 weeks of chow (dashed columns) or HFD (solid columns). **(g)** *Ex vivo* lipolysis in adipose tissue explants from 6-8 week-s old mice were fed with a chow diet for 12 weeks. Adipose tissue explants were isolated, and lipolysis was stimulated with 10µM isopreterenol for 2 hours (mean±SEM; n=6-7, Student's t-test).

Figure 2.17


a
LIVER
Ctrl
Tg
Chow
HFD
b
mg g-1
100
75
50
25
0
***
**
**
Ctrl Tg Ctrl Tg
Chow HFD
c
Ctrl
Tg
Relative mRNA expression
1.5
1.0
0.5
0.0
*
**
*
Plin2
Cidec
Srebp1c
Fasn
Acly
d
Total Cholesterol (mg dl-1)
400
300
200
100
0
*
Ctrl Tg Ctrl Tg
Chow HFD
e
Total triglyceride (mg dl-1)
125
100
75
50
25
0
Ctrl Tg Ctrl Tg
Chow HFD
f
FFA (mmol l-1)
2.0
1.5
1.0
0.5
0.0
*
Ctrl Tg Ctrl Tg
Chow HFD
g
Glycerol Release
(ng glycerol
per mg tissue min-1)
0.25
0.20
0.15
0.10
0.05
0.00
Ctrl
Tg
Isopreterenol
-
+
-
+
iWAT
eWAT

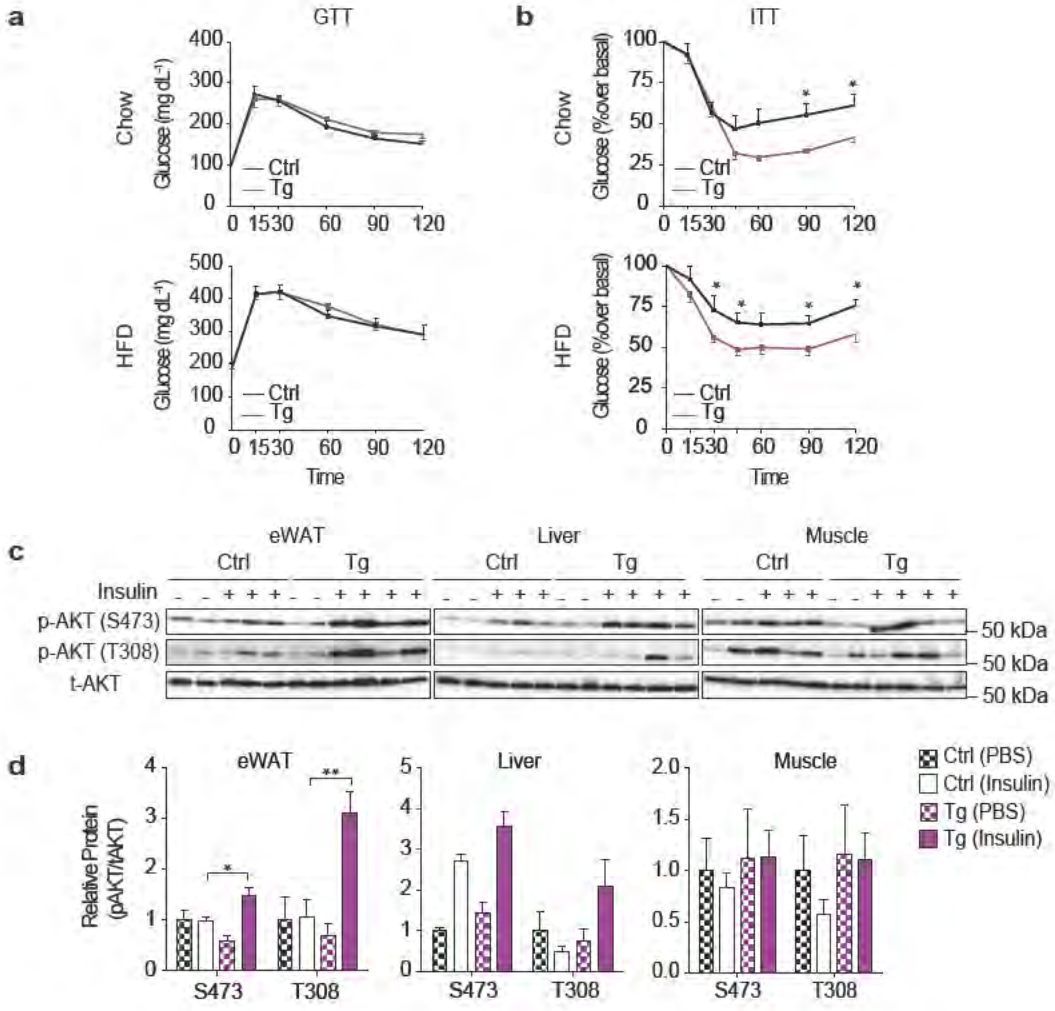
Improved insulin signaling in *Tnmd*-overexpressing mice

Because *Tnmd* transgenic mice displayed decreased adipose tissue inflammation and less liver triglyceride content, we hypothesized that these animals might demonstrate improved insulin sensitivity. Although *Tnmd* transgenic mice displayed unaltered glucose tolerance in an i.p. glucose tolerance test on chow or HFD (Figure 2.18, a), they were remarkably more insulin responsive than their control littermates on both chow and HFD during an insulin tolerance test (Figure 2.18, b). These data suggest that adipose-specific overexpression of TNMD improves systemic insulin sensitivity.

In order to determine whether insulin sensitivity signaling was also enhanced in insulin-responsive tissues in *Tnmd* transgenic mice at the molecular level in insulin-responsive tissues, HFD-fed control and *Tnmd* transgenic mice were injected with either insulin or PBS. Then 15 minutes later, muscle, liver and eWAT were isolated from animals, and analyzed for phospho-Akt levels were analyzed as an indicator of insulin signaling. Akt phosphorylation at both S473 and T308 sites as detected by specific anti-phosphoserine and anti-phosphothreonine antibodies was significantly increased in *Tnmd* transgenic mouse eWAT compared with littermate controls. Moreover, a trend towards an increased Akt phosphorylation at these sites was also observed in both liver and muscle, suggesting that in addition to improving adipose tissue insulin sensitivity, adipose TMND might also improve insulin responsiveness in other peripheral tissues (Figure 2.18, c, d).

Figure 2.18: TNMD increased Akt phosphorylation in eWAT and improved systemic insulin sensitivity.
(a) Glucose tolerance and **(b)** insulin tolerance tests were performed in male control (Ctrl) or transgenic (Tg) mice after 12 weeks of chow or HFD (mean±SEM; Chow n=12 (control), n=10 (transgenic), HFD, n=11 (control), n=13 (transgenic), *p<0.05, **p<0.01, ***p<0.001, by Student's t-test) **(c,d)** Male control (Ctrl) and transgenic (Tg) mice that were fed with HFD for 12 weeks. Mice were fasted for 4 hours and tissues were harvested 15 minutes after PBS or insulin injection. **(c)** Western blot analysis and relative protein expression levels for p-Akt (S473), p-Akt (T308) and Total Akt in eWAT, liver, and muscle tissue lysates. **(d)** Densitometry analysis of **(c)** (mean±SEM; n=4 (control and transgenic, PBS), n=5 (control, insulin), n=7 (transgenic, insulin), *p<0.05, **p<0.01, ***p<0.001, by Student's t-test).

Figure 2.18


a
GTT
Chow
HFD
Glucose (mg dL-1)
Ctrl
Tg
Time
b
ITT
Glucose (%over basal)
c
eWAT
Liver
Muscle
Ctrl
Tg
Insulin
p-AKT (S473)
p-AKT (T308)
t-AKT
50 kDa
d
Relative Protein (pAKT/tAKT)
S473
T308
Ctrl (PBS)
Ctrl (Insulin)
Tg (PBS)
Tg (Insulin)

To further assess what tissues contributed to the enhanced insulin sensitivity in *Tnmd* transgenic mice, hyperinsulinemic-euglycemic clamps were performed. The clamp data demonstrated that although glucose levels during the clamp were similar, *Tnmd* transgenic animals had higher insulin sensitivity compared with control littermates as assessed by glucose infusion rate (Figure 2.19, a, b). This difference could be attributed to improved hepatic insulin sensitivity because *Tnmd* transgenic animals displayed decreased hepatic glucose production (Figure 2.19, c), whereas no differences were observed in tissue-specific glucose uptake (Figure 2.19, d, f). Collectively, these data suggest that *Tnmd* overexpression in murine adipose tissue improves systemic insulin sensitivity.

Figure 2.19

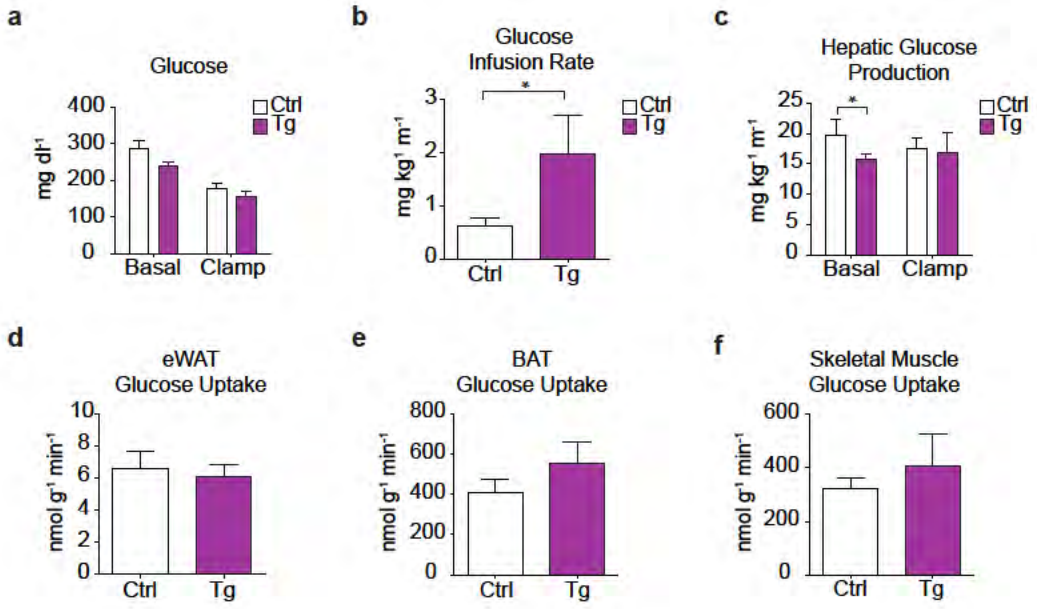


Figure 2.19: TNMD improved glucose homeostasis in mice with diet induced obesity.
Male control (Ctrl) and transgenic (Tg) mice were fed HFD for 12 weeks. Mice were fasted for 4 hours before the clamp study. **(a)** Glucose levels before and during the hyperinsulinemic-euglycemic clamp (mean±SEM; n=8 (control), n=4 (transgenic)). **(b)** Glucose infusion rate **(c)** Hepatic glucose production measured during clamp (mean±SEM; n=8 (control), n=4 (transgenic), *p<0.05, **p<0.01, ***p<0.001, by Student's t-test). Glucose uptake by **(d)** Epididymal adipose tissue, **(e)** Brown adipose tissue (interscapular), **(f)** Skeletal muscle (gastrocnemius) (mean±SEM; n=8 (control), n=4 (transgenic)).

Discussion

Identification of factors that modulate pathological consequences of obesity is a vital step towards development of novel therapeutic approaches to treatment of insulin resistance and other aspects of metabolic syndrome. In this study, we demonstrated that insulin resistant obese individuals have increased *TNMD* expression compared with insulin sensitive controls in the omental adipose depot, even when matched for BMI (Figure 2.1). Previous studies demonstrated that *TNMD* is highly expressed in human adipose tissue and its expression is further increased in obese conditions (290). Furthermore, genetic studies that investigated an association between SNPs and various metabolic markers suggested a potential role for this gene in adipose tissue in disease (296). Though no difference in *TNMD* expression was observed in insulin resistant versus insulin sensitive patients in subcutaneous adipose tissue depots, previous studies that analyzed subcutaneous adipose tissue biopsies demonstrated a correlation of *TNMD* expression, fasting serum insulin levels and HOMA-IR in obese patients (290, 318). *TNMD* expression is predominantly in adipocytes compared to the SVF (Figure 2.2), suggesting that the expression differences observed in insulin sensitive versus insulin resistant individuals mainly result from expression changes in primary adipocytes.

A key finding in this study is that TNMD is required for differentiation of human SGBS and mouse 3T3-L1 preadipocytes (Figure. 2.4, Figure 2.5).

Because *TNMD* expression is readily stimulated after adipogenic induction and continues to increase during differentiation in human cells, we supposed hypothesized that the absence of *TNMD* in preadipocytes would impair early differentiation. Consistent with this notion, when *TNMD* expression was silenced, expression of transcription factors involved in adipogenesis such as *C/EBPA* and *PPARG* was were decreased at early time points of differentiation (Figure 2.8), and adipogenesis was impaired. While the exact function of TNMD in this process is unclear, TNMD is hereby identified as a novel required factor in early stages of adipocyte differentiation. *TNMD* expression is actually decreased two days after induction of 3T3-L1 cell adipogenesis. Therefore, unlike the case in human cells, its expression is actually higher in mouse preadipocytes when compared to mature adipocytes. However, silencing of TNMD prior to induction was sufficient to inhibit the adipogenesis of these mouse preadipocytes, indicating that TNMD is required for the initiation of adipogenesis in both species.

Because it appeared TNMD had a potential role in human cells and human patients but was expressed at low levels in mouse adipose tissue, we sought to address the role of TNMD in adipose tissue by generating a mouse model with higher increased adipose tissue specific *Tnmd* expression. Such adipose-specific *Tnmd* expression in transgenic mice increased adipogenic and lipogenic gene as well as protein expression in eWAT upon high fat diet feeding. Notably, PPARγ, one of the major regulators of glucose metabolism and adipocyte function (197, 206, 330), was significantly upregulated in the eWAT of

HFD-fed *Tnmd* transgenic mice compared with their control littermates (Figure 2.14). Because activation of PPARγ has many beneficial effects on adipose tissue including improving lipid metabolism and decreasing serum FFAs (331-333), it can be inferred that TNMD strongly influences adipogenesis through by regulating PPARγ expression *in vivo.* Consistent with these findings *in vivo*, silencing *TNMD* significantly reduced adipocyte differentiation and adipogenic gene expression including *PPARG* in human and mouse preadipocytes *in vitro*.

Many recent studies have described that the ECM has a critical function to regulate adipose tissue homeostasis in obesity (226, 229, 233, 334, 335), and exogenous signals regulated by ECM proteins are involved in determining the fate of mesenchymal progenitor cells. For example, ECM stiffness and composition regulates Wnt and TGFβ signaling, which has an inhibitory or, in some cases, a stimulatory role in adipogenesis (114). Adipose tissue ECM also provides a suitable environment for changes in cell shape during adipogenesis and cell expansion (336-339). In this study, we noted a reduction inreduced collagen staining in *Tnmd* transgenic adipose tissue, and collagen and matrix metalloproteinase gene expression was also significantly reduced in *Tnmd* overexpressing adipose tissue (Figure 2.15). These results suggest that TNMD may promote beneficial adipogenesis at least in part by modulating properties of the ECM in adipose tissue.

In addition to enhancing insulin signaling in eWAT, beneficial effects of TMND in liver was also observed, which enhanced systemic insulin sensitivity

(Figure 2.18, 2.19). *Tnmd* transgenic mice had reduced hepatic lipid deposition and were more responsive to insulin even in lean, chow-fed conditions. Furthermore, hepatic glucose production was reduced in TNMD transgenic animals (Figure 2.17). However, it is not established whether these peripheral effects are due to the improved lipid sequestration and decreased inflammation in eWAT. Future studies will investigate the mechanisms by which adipose TNMD function might cause beneficial signaling to other tissues.

Tnmd transgenic mice had smaller BAT with fewer lipid droplets after HFD (Figure 2.16). Furthermore, thermogenic genes *Ucp1, Prdm16* and *Pparg* were upregulated in transgenic animals compared with their littermate controls (Figure 2.16, b). PRDM1*6* and PPARγ are not only involved in BAT differentiation but also in BAT maintenance along with UCP1 (98, 340). However, though TNMD was overexpressed in BAT in this model, the thermogenic capacity of the animals as assessed by RER remained unchanged (Figure 2.16). Future studies will utilize thermoneutral or cold challenge conditions to assess whether TNMD has a role in regulation of BAT energy expenditure.

TNMD expression was higher in the human insulin resistant cohort in our study, yet paradoxically mice overexpressing *Tnmd* in adipose tissue displayed improved insulin sensitivity (Figure 2.18, 2.19). In humans, omental fat from insulin resistant subjects displays more inflammation and larger adipocytes compared with BMI-matched insulin sensitive subjects (300). It is possible that the increased inflammation in these insulin resistant subjects arises from

enhanced adipocyte death in the insulin resistant omental fat. Indeed, a correlation between cell death, insulin resistance, and adipocyte size has previously been reported (341). Thus, we speculate that TNMD might be increased in insulin resistant omental fat to increase adipocyte replenishment in these conditions (212, 325). Indeed, increased preadipocyte proliferation was observed in TNMD transgenic animals (Figure, 2.13), which could promote healthy tissue expansion. It is also possible that either the omental fat microenvironment or endocrine signals associated with the insulin resistant state can contribute to *TNMD* overexpression, perhaps as a compensatory mechanism to promote adipogenesis and increase insulin responsiveness. Finally, most of the insulin resistant human subjects in our cohort had been treated for different amounts of time with various medications including thiazolidinediones before bariatric surgery and tissue collection. Thus, we cannot rule out the possibility that *TNMD* expression was increased in insulin resistant subjects as a result of these medications.

In summary, our study reveals that the gene *TNMD*, which is highly expressed in human adipose tissue, encodes a protective adipose tissue factor that promotes preadipocyte proliferation, adipogenesis, adipose tissue health, and insulin responsiveness *in vivo*. The data presented herein support the hypothesis that TNMD contributes to beneficial visceral adipose tissue expansion that protects against metabolic dysfunction. Because adipose TNMD expression

improves insulin sensitivity systemically, it may have potential as a therapeutic target to protect metabolic homeostasis in obesity.

CHAPTER III

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The consequences of obesity related to adipose tissue dysfunction include insulin resistance and impaired glucose homeostasis. Therefore, identifying novel mechanisms that regulate adipose tissue function in the context of obesity is crucial to further our understanding of the pathogenesis of metabolic diseases. The work presented here suggests that TNMD enhances adipose tissue function. In a genome wide microarray study, TNMD was elevated in omental adipose tissue of insulin resistant patients compared with insulin sensitive subjects of similar BMI (Figure 2.1 a, c-e). Furthermore, previous studies demonstrated that TNMD expression is very high in human adipose depots and further increases upon obesity (290). Therefore, I aimed to address two following three questions:

1. Is TNMD required for adipocyte differentiation?
2. Does TNMD promote adipose tissue expansion and enhance adipose tissue function *in vivo*?

To address the first question, I utilized both human (SGBS) and mouse (3T3-L1) cells. Using siRNA-mediated knockdown, I demonstrated that TNMD is required for adipocyte differentiation in both human and mouse cells as assessed by multiple biochemical methods. My results indicated that TNMD is also necessary for the induction of adipogenic gene expression during adipogenesis (Figure 2.4, 2.5). Gene chip analysis and qRT-PCR validation studies revealed that TNMD depletion attenuated adipogenic transcription factor and target gene induction in the first day of differentiation (Figure 2.7, 2.8).

To address the second question, I generated a transgenic mouse model that expresses a C-terminal FLAG tag on TNMD specifically in adipose tissue using the adiponectin promoter. Specific and significant expression of TNMD was determined in both white and brown adipose depots (Figure 2.10). For metabolic characterization, I fed transgenic and control mice either chow (normal) or HFD and measured weight gain over time. No difference in body weight was observed between transgenic and control littermates (Figure 2.11). However, eWAT weight was significantly increased in the transgenic animals fed with HFD (Figure 2.12). I then assessed whether the increase in eWAT weight was mediated by adipocyte hyperplasia or hypertrophy by measuring cell size. No cell size difference was observed in WAT depots isolated from transgenic and control animals (Figure 2.12). These data suggested that eWAT expansion driven by TNMD overexpression occurs via hyperplasia but not hypertrophy. Lipogenic gene expression was consistently increased in the eWAT of transgenic animals suggesting an increased capacity for fat sequestration (Figure 2.13). Furthermore, histology analysis indicated a decrease in immune cell content within eWAT of transgenic mice compared with control animals (Figure 2. 12). Consistent with these results, using trichrome staining, reduced collagen accumulation was also observed in the eWAT of transgenic mice (Figure 2.15). By measuring triglyceride levels and analyzing histology samples, I also assessed lipid deposition in peripheral tissues such as liver observed reduced hepatic steatosis in TNMD transgenic animals compared with controls (Figure

2.17). These results suggested that increased capacity of eWAT for lipid storage prevented free fatty acid deposition in non-adipose tissues. Systemic insulin resistance was also improved in the TNMD overexpressing mice (Figure 2. 18, 2.19).

In conclusion, the data in this thesis suggests a role for TNMD in adipocyte differentiation and healthy adipose tissue expansion during obesity. Furthermore, these results provide a novel model where eWAT is expanded, yet systemic insulin responsiveness is improved, which supports the notion that adipose tissue health matters more than adipose tissue mass in the context of obesity.

Figure 3.1

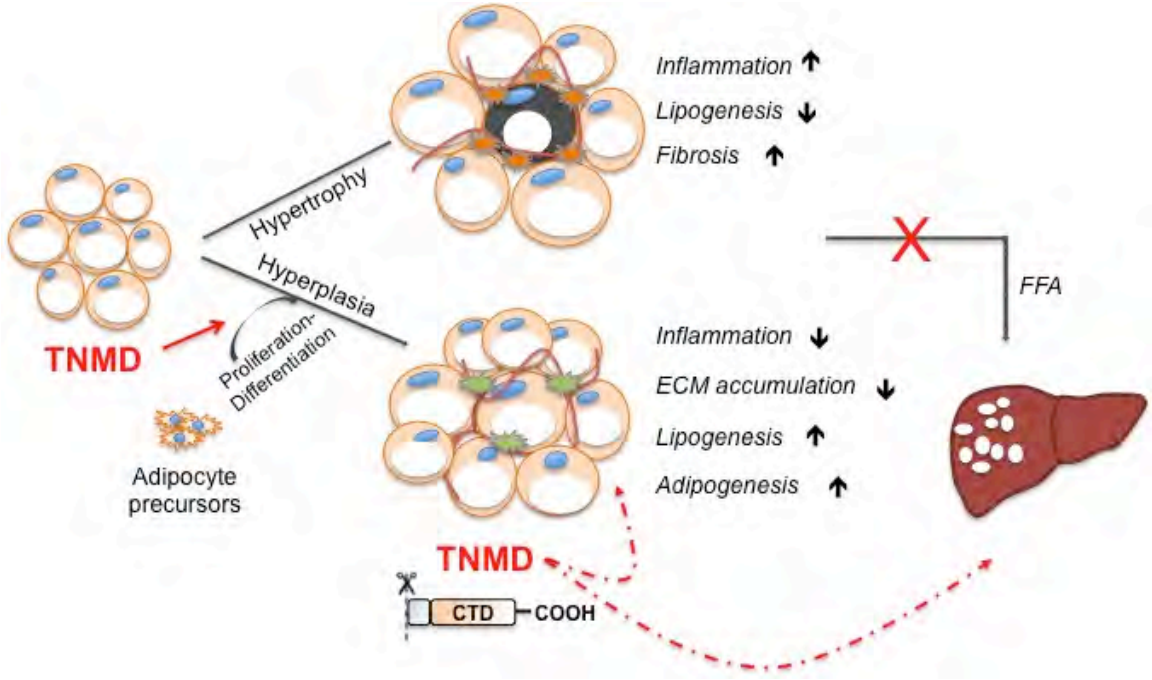


Figure 3.1: A model of TNMD action in adipose tissue

TNMD as a novel regulator of adipogenesis and beneficial adipose tissue expansion

To identify novel molecules that are involved in promoting insulin resistance or conversely maintaining insulin sensitivity in obese subjects, our laboratory performed a microarray analysis using whole adipose tissue samples isolated from subcutaneous and visceral adipose depots of morbidly obese patients who underwent bariatric surgery. Using the same approach, our laboratory previously showed that the inflammatory gene signature, especially inflammatory chemokines such as CCL2, CCL3 and CCL4 were significantly increased in omental but not subcutaneous fat of insulin resistant patients compared with insulin sensitive subjects. These data demonstrated that independent of BMI, macrophage infiltration and chemokine secretion is associated with insulin resistance in humans (300). In the same study, many genes were differentially expressed between insulin resistant and insulin sensitive patients. Here, I focused on TNMD because 1) it was significantly upregulated in insulin resistant omental adipose tissue, which was verified by qRT-PCR and Western Blot analysis (Figure 2.1), and 2) TNMD expression was limited almost exclusively to primary adipocytes in human adipose tissue (Figure 2.2). Adipose tissue immune cell content rapidly increases during obesity and is involved in the pathogenesis of insulin resistance (223, 244). Therefore, it was important to rule out that the increase in TMND expression that was observed in whole adipose tissue samples was not due to an increase in the immune cell

population. The predominant expression of TNMD in primary adipocytes suggested that TNMD is concentrated in this cell type. Further experiments are needed to compare TNMD expression in isolated primary adipocytes from insulin resistant and insulin sensitive subjects.

Surprisingly, no difference in TNMD expression between insulin resistant and insulin sensitive subjects was observed in subcutaneous adipose tissue, which could be attributed to differences in gene expression among white adipose depots (89). Interestingly, TNMD is responsive to glucocorticoid treatment *in vitro* (342). Even though systemic cortisol levels do not change in obese subjects, increased cortisone levels have been demonstrated in obese visceral adipose tissue. 1-beta hydroxysteroid dehydrogenase 1 (HSD1), a reductase enzyme that generates cortisol *in vivo*, has higher activity in stromal cells isolated from omental adipose tissue compared with those isolated from subcutaneous depots (94). Cortisol levels or HSD1 activity in omental fat of human subjects from both groups has not been measured. It would be interesting to assess whether TNMD expression is upregulated by changes in local glucocorticoid levels in omental adipose depots of insulin sensitive vs. insulin resistant patients.

Because the SVF includes adipocyte precursors, the very low expression of TMND in this fraction and comparatively high expression in primary adipocytes suggested that TNMD is responsive to adipogenic differentiation. To address this question, I utilized SGBS preadipocytes as a relevant human cell culture model. Indeed, TNMD was stimulated several hundred fold upon differentiation.

Adipogenic differentiation was confirmed by assessing the expression of adipogenesis markers such as *ADIPOQ* and *PPARG* (Figure 2.3, a). Validation of these results using other human adipocyte cell culture models such as human adipose derived stromal cells (hADSCs) could be performed in the future.

TNMD is induced very early in the adipocyte differentiation program and has a similar expression pattern to C/EBPα (Figure 2.3, b). TNMD expression started to increase 18 hours after induction and continued to increase throughout adipogenesis in human cells. These results suggest 1) TNMD is an adipogenic gene that is involved in adipocyte differentiation and has a function in mature adipocytes, 2) TNMD expression is regulated by adipogenic transcription factors C/EBPα and PPARγ or 3) TNMD acts upstream of adipogenic transcription factors. To test the first hypothesis, TNMD was silenced in preadipocytes two days prior to adipogenic induction, and silencing TNMD significantly attenuated adipogenesis in SGBS cells (Figure 2.4). Because TNMD is upregulated by differentiation, diminished adipogenesis itself could also contribute to low TNMD expression compared with control cells transfected with scrambled RNA (Figure 2.4, e). Thus, these results suggest that TNMD induction is required for adipogenesis and adipogenic gene expression. Mouse adipocyte cell line 3T3-L1s are by far the most common cell line used to study adipogenesis and adipocyte biology (138). TNMD, however, demonstrated a different expression profile in these cells. As opposed to human SGBS preadipocytes, TNMD expression decreased after the second day of differentiation in 3T3-L1s (data not

shown). However, TNMD knockdown diminished differentiation in 3T3-L1 cells as well. This difference between human and mouse adipocytes in terms of TNMD expression during differentiation is interesting and suggests that even at low levels of expression, its effects are significant. Further studies will address how TNMD is regulated in different species. Such different expression patterns between mouse and human raised an issue about potential off-target effects of siRNA-mediated knockdown. Rescue experiments using expression vectors with mutated siRNA target sites would further support our findings. Alternatively, different oligos targeting TNMD gene could be used to rule out the possibility of off-target phenotype. However, overexpressing genes by transfection is technically challenging in SGBS and 3T3-L1 cells yet overexpression via lentiviral or adenoviral infection could create artificial results due to unnecessarily high expression of the target gene or off target effects of the virus.

Even though *in vitro* results strongly suggest that TNMD is required for adipogenic differentiation, mechanistic details on how TNMD regulates adipogenesis and adipogenic gene expression are still unclear. Hence, the molecular pathways that are altered upon TNMD knockdown were investigated in unstimulated and stimulated cells using microarray analysis. These experiments further supported the finding that silencing TNMD attenuated stimulation of adipogenic genes (Figure 2.7, a). The microarray data for some transcription factors such as C/EBPα were verified at both mRNA and protein levels. These findings suggest that TNMD might be an upstream regulator of this transcription

factor. However, experiments to address whether the reduction in C/EBPα expression is the result or the cause of diminished adipogenesis could be performed in the future.

Next, I asked whether the phenotype observed upon TNMD depletion is cell autonomous or non-cell autonomous because TNMD has been predicted to be a plasma membrane protein with a large extracellular portion (275, 285). Considering that cell-cell contact is required for adipogenic differentiation (114), I hypothesized that TNMD might be involved in the interaction of adjacent cells. TNMD also has a putative cleavage site, and studies suggested that the cleaved C-terminal portion is active (285). Interestingly, I also demonstrated that when overexpressed in SGBS preadipocytes, TNMD is cleaved and the C-terminal peptide is released into culture media (Figure AI-1). Conceivably this peptide might act on neighboring cells such as adipocytes precursors, or could reach other tissues through circulation. To address these questions, I co-cultured non-transfected cells and siTNMD transfected cells together to observe the consequence on adipogenesis. In this experiment, co-culturing non-transfected and TNMD silenced cells resulted in an approximate 50% reduction in Oil Red O staining, which suggested a cell autonomous role for TNMD in adipogenesis. Even though these data do not rule out the possibility that TNMD is cleaved and secreted, it provides evidence that the secreted product, at least at endogenous levels, is not sufficient to rescue reduced adipogenesis in the absence of TNMD. Further studies using conditioned media from TNMD overexpressing cells could

also be performed to assess whether TNMD acts in a cell autonomous manner to promote adipogenesis.

Because TNMD expression increased remarkably during differentiation, I hypothesized that TNMD may have additional effects on adipocyte biology. Future studies using human *in vitro* adipocyte models are needed to address whether TNMD functions in the metabolic processes such as lipogenesis, lipolysis or glucose uptake and if so, what is the mechanism whereby TNMD regulates these processes.

Adipose tissue is a complex organ in which adipocytes interact with other cells types in addition to the extracellular matrix (24, 223). I aimed to address my metabolic questions in the context of whole adipose tissue. Therefore, an *in vivo* model was developed. Expression of *Tnmd* in mouse adipose depots is low or below detection (290). Therefore, an adipocyte-specific adiponectin promoter-driven *Tnmd* overexpression model was generated. qRT-PCR and Western Blot analysis of TNMD confirmed very low or undetectable levels of TNMD in mouse adipose depots (Figure 2.10, data not shown). Moreover, *Tnmd* transgenic mice displayed a significant and specific expression of *Tnmd* in eWAT, iWAT and BAT (Figure 2.10). Surprisingly, *Tnmd* overexpression in adipose tissue did not affect total body weight gain (Figure 2.11, a). However, individual adipose depots from animals that were fed HFD revealed that TNMD overexpression caused a significant increase eWAT but not subcutaneous depot expansion (Figure 2. 11, b). These results raised two questions: 1) Did eWAT in transgenic animals

expand via hypertrophy or hyperplasia? and 2) Does TNMD control adipose tissue expansion? To pinpoint the mechanism of adipose tissue expansion, cell size was measured in histology sections, and the average cell size from both adipose depots (eWAT and iWAT) was unchanged (Figure 2.12, a). These results suggested that adipocyte number was increased in HFD-fed transgenic animals. Thus, earlier time points were investigated during HFD feeding to observe the formation of new and smaller adipocytes. Adipose depot weight and adipocyte size was assessed in animals fed HFD for only 4 weeks, and no difference in either adipose depot weights or adipocyte size was observed. These data suggest that hyperplasia occurs at the late time points of the HFD challenge. This conclusion is consistent with two models that have been recently published. In the first model, Scherer and colleagues demonstrated that eWAT expands by both hypertrophy and hyperplasia in mice, the latter of which occurs after 8 weeks of HFD feeding (133). In the second model, preadipocyte proliferation was increased in the first week of HFD, but newly differentiated adipocytes could not be detected for 8 weeks (220). It is unclear when preadipocytes complete differentiation and start expanding in size during diet induced obesity; addressing this question will be highly informative.

Reduced tenocyte proliferation has been reported in *Tnmd* null mice (277). Therefore, I hypothesized that TNMD might also promote preadipocyte proliferation, and *in vivo* BrdU incorporation into preadipocytes isolated from *Tnmd* overexpressing mice was indeed increased (Figure 2.13). These results

suggested that enhanced preadipocyte proliferation in TNMD transgenic animals resulted in hyperplastic eWAT. One could argue that the adiponectin promoter would drive *Tnmd* expression in mature adipocytes rather than precursors; thus, effects in preadipocyte proliferation cannot be explained by *Tnmd* overexpression. Although cell culture experiments suggested that the effects of TNMD were cell autonomous, it remains to be tested whether TNMD increased *in vivo* preadipocyte proliferation in a cell autonomous manner. Another important question is whether TNMD promotes *in vivo* differentiation of preadipocytes that reside in the eWAT. Future studies using *in vivo* tracer models can address this question. Alternatively, tracing BrdU labeled adipocytes would be informative to determine whether labeled preadipocytes actually make new adipocytes in the later time points of HFD challenge.

Adipose tissue *de novo* lipid synthesis is reduced in obesity (45). To investigate whether adipogenic and lipogenic gene expression were increased in eWAT of *Tnmd* transgenic animals, I performed qRT-PCR and Western Blot analysis, and the expression of lipogenic genes such as *Srebp1c, Fasn* and *Acly* was significantly increased in transgenic animals compared to control littermates (Figure 2. 14, a). Consistent with our *in vitro* adipogenesis experiments, PPARγ was also elevated in the eWAT of transgenic animals (Figure 2.14, b,c). These data suggest that TNMD enhances lipid synthesis in eWAT, thereby promoting efficient lipid sequestration in this depot during obesity. However, it should be taken into consideration that adipocyte-specific gene expression in whole

adipose tissue samples could appear to decrease when the number of other cell types elevates. Therefore, increased immune cell infiltration in the control eWAT might contribute to the apparent decrease in adipocyte-specific gene expression in whole adipose tissue. This possibility could be ruled out by assessing gene expression profiles in isolated SVF and primary adipocytes. Even though no difference in tissue weight or cell size was observed, it would be informative to determine whether *de novo* lipogenesis is improved in subcutaneous adipose depots. *Ex vivo* lipogenesis assays using eWAT and iWAT isolated from chow fed animals did not reveal any difference in lipogenesis between the transgenic and control mice. However, there were some potential pitfalls with this experiment. First, *ex vivo* conditions do not always fully represent the *in vivo* tissue environment and could create artificial results. Second, chow animals were used for this experiment, and differences might only be observed in animals challenged with HFD.

TNMD may have anti-angiogenic effects (283, 285). One could expect that overexpression of a putative anti-angiogenic protein can blunt vessel dissemination during adipose tissue expansion. Therefore, the role of TNMD in adipose tissue angiogenesis was elucidated by isolectin staining of the vasculature and vessel density quantification. Consistent with previous results reported in *Tnmd* knockout mice, there was no effect of adipose-specific *Tnmd* overexpression on the adipose tissue vasculature (Figure 2.15) (277). These

results suggest that TNMD promotes eWAT expansion independent from modulating the tissue vasculature.

TNMD has been extensively studied in the context of tendon tissue. Studies show that TNMD knockout affects collagen matrix formation and structure (277). Because the ECM can affect adipocyte differentiation, metabolism and expansion (223, 336, 337), I investigated the expression of ECM components by qRT-PCR and structure by Masson's Trichrome staining in adipose tissue and demonstrated that collagen accumulation was reduced in *Tnmd* overexpressing mice. Trichrome staining does not distinguish types of collagen; therefore, it would be useful to determine which types of collagen are differentially expressed in the control and TNMD transgenic adipose tissue, as collagen VI has adverse metabolic effects in adipose tissue (233, 335). Gene expression analysis partly confirmed reduced collagen expression in the eWAT of transgenic animals. Interestingly, the expressions of some matrix metalloproteinases (MMPs) were also downregulated in the transgenic animals (Figure 2.15, d). This result was rather contradictory to the reduction in collagen expression because tissue MMP depletion is often characterized by collagen accumulation (159). The decrease in MMP12 (macrophage metalloelastase) could be attributed to a reduction in macrophage content in *Tnmd* overexpressing eWAT (Figure 2.12, a Figure 2.15, e). However, MMP activity inhibitors (TIMP) expression levels need to be analyzed. Interestingly, TGFβ, SMAD2 and SMAD3 mRNA levels were also reduced in the transgenic animals. Although the *in vivo*

and *in vitro* effects of TGFβ on adipogenesis and adipose tissue formation is somewhat contradictory (114), it could be interesting to explore whether TNMD promotes hyperplasia in adipose tissue via modulating TGFβ signaling.

As mentioned above, *Tnmd* overexpression also resulted in reduced immune cell infiltration and crown like structure formation in adipose tissue. mRNA levels of proinflammatory molecules such as *Ccl2* were also diminished (Figure 2.12). There is a vicious cycle in obese adipose tissue whereby cellular stress in adipocytes causes pro-inflammatory cytokine secretion, which can attract macrophages, in turn furthering adipose tissue inflammation (262). Thus, it would be interesting to address whether TNMD lessens cellular stress by improving the tissue environment in favor of healthier expansion, or alternatively whether TNMD regulates inflammatory pathways and decreases inflammation in obese adipose tissue.

Tnmd was also overexpressed in BAT of transgenic mice (Figure 2. 10). Reduced lipid deposition was observed as assessed by histology, consistent with reduced BAT weight in the transgenic animals. Furthermore, *Ucp1* expression increased in the *Tnmd* overexpressing mice. However, metabolic cage studies detected no difference in respiratory exchange rate or oxygen consumption. Though BAT seemed to have higher activity in transgenic animals, it did not contribute to systemic energy expenditure at room temperature. Performing the metabolic experiments in thermoneutral conditions in which cold stress is

eliminated for mice could give a better insight into whether BAT is involved in the systemic metabolic actions of TNMD.

It was also important to explore whether apparent healthy expansion of visceral adipose tissue resulted in an inhibition of peripheral lipid deposition. Histological examination and reduced triglyceride content clearly demonstrated that hepatic steatosis was prevented in the transgenic animals, which was consistent with reduced whole liver weight. These data suggest that better sequestration of the lipids in eWAT protected the liver from TG accumulation, even in very obese conditions. Genes controlling *de novo* lipogenesis and lipid storage were measured in this tissue. Hepatic glucose production in the transgenic animals was also attenuated in the basal state (Figure 2.19, c). Therefore, it would be interesting to investigate whether gluconeogenesis was altered in the transgenic animals.

Next, I addressed systemic metabolic health in *Tnmd* transgenic mice. Even though no difference was observed between genotypes in a GTT, *Tnmd* overexpressing mice were more insulin sensitive as assessed by ITT (Figure 2.18). Furthermore, increased insulin-stimulated AKT phosphorylation was observed in eWAT (Figure 2.18, c, d). Healthier adipose tissue expansion is associated with improved systemic insulin responsiveness, and the ITT consistently demonstrated a profound metabolic difference between control and transgenic animals. Insulin-dependent, tissue-specific glucose uptake in was assessed during a hyperinsulinemic-euglycemic clamp study. In this study, a

trend to increased glucose uptake in BAT and skeletal muscle was observed in TNMD transgenic mice compared with controls. Interestingly, the liver was more insulin sensitive in *Tnmd* transgenic mice than controls, and lower hepatic glucose production was also observed in basal conditions. Furthermore, there was a significant increase in the glucose infusion rate during the clamp in transgenic mice compared to control littermates (Figure 2.19). Therefore, improved insulin signaling in the eWAT of *Tnmd* transgenic animals is a reflection of healthier adipose tissue, and the clamp study demonstrates that the liver is also a contributing factor. Inter communication between eWAT and liver should also be considered.

In conclusion, the data in this thesis provide evidence for a beneficial role of TNMD in adipocyte differentiation and adipose tissue function. This work also advances understanding of adipose tissue expansion during obesity. Visceral adipose tissue has long been associated with metabolic diseases (88, 266, 304). Here, selective visceral adipose depot expansion was actually beneficial because adipocyte number and fat sequestration was maximized while cellular stress was abated. Thus, TNMD could be an important therapeutic target to promote the healthy visceral adipose tissue expansion during obesity, and more importantly, to reverse adipocyte dysfunction in the disease state.

Future Directions

TNMD has been widely used as a differentiation marker for tenocytes (273, 277, 286). However, TNMD's function has not been fully understood. Defining the intracellular localization of TNMD might give insight into this protein's function. Based on sequence TNMD is predicted to be a type II transmembrane protein (275, 343). However, contradictory results on the intracellular localization of TNMD have been reported. It has been detected in various locations in the cell such as plasma membrane, golgi and nuclear envelope. Even though endogenous protein has been detected in some of these studies, proper controls including TNMD null cells have not been used (286, 288). Moreover, many of these studies utilized an overexpression approach, which can give rise to artificial results. Therefore, careful analysis of intracellular localization of TNMD in adipocytes with a specific antibody that can detect the endogenous protein will be useful in terms of understanding its possible functions. Moreover, fractionation studies using human adipose tissue where TNMD is expressed at high levels will be helpful to assess localization.

Future work identifying the molecular mechanisms by which TNMD achieves its metabolic effects systemically and locally will be of great importance. Unbiased techniques such as RNAseq or proteomics in combination with genetic studies including mutation analyses targeting SNPs in the human *TNMD* gene that are associated with metabolic parameters (potentially using CRISPR technology) will be very informative.

Even though *in vitro* adipogenesis has been very well studied using cell culture systems, *in vivo* mechanisms that are implicated in adipogenesis are incompletely understood (114). We were able to demonstrate that TNMD contributes to hyperplastic adipose tissue expansion through promoting preadipocyte proliferation in the first week of HFD. However, the question whether TNMD also stimulated adipogenesis in the visceral adipose depot is yet to be addressed. Crossing *Tnmd* transgenic mice with tamoxifen inducible Adiponectin-CreER mT/mG (220) reporter line or doxycycline inducible Cre-LacZ reporter line (133) could be the proper approach to address this question. Furthermore, administering the potentially secreted portion of TNMD to these reporter lines will be helpful to determine the effect of TNMD on adipogenesis *in vivo*. In both cases, observing new adipocyte formation at greater levels in transgenic or TNMD administered animals would further prove that TNMD promotes hyperplastic adipose tissue expansion *in vivo*.

The most significant future work on TNMD in terms of impact on the field might be to determine whether it is a novel adipokine, a secreted factor that acts locally or systemically to affect adipose or other tissue function. Important remaining questions related to this include assessing whether TNMD is in fact secreted, and whether the putative secreted peptide functions locally or circulates systemically. If the peptide circulates, does it function as an endocrine factor that regulates peripheral tissue function? These questions could be answered using parabiosis experiments in which the circulation of a wild type

mouse is attached to a *Tnmd* transgenic mouse. If TNMD acts as a secreted factor or alternatively stimulates the secretion of other factors from eWAT, metabolic improvements should also be observed in the wild type animals in obese conditions. If no effect is observed in parabiosis experiments, the data would suggest that TNMD might act in a paracrine fashion. Even though TNMD is likely to be cleaved from its putative furin site, identifying the exact sequence of this secreted portion will be important in terms of producing the recombinant peptide and assessing its physiological effects. Alternatively, generating a transgenic mice with non-cleavable furin site or taking a loss-of-function approach by using neutralizing antibodies will give better insight into the possible role of TNMD as a beneficial adipokine.

APPENDIX I

Introduction, Results and Discussion

Adipose tissue is one of the biggest endocrine organs in the body (62). Many studies have demonstrated that peptide hormones secreted from adipocytes have various biological effects (262). Adipose driven cytokines-also known as adipokines- regulate not only adipose tissue function but also metabolic homeostasis in the peripheral tissues such as muscle and liver (344). Moreover, ECM proteins that are processed and released from adipocytes into the adipose tissue microenvironment can play roles in the adipocyte expansion and preadipocyte proliferation (345). Full characterization of adipocyte secretome and ECM composition is unlikely complete. Therefore, identifying novel adipokines or ECM-related proteins that are implicated in the adipose tissue's metabolic function is of great importance.

Recent studies suggested a metabolic role for TNMD (290, 296, 318). We also identified it as a novel regulator of adipogenesis and visceral adipose tissue expansion in diet induced obesity. TNMD promoted preadipocyte proliferation and hyperplastic adipose tissue expansion upon high fat diet feeding and mice overexpressing TNMD selectively in the adipose tissue demonstrated improved systemic insulin sensitivity in obese conditions (Chapter II). Studies also suggested a role for TNMD in the tendon tissue as TNMD null mice demonstrated disorganized collagen fibrils in the tendon tissue (277). We also demonstrated a reduced collagen accumulation in *Tnmd* overexpressing adipose

tissue (Chapter II). In the light of these findings, we sought to address two important questions:

1) Is TNMD processed and secreted into local environment or blood circulation?
2) Does TNMD interact with ECM components?

In order to address the first question, we overexpressed TNMD with N-terminal 3HA and C-terminal Flag tag using an adenoviral construct. We used 3HA expressing empty virus infected and non-infected cells as controls. Cells were incubated in a serum free media supplemented with 33 uM biotin, 17 uM pantothenic acid for 48 hours after the infection (Figure A.1, a). Overexpression of full length TNMD was confirmed in lysates using an antibody against a peptide in the extracellular domain of TNMD (Figure A.1, b). We also analyzed the concentrated media by Western Blot. Surprisingly, we detected a band around 15 kDa in the media isolated from TNMD overexpressing cells but not from control cells. These results suggest that TNMD might be cleaved by endopeptidases and secreted into extracellular environment (Figure A.1, c). Interestingly, we also observed a similar size band in the lysates and this band was slightly smaller than the one in the media, which suggest that TNMD might be processed inside the cell and secreted through canonical secretion pathway. Moreover, we were able to detect the cleaved product with Flag antibody, whereas no band was detected using HA antibody. These results provided evidence that C-terminal domain of TNMD is secreted into media.

Figure A1.1

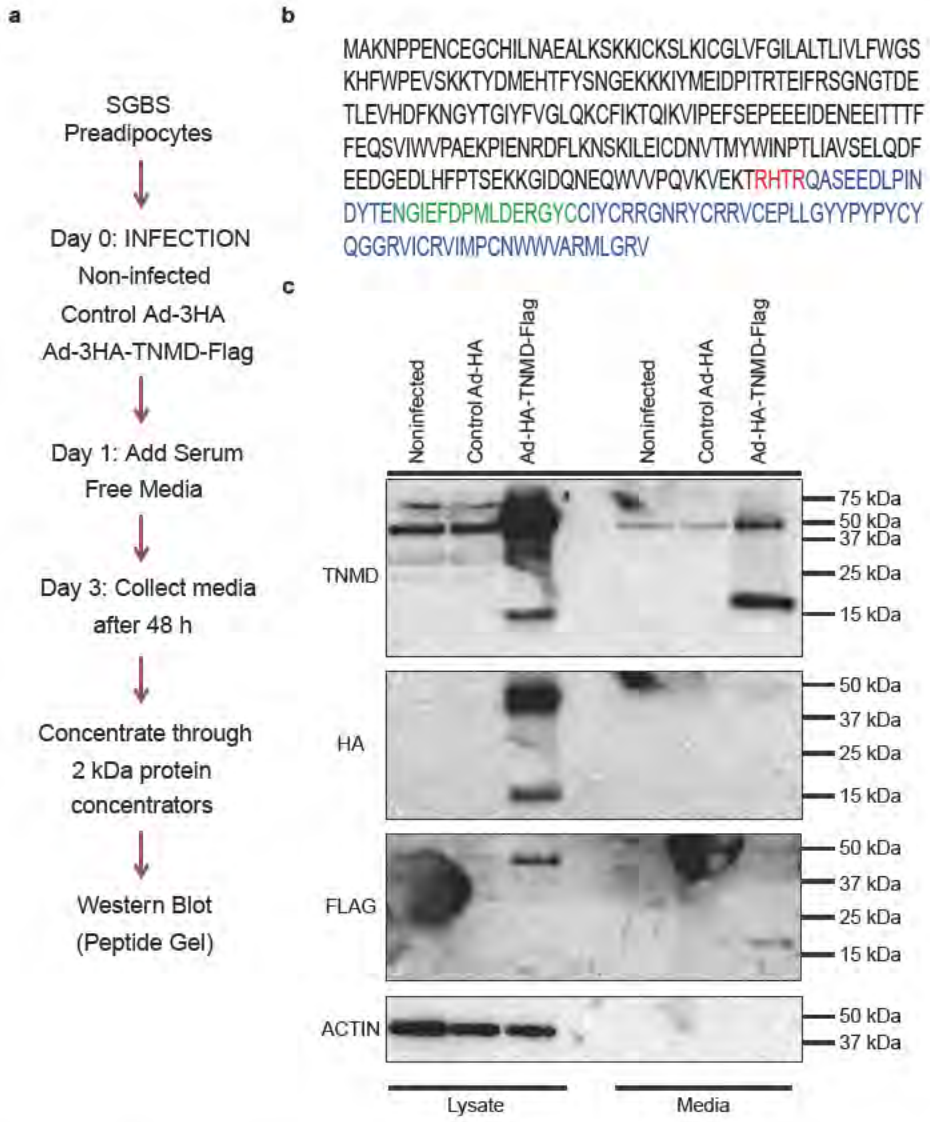


Figure A1.1: TNMD is processed and excreted into media
SGBS cells were plated in 15 cm dishes and infected with indicated adenoviral constructs **(a)** Schematic demonstration of experimental flow **(b)** Amino acid sequence of full length TNMD. Putative furin cleavage site is indicated in red, predicted secreted portion of TNMD is represented in blue and peptide against which TNMD antibody is generated is indicated in green **(c)** Immunoblot analysis of lysates and media collected from non-infected, Control Ad-3HA and Ad-3HA-TNMD-Flag infected SGBS preadipocytes. Representative image of three independent experiments.

We have demonstrated that TNMD overexpression in the adipose tissue altered the collagen accumulation and the expression of ECM proteins at the mRNA level. A role for tenomodulin in modulating ECM interactions was also suggested by proteomics data obtained in SGBS cells. We overexpressed TNMD using an adenoviral vector expressing TNMD with HA (N-terminus) and Flag (C-terminus) tags and immunoprecipitated TNMD-associated proteins to identify potential binding partners. In proteomics analysis, we found several proteins that are either located in adipose tissue ECM or involved in processing ECM proteins (Table A.1). Even though validation studies are needed, these results suggest that TNMD may contribute to healthy visceral adipose tissue expansion through direct interaction with extracellular matrix proteins or function as an extracellular matrix protein that generates a local environment to favor adipocyte expansion and preadipocyte proliferation.

Table A.1

Protein Name	Gene Symbol	Spectral Counts	
		Ad-HA-Control	Ad-3HA-TNMD–Flag
TGFβ-induced protein ig-h3	*TGFBI*	2	29
Thrombospondin 1	*THBS1*	3	17
Periostin	*POSTN*	0	12
Metalloproteinase inhibitor 3	*TIMP3*	2	10
Ugl-Y3 (Fibronectin Precursor)	*FN1*	3	10
Plasminogen activator inhibitor 1	*SERPINE1*	0	8
Protein-lysine 6-oxidase	*LOX*	0	8
Glypican 1	*GPC1*	0	6
Galectin 7	*LGALS7*	2	6
Connective tissue growth factor	*CTGF*	0	5
Tenascin C	*TNC*	0	4
Emilin 1	*EMILIN1*	0	4
Collagen alpha-2 (VI) chain	*COL6A2*	0	3
Matrix metalloproteinase 14	*MMP14*	0	2
Galectin-3-binding protein	*LGALS3BP*	0	2
Metalloproteinase inhibitor 1	*TIMP1*	0	2

Table A.1: ECM proteins or ECM-protein processing enzymes interacting with TNMD

Whether TNMD is circulated in the body is yet to be addressed. We are currently analyzing serum samples obtained from *Tnmd* overexpressing mice. Detecting TNMD in the serum and addressing the question whether TNMD that potentially secreted from visceral adipose tissue acts on liver will provide an insight into our understanding of how TNMD regulates systemic lipid and glucose metabolism. Moreover, characterizing the secreted portion of TNMD will be very informative for design and production of a recombinant peptide to test the physiological function of secreted TNMD. Given that overexpressing TNMD in adipocytes improved systemic insulin resistance in obese mice and that secreted portion potentially posses a physiological function, TNMD could be considered as a novel therapeutic target.

Experimental Procedures

Cell Culture

Simpson Golabi Behmel Syndrome (SGBS) cells were cultured in DMEM/F12 media supplemented with 10% fetal bovine serum, 33 uM biotin, 17 uM pantothenic acid, 100 units ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin. Cells were seeded in 15 cm dishes and infected with Ad-3HA-TNMD-Flag or Ad-3HA-Control virus. Infection media was changed after overnight incubation. Cells were washed and incubated in serum free media supplemented with 33 uM biotin, 17 uM pantothenic acid, 100 units ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin for 48

hours. Media was collected and centrifuged for 5 min at 1500 rpm. Supernatant was concentrated in protein concentrators with 2000 Da molecular weight cut off (Vivaspin 2, Sartorius).

Western Blotting

Cell lysates were prepared using RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% sodium deoxycholate, 1% NP-40, 0.2 % SDS, 50 mM EDTA) containing 1X HALT protease and phosphatase inhibitors (Thermo Scientific). Total protein was separated on 15% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk solution in TBS-T and immunoblotted with an antibody generated against NGIEFDPMLDERGYC peptide from C-terminus of TNMD (Rockland, 1:5000), HA (Cell Signaling, 1:1000), FLAG (Cell Signaling, 1:1000) and actin (Sigma, 1:5000) antibodies.

Immunoprecipitation and Proteomics Analysis

SGBS cells were infected with Ad-3HA-TNMD-Flag or Ad-3HA-Control virus. After 48 hours, cells were lysed with lysis buffer (1% NP40, 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1X HALT protease and phosphatase inhibitors). 1 mg total lysate from each sample was incubated overnight with HA antibody (Cell Signaling, 3724) at 4^0C, which was followed by incubation with Protein A for an additional hour. Samples were loaded on SDS-PAGE (Biorad Mini-Protean

TGX 4-20%). Proteomics analysis was performed by UMass Medical School Proteomics and Mass Spectrometry Core Facility.

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