Identification of the transcription factor ZEB1 as a novel modulator of adiposity

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DEDICATION

This thesis is dedicated to my family whose love and support has enabled this accomplishment. I thank my parents who have always encouraged me and have stood behind me every step of the way. It is from them that I have developed my work ethic and scientific curiosity. I would also like to recognize my brother who despite our long-distance relationship throughout most of graduate school would continuously send his love and support over the phone. This dedication would not be complete without thanking my best friend who sat by me while I read all my papers and listened to my ideas regardless of their content. Finally I would like to dedicate this to my husband who especially in these final stages has been patient and endured the roller coaster of emotions driven by experimental pitfalls and successes. He is the rock I lean on and has kept me focused on what is important in life.

ABSTRACT

Obesity and its subsequent metabolic disorders have become global health problems. While this is largely due to environmental influences, the propensity to gain weight also has a significant genetic component. In an effort to identify genes that contribute to obesity, several genome-wide scans on obese patient populations have been performed. One consistent location that displayed linkage to obesity is chromosome 10p11-12. A likely candidate gene within this region is the *TCF8* gene, which encodes the ZEB1 transcription factor.

To test whether *TCF8* is an anti-obesity gene, DNA from obese patients was genotyped using single nucleotide polymorphisms throughout the *TCF8* genetic locus. Logarithm of the odds for linkage of *TCF8* to childhood obesity was high in two regions. Sequencing of a subset of the patient DNAs revealed a polymorphism that results in an amino acid change within the coding region of 50% of the patients. More tests will be required to determine whether the polymorphism has a functional consequence.

In addition, *TCF8+/-* and wild type (WT) C57BL/6 mice were fed a high fat diet or regular chow diet for 20 weeks and their body weights, body composition, and metabolic parameters measured. *TCF8 +/-* female mice were significantly heavier on both diets due to an increase in fat. Increased adipose mass was the result of increased adipocyte size and was sufficient to cause metabolic consequences. Interestingly, this phenotype was not observed in male or female mice treated with the specific anti-estrogen Faslodex, suggesting that ZEB1 is mediating some of estrogen's anti-obesity effects. Expression of several known

estrogen-regulated genes important in lipolysis and lipogenesis measured in *TCF8 +/-* and WT suggests that ZEB1 modulates the flux of lipids in adipocytes.

This thesis identifies *TCF8* as an anti-obesity gene in mice and potentially in humans. Loss of one copy of the *TCF8* gene is sufficient to increase adiposity and subsequent metabolic consequences. This is a novel observation as no one has previously proposed a role for ZEB1 in adipose tissue. In addition, this data contributes to our understanding of sexual dimorphism in metabolism.

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ABBREVIATIONS

36B4	Acidic ribosomal phosphoprotein PO
ACC1	Acetyl CoA carboxylase 1
AMPK	Adenosine monophosphate-activated protein kinase
BMI	Body mass index
BMP	Bone morphogenetic protein
C/EBP	CCAAT/ enhancer binding protein
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesynchymal transition
ER	Estrogen receptor
FAS	Fatty acid synthase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GTT	Glucose tolerance test
HFD	High fat diet
HSL	Hormone sensitive lipase
IP	Intraperotineal
ITT	Insulin tolerance test
JAK/ STAT	Janus-family kinase/ signal transducer and activator of transcription
LOD	Linkage of the odds
LPL	Lipoprotein Lipase
MEF	Mouse embryonic fibroblast
MiR	Micro ribonucleic acid
NCBI	National Center for Biotechnology Information
ηg	Nanogram
NPY	Neuropeptide Y
PCR	Polymerase chain reaction
POMC	Proopiomelanocortin
PPARγ	Peroxisome proliferator gamma
Rb	Retinoblastoma protein
RCD	Regular chow diet
SCD1	Stearoyl-CoA desaturase 1
SDF1α	Stromal derived cell factor 1
SEM	Standard error of the means
SNP	Single nucleotide polymorphism
TCF8	Transcription factor 8
TIP160	Tat-interacting protein 160
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
μg	Microgram
ZEB1	Zinc finger ebox binding protein

CHAPTER I

INTRODUCTION

The *TCF8* gene

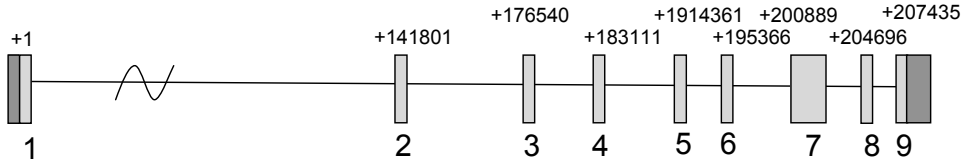
The human *TCF8* gene on chromosome 10p11.2 encodes the transcription factor Zinc Finger E-Box binding homeodomain protein 1 (ZEB1, δEF1, AREB6, zfhx1a) [1]. TCF8 is composed of 9 exons [2] and has an unusually large first intron of 141,743 bp (Figure 1). Little is known regarding ZEB1 transcriptional regulation. The 5' region of the gene has no TATA box but is GC rich. While a transcription start site has not been described, the region 219 bp upstream of exon 1 is important for regulation of the gene [3].

To date, there are few known regulators of *TCF8*. The steroid hormones androgen, estrogen, and progesterone were the first regulators of *TCF8* identified. *TCF8* induction by estrogen was originally shown in the chick oviduct [4]. Subsequently, it was shown to be estrogen-regulated in a number of tissues including human ovary [5], uterine myometrium [6], mouse pituitary [7], and mouse adipose tissue (J. Saykally, unpublished data). In addition, ZEB1 increases in the presence of progesterone in human myometrial cells and has a synergistic effect when estrogen is added [6]. The mechanism of regulation by steroid hormones is yet to be determined, but the time course of ZEB1 upregulation following estrogen treatment suggests that it is a primary response [4]. While several estrogen response element (ERE) half site sequences exist in the proximal promoter, genome wide chromatin immunoprecipitation studies suggest that the closest ERα bound ERE is located 92 Kb downstream of the *TCF8* gene (data mining from [8]).

Figure 1. A genomic map of the *TCF8* gene. A line diagram for 208,595 base pairs of the *TCF8* genomic locus located on chromosome 10p11.2. The TCF8 gene has nine exons with a total coding region of 3372 bp. Exons 5,6,and 7 encode the N-terminal zinc finger cluster. Exons 8 and 9 encode the C- terminal zinc finger cluster. The central homeodomain is 192 bp within exon 7. TCF8 has a large first intron and a 3' UTR region that is important for regulation by microRNAs.

+1 = translation start site

= Exon = UTR



Exon	Length
5' UTR	23
1	58
2	201
3	63
4	162
5	203
6	106
7	1811
8	181
9	593
3' UTR	1934

Intron	Length
1	141,743
2	34,538
3	6,508
4	8,163
5	3,727
6	5,417
7	1,996
8	2,558

With our current knowledge regarding the distances at which steroid hormones can bind to regulate genes, it is possible that this is the site through which *TCF8* is regulated. However, this study was done in MCF7 cells where *TCF8* is not regulated by estrogen, making it plausible that the genome-wide screens did not identify the active ERE for *TCF8*. Alternatively, one of the putative ERE half sites, SP1 sites, AP1 sites or a combination allows estrogen receptor to bind directly or indirectly to the promoter [9-11]. Androgen has been shown to bind to two androgen response elements located in the proximal promoter region and upregulate ZEB1 levels in a prostate cancer cell line (B. Anose and M. Sanders unpublished data).

TCF8 is also regulated by other extracellular signaling molecules. Work done in a prostate cancer cell line identified up-regulation of *TCF8* by Insulin-like growth factor-1 (IGF-1) [12]. IGF1 binds a cell surface receptor and activates the mitogen-activated protein kinase and phosphoinositide-3-kinase pathways to regulate downstream events [12]. Its primary function is to stimulate cell growth. ZEB1 is also induced by transforming growth factor-β (TGFβ) signaling indirectly through an increase in E26 transformation-specific (Ets1) transcription factor [13]. TGFβ is a secreted protein that binds receptors on the cell surface initiating a phosphorylation cascade that involves the recruitment of SMAD proteins and subsequent upregulation of genes that inhibit cellular proliferation and promote apoptosis [14]. The bone morphogenetic proteins (BMPs) family of ligands also activate the TGFβ signalling pathway and *TCF8*. BMPs promote differentiation of mesynchymal tissues such as bone, chondrocytes, and adipocytes [15-17].

More recently, it has been reported that *TCF8* is negatively regulated by microRNAs (miR). A number of miRs of the miR200 family (miR200c, miR200a, miR200b, and miR141) decrease *TCF8* expression [18, 19]. MiR200c, located on chromosome 12, was the first miR to be identified as a negative regulator of *TCF8*. An inverse correlation between levels of TCF8 and miR200c mRNA expression occurs in immortalized cell lines. In cell types where *TCF8* has high expression such as the HT1080 cell line, very little miR200c expression was observed [18]. Conversely, in MCF7 cells where ZEB1 mRNA is almost undetectable, miR200c was expressed at a high level [18, 19]. Anti-microRNA oligonucleotide were used to knockdown miR200c in MCF7 cell lines and the only transcript that increased appreciably was that for ZEB1 [18]. Two perfect miR200c seed sites (CAGUAUU) exist in the ZEB1 mRNA 3' untranslated region (UTR), as well as other imperfect seed sites that could facilitate binding.

ZEB1 mRNA was later shown to be regulated by miR200b, which is located on chromosome 1 and shares a promoter with miR200a and mir429 [20]. MiR200a and miR429 may work in a cooperative manner to repress ZEB1 mRNA as well but do not appear to act independently. The miR200 family of microRNAs have sequence homology and a similar seed sequence to which they bind. ZEB1 has 3 miR200a, 5 miR200b, and 1 miR205 predicted sites in its 3' UTR [20].

In summary, the *TCF8* gene located at chromosome 10p11.2 encodes the ZEB1 transcription factor. Little is known regarding its regulatory regions. Nonetheless, a number of factors have been identified as positive or negative

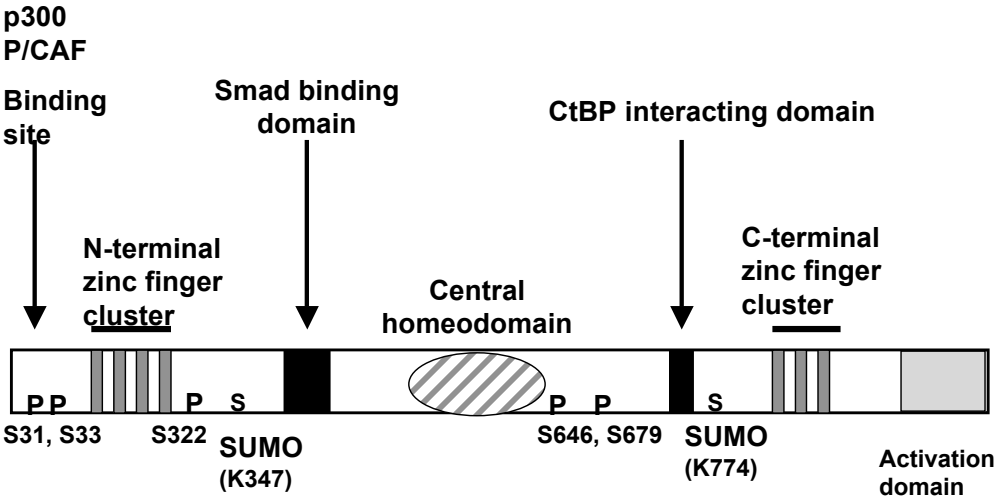
regulators of *TCF8* transcription. ZEB1 expression is increased by steroid hormones, IGFI, TGFβ, and BMPs, and decreased by miRs and negative autoregulation (to be discussed later).

ZEB1 and its function

The ZEB1 protein was originally identified in chicken as a repressor of the δ1-crystallin gene in 1993 and termed δEF1 [21]. Shortly thereafter, ZEB1 was cloned from HeLa cell DNA as a B-cell specific repressor of the IgH enhancer [22] and identified as the full length version of a previously cloned smaller protein known as Nil-2-a [23]. ZEB1 is a 125 kDa protein that has 4 N-terminal and 3 C-terminal evolutionarily conserved zinc fingers through which it binds E-boxes with the consensus sequence CACCT (Figure 2) [22]. It also has a central homeodomain that is thought to facilitate protein-protein interactions [24]. Some post-translational modifications have been identified including phosphorylation [25] and sumoylation [26]. ZEB1 is found in *C.elegans* [27], *Drosophila* [28], and vertebrates [2, 21, 24]. Homologs have high sequence homology of the zinc fingers, especially between mouse and human. The mouse gene is located on chromosome 18 and only has 8 exons as it is missing the third exon [2].

ZEB1 is a transcription factor that both positively [4] and negatively [29] regulates gene expression, as reviewed in [24]. The mechanisms by which ZEB1 activates versus represses transcription are poorly understood. What is known suggests that the mechanism may be cell-type and gene-specific.

Figure 2. Structural elements of the ZEB1 protein. The human ZEB1 protein is composed of 1124 amino acids. ZEB1 binds to E-boxes (CACCTG) to repress or activate transcription via the zinc finger clusters (gray bars) at the N and C-termini of the protein. A centrally located homeodomain (hashed bars) is proposed to facilitate protein-protein interaction. A C-terminal 'acid blob' activation domain is required for gene activation. Postranslational modifications are marked with a P for phosphorylation or S for sumoylation indicating the location of modified amino acids in the ZEB1 protein.



Region	Amino Acid
Zinc Finger	170-193
Zinc Finger	200-222
Zinc Finger	240-262
Zinc Finger	268-292
Homeodomain	581-640
Zinc Finger	904-926
Zinc Finger	932-954
Zinc Finger	960-981

ZEB1 can negatively regulate through competitive binding [30]. In addition, ZEB1 has been can directly bind to promoters and recruit co-repressors such as C-terminal binding protein (CtBP) [31], CtBP2, Tat-interacting protein (TIP 160) [32], and histone modifiers such as histone deacetylases and histone methyltransferases [31].

While most identified target genes are down-regulated, ZEB1 can also activate transcription as in the case of the ovalbumin gene [4, 33] and vitamin D3 receptor gene [34]. ZEB1 positively regulates genes by binding co-activators p300 and P/CAF [35]. In some cases, these positive co-activators bind and physically block the binding of CtBP, preventing down-regulation [24]. Through E-box consensus sequences in its own promoter ZEB1 is able to negatively regulate its own expression [3]. While this phenomenon is probably context- and cell-specific, the autoregulation suggests that *TCF8* is tightly regulated.

ZEB1 has several biological functions within the developing embryo as well as the adult. ZEB1 is primarily expressed in mesynchyme of the mouse embryo and is crucial in development [21]. This is emphasized by the perinatal lethality of the TCF8 knockout mouse [36]. In the adult, ZEB1 expression is fairly ubiquitous but higher in mesenchymal tissues such as uterus, lung, colon, and adipose tissues [5, 37]. No large-scale scan for ZEB1 regulated genes has been published, but a number of targets have been identified [38-51]. Numerous downstream targets and wide-spread tissue localization allows for diverse effects of ZEB1.

ZEB1 is an important factor in the development and differentiation of several cell lineages including the immune system. The TCF8 knockout mouse is perinatal lethal limiting experiments on adults[36]. A ZEB1 mutant mouse missing only the C-terminal zinc fingers of the protein was generated [38]. About 20% of these mice survive into adulthood, and display improper development of the thymus and impaired T-cell number with a total population of T-cells only 1% that of wild type (WT) [38]. This defect has been attributed to ZEB1 targeting of the interleukin-2 [23] and CD4 genes [39], two factors important in T-cell function. While the phenotype is not obvious in the knockout mouse, ZEB1 also reduces angiogenesis through the ultimate activation of R-Ras. ZEB1 binds the calcium and diacylglycerol (DAG) responding GEF CalDag-GEFII1 gene, a known repressor of R-Ras [40, 41].

The differentiation of several mesenchymal lineages is also regulated by ZEB1. Pluripotent mesenchymal stem cells can differentiate into osteocytes, myocytes, chondrocytes, and adipocytes [42]. The *TCF8* knockout mouse displays impaired bone development with long bones shortened and increased in girth, a fused rib cage, and overgrowth of the vertebrae [36]. In addition, studies done in C2C12 cells suggest that ZEB1 prevents their differentiation into osteoblasts through the downregulation of BMP-2 [17] and of liver/bone/kidney alkaline phosphatase (BLK-ALK) [43]. Conversely, ZEB1 has also been shown to downregulate osteocalcin [44]. While osteocalcin is a marker gene in osteoblasts, its expression early in development limits bone formation. Loss of

this factor would lead to increased bone formation, as seen in the knockout mouse.

ZEB1 has opposing roles in different types of muscle. It has been shown to prevent myogenesis through downregulation of alpha 7 integrin, an important factor in skeletal muscle development [45]. In addition, in C2C12 cells ZEB1 prevents myotube formation through downregulation of the p73 gene, a p53 family member [30]. In the case of the p73 gene, ZEB1 negatively regulates p73 transcription through competitive binding to the E-box. ZEB1 displaces the crucial transactivator MyoD and as a result the p73 gene is repressed [30].

In vascular smooth muscle cells, however, ZEB1 promotes smooth muscle cell differentiation. Through interaction with Smad3 and Serum Response Factor, ZEB1 activates some of the necessary smooth muscle program genes to increase vasculature [46]. ZEB1 null embryos have suppressed differentiation of smooth muscle cells. *TCF8+/-* mice display increased neointimal/ medial wall area, suggesting that ZEB1 is normally protective of vascular disease [46].

ZEB1 also negatively regulates collagen, a mesenchymal protein that composes the extracellular matrix in connective tissues. There are several collagen proteins that are expressed in a tissue-specific manner. ZEB1 represses type II collagen (col2aI) during chondrocyte development [47]. It also represses pro-collagen type 1 alpha 2 gene (col1a2) expression in vascular smooth muscle cells through binding competition in the enhancer [16]. Collagen I is repressed through ZEB1 binding a repressor element in the upstream promoter in osteoblasts [48].

In addition to opposing differentiation in the mesenchymal tissues discussed above, ZEB1 is pro-proliferative. ZEB1 is highly expressed in cancer cells where proliferation is uncontrolled, as reviewed in [24]. Conversely, the loss of ZEB1 results in cellular senescence in mouse embryonic fibroblast (MEF) cells [49]. The senescence observed in the *TCF8-/-* MEFs corresponds to an increase in cyclin dependent kinase inhibitors [36]. ZEB1 expression is kept in check in proliferating cells through negative regulation by the retinoblastoma protein (Rb) and E2F1, known inhibitors of the cell cycle. The *TCF8* knockout mouse has impaired neuronal crest closure and cleft palate formation, presumably due to a combination of migratory and cell cycle defects [36]. Similarly, ZEB1 is linked to survival in the central nervous system (CNS) [50]. Through the down-regulation of pro-apoptotic p73 and resulting increase in p63 expression, ZEB1 activates a pro-survival pathway [50]. In contrast, ZEB1 can bind SMADs 1, 2, & 3 to activate transcription of proteins such as p21, p15, which are cyclin dependent kinase inhibitors [51]. This effect can only be seen in conjunction with TGFβ signaling. The tissue and context may be very important for ZEB1 regulation of the cell cycle.

In humans, few diseases to date have been associated with ZEB1. This is probably because severe disruptions in the protein may be lethal. Mutations in one copy of the *TCF8* gene can cause posterior polymorphous corneal dystrophy (PPCD)[52, 53]. This may be through the downregulation of collagen type IV alpha 3 (COL4A3). Mutant ZEB1 is not able to repress COL4A3 and the result is overgrowth of the cornea [52]. While *TCF8* is not the only gene that contributes

to this disease state, almost half of people who have PPCD have a mutation in ZEB1 [52, 53]. This disease has also been recapitulated in heterozygous mice [54].

In addition to a mutation in the gene, altered expression of ZEB1 is tied to cancer in humans [55-57]. Several of the genes that are repressed by ZEB1 function to oppose cell migration. While the most notorious gene is E-cadherin [55], other cell-cell adhesion genes are also down-regulated by ZEB1 [56, 57]. E-cadherin is a cell-cell adhesion protein, and loss of this protein is necessary for epithelial to mesenchymal transition (EMT) [12]. While cellular migration is essential to a developing embryo, it is harmful when activated inappropriately. Increased levels of ZEB1 expression correlate with several mesenchymal carcinomas, as reviewed in [24]. Whether this is causal or a result of a tumorgenic environment has not been determined. However, upregulation of ZEB1 in carcinomas initiates EMT, resulting in metastasis of the tumors [58-63].

In summary, ZEB1 is a transcription factor that binds DNA at E-box consensus sites via zinc finger clusters to positively or negatively regulate gene transcription. Several target genes have been identified through which ZEB1 regulates developmental processes. It inhibits the differentiation of mesenchymal tissues such as bone [17, 36, 43, 44], muscle [30, 45], and collagen [16, 47, 48], and promotes T-cell maturation [38], vascularization [46], and epithelial to Mesenchymal transition [24]. Mutations in the *TCF8* gene contribute to PPCD, a disease resulting in overgrowth of the cornea [52, 53]. Loss of ZEB1 can result in cellular senescence *in vitro* [49], while increased

expression is observed in several cancers and increases metastatic potential by increasing EMT [58-63].

Adipose physiology

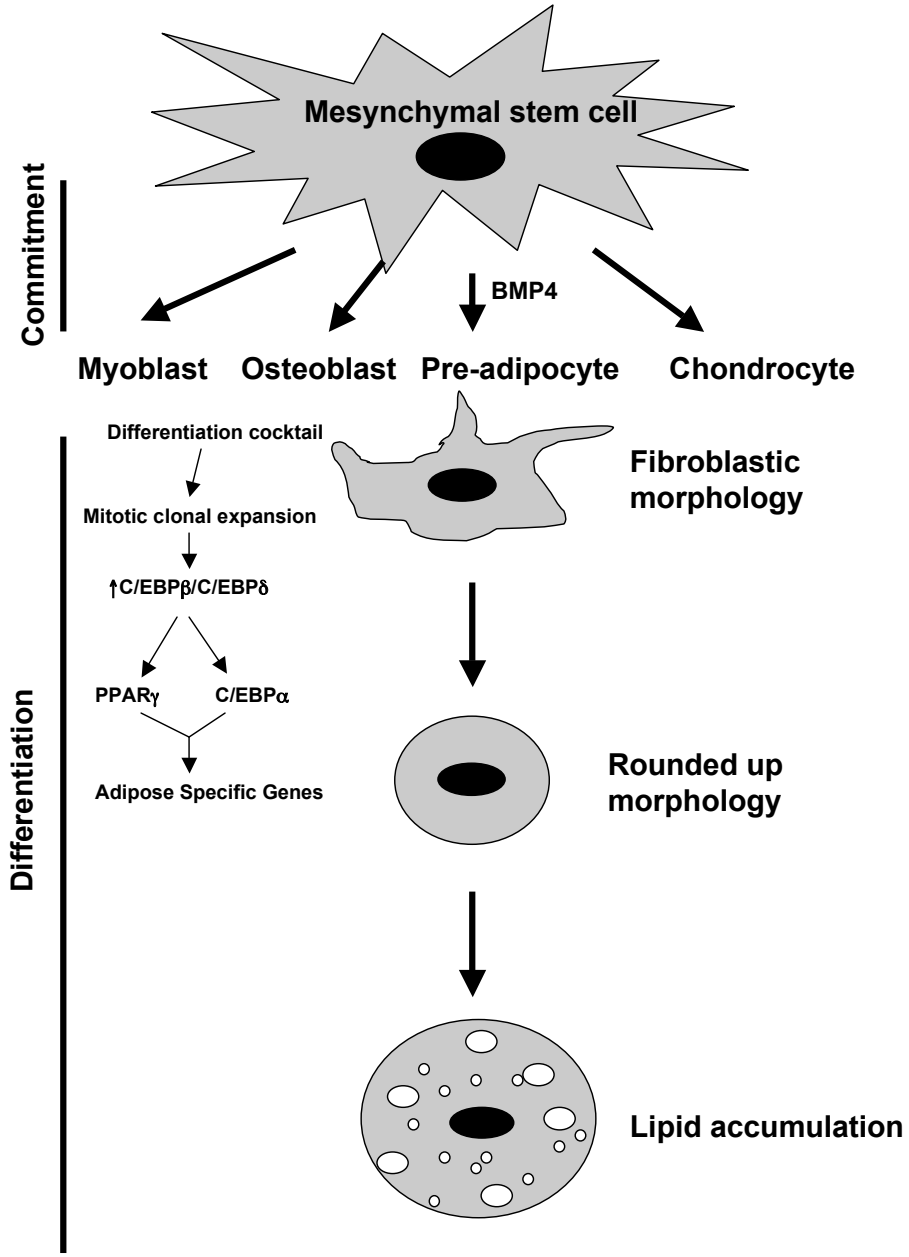
Adipose tissue is an endocrine organ that stores lipid as an energy source and participates in regulating energy homeostasis throughout the body. There are two types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT). While both BAT and WAT store lipid, their origin, location, and function are quite different as reviewed in [64]. BAT is plentiful in human infants, but found to a lesser degree in adults. BAT originates from the muscle lineage and has small lipid droplets dispersed throughout the cell [65]. The function of BAT is to transfer lipid energy into heat via thermogenesis. This is accomplished by uncoupling protein 1 (UCP1) [66, 67].

White adipose tissue was thought to exist primarily as an energy depot. However, it is also an active regulator of energy homeostasis [64, 68, 69]. Through the production and release of hormones and cytokines termed “adipokines”, WAT regulates several metabolic processes throughout the body. WAT increases through both adipocyte hyperplasia and hypertrophy [64, 68, 69]. New adipocytes are generated from pluripotent mesenchymal stem cells located in the adipose stroma and in bone marrow [42]. The development of new adipocytes is a multi-step process whereby the cell is committed to the pre-adipocyte lineage and then triggered to differentiate into a mature adipocyte, as reviewed in [64, 68, 69]. The factors that trigger a pluripotent stem cell to commit

to a pre-adipocyte *in vivo* are unknown. *Ex vivo,* this can be accomplished by addition of bone morphogenetic protein 4 (BMP4) [15, 70, 71]. The process of differentiation from pre-adipocyte to adipocyte is better understood, with several adipogenic regulators identified. NIH 3T3-L1 cells are a pre-adipocyte cell line that can be treated to differentiate into mature adipocytes in cell culture, as reviewed in [64, 68, 69]. Cells are grown to confluency and subsequent growth arrest. Following treatment with a differentiation cocktail that includes methyl-buytlxanthine, dexamethasone, and insulin, cells re-enter the cell cycle and go through rounds of mitosis termed “mitotic clonal expansion”, resulting in the upregulation of CCAAT enhancer binding proteins (C/EBP) β and δ, which in turn increase C/EBPα (Figure 3). While the C/EBPα knockout mouse shows impaired adipose development, and C/EBPα is characterized as an important component in adipogenesis [72], it is not essential to adipose development [73]. The one factor that has been determined necessary and sufficient for differentiation is Peroxisome Proliferator-Activated Receptor-γ (PPARγ). PPARγ is a nuclear receptor that heterodimerizes with RXR to regulate genes. Following mitotic clonal expansion, PPARγ is upregulated by C/EBP β & δ. PPARγ has two isoforms, PPARγ1 and PPARγ2, due to both alternate promoter utilization and alternate splicing. PPARγ2 is the predominant form found in adipose tissue [74]. The endogenous ligand for PPARγ is still controversial, although several lipid-like ligands have been shown to bind the receptor, as reviewed in [69]. The PPARγ knockout mouse does not survive due to a placental defect in the mother [75, 76]. PPARγ+/- mice are resistant to obesity even when fed a high fat diet.

Genome-wide chromatin immunoprecipitation revealed PPARγ and C/EBPα function together to regulate several downstream targets, changing the expression profile in the pre-adipocyte to that of an adipocyte (Figure 3) [77].

Figure 3. Schematic of adipocyte differentiation. Adipocytes are derived from multipotent mesynchymal stem cells that reside in adipose stroma. Cells are treated with BMP4 in vitro to commit them to the pre-adipocyte lineage. Cells are grown to confluency to achieve contact inhibition, where they subsequently exit the cell cycle. Treatment with a differentiation cocktail including dexamethasone, insulin, and isobutylxanthine triggers the cells to re-enter the cell cycle and undergo two rounds of mitotic clonal expansion. A transcriptional cascade upregulates adipogenic genes and the cell enters terminal differentiation and morphological changes occur.


Mesynchymal stem cell
Commitment
BMP4
Myoblast
Osteoblast
Pre-adipocyte
Chondrocyte
Differentiation cocktail
Mitotic clonal expansion
↑C/EBPβ/C/EBPδ
PPARγ
C/EBPα
Adipose Specific Genes
Differentiation
Fibroblastic morphology
Rounded up morphology
Lipid accumulation

Lipid storage and metabolism

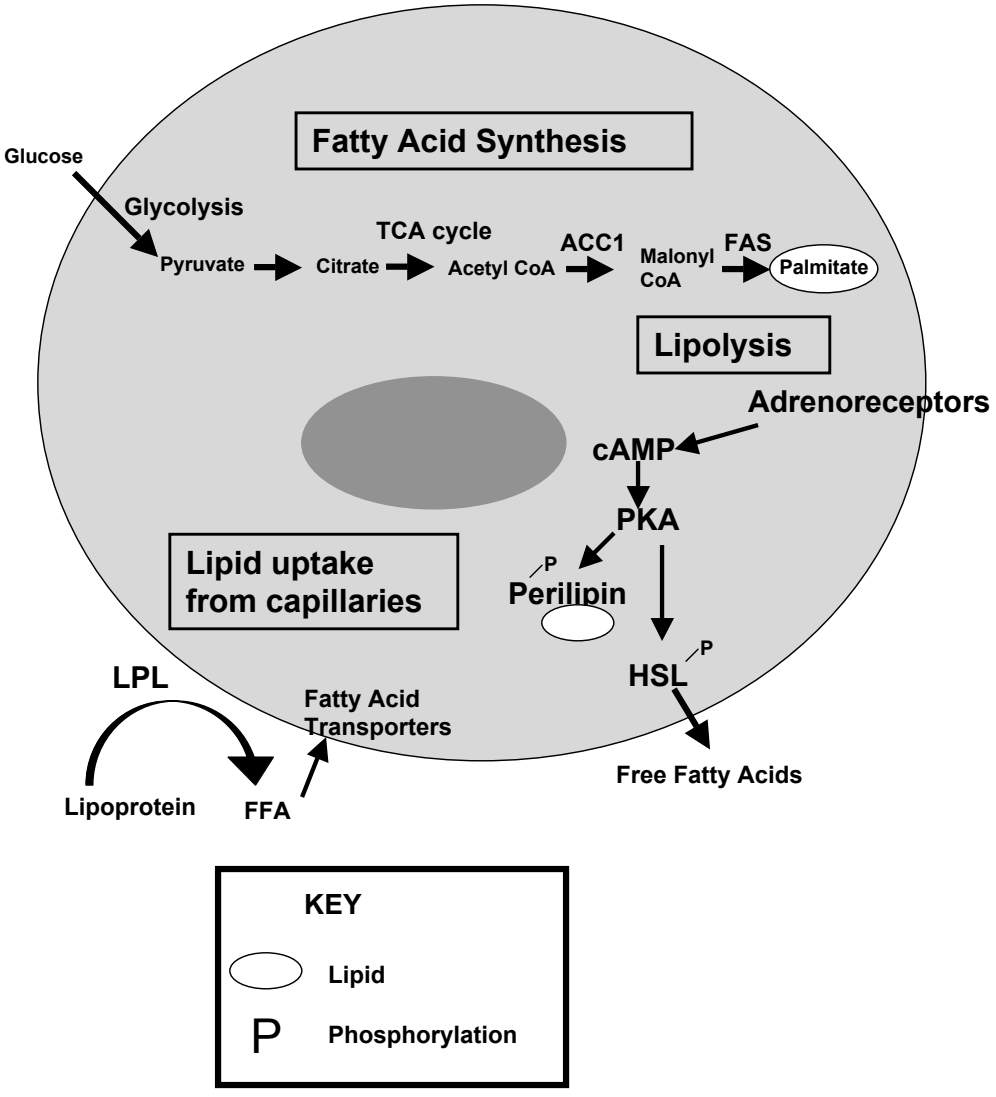
Adipose size is determined by the rate of lipogenesis, the accumulation of lipids, and the rate of lipolysis, which is the breakdown of lipid to be used as energy (Figure 4). Lipid accumulation within the adipocyte is a complex process that is promoted by one crucial enzyme, liproprotein lipase (LPL) [78]. LPL is found in capillary walls and functions to hydrolyze triglycerides, removing the glycerol backbone from fatty acids and allowing their uptake into the cell. Free fatty acids may diffuse passively into the adipocyte or be transported into the cell by fatty acid transporters [78].

To efficiently store energy, adipocytes can also synthesize fatty acids from acetyl coA. Acetyl coA carboxylase (ACC1) is the rate limiting enzyme that initiates fatty acid synthesis by converting acetyl coA to malonyl coA (Figure 4). Subsequent reactions involving fatty acid synthase convert malonyl coA into palmitate, which is then stored within the adipocyte.

Lipolysis is the breakdown of lipids and is dependent upon the enzyme hormone sensitive lipase (HSL) [79]. This enzyme catalyzes the hydrolysis of triglycerides allowing for their breakdown and subsequent use for energy. HSL increases following β-adrenergic signaling. The increase in intracellular cAMP initiates a phosphorylation cascade that activates HSL [80, 81].

There are several adipose depots distributed throughout the human body and the location of fat deposition impacts a person's health [82, 83]. Subcutaneous fat pads are those just under the skin throughout the body.

Figure 4. Lipogenesis and lipolysis in the adipocyte. Adipocytes can accumulate lipid through two mechanisms 1) fatty acid synthesis where they convert glucose into a fatty acids or 2) lipid uptake from the bloodstream. In fatty acid synthesis ACC1 converts acetyl coA into malonyl coA which is converted into palmitate by FAS and stored as lipid in the adipocyte. Lipid uptake requires the LPL enzyme to hydrolyze liproproteins from the blood stream releasing fatty acids to either diffuse or be transported into the adipocyte. Lipolysis, or the breakdown of lipids, is stimulated by catacholmine binding to adrenergic receptors on the cell surface increasing intracellular cAMP, which activates protein kinase A. Protein kinase A phosphorylates perilipin and HSL. Perilipin coats the lipid droplets within adipocytes in its unphosphorylated state. Upon its phosphorylation, perilipin releases the lipids to be broken down by HSL. Hydrolysis of triglycerides by HSL releases the fatty acids into the blood stream for transport to peripheral tissues for oxidation.


Fatty Acid Synthesis
Glucose
Glycolysis
Pyruvate
TCA cycle
Citrate
Acetyl CoA
ACC1
Malonyl CoA
FAS
Palmitate
Lipolysis
Adrenoreceptors
cAMP
PKA
P
Perilipin
Lipid uptake from capillaries
P
HSL
LPL
Fatty Acid Transporters
Lipoprotein
FFA
Free Fatty Acids
KEY
Lipid
P
Phosphorylation

Visceral fat pads are those surrounding internal organs such as the heart (pericardial), kidney (perirenal), and stomach/ intestine (omental), and thus are located in the intra-abdominal region. Visceral adipose tissue is located centrally on a human and leads to what is termed an “apple shape”, which increases the risk of cardiovascular and metabolic disease [82, 83]. Energy metabolism is regulated differently in different fat pads, as reviewed in [84]. Visceral adipose tissue is more vascularized and inervated than subcutaneous adipose [85]. However, subcutaneous tissue has larger adipocytes and is more advantageous for energy mobilization in a time of need. Viseral fat can be mobilized more rapidly for a short-term event, whereas subcutaneous fat is an energy source for chronic events as reviewed in [84]. Males tend to accumulate more visceral fat while pre-menopausal females tend to accumulate more subcutaneous fat [86]. This is largely due to steroid hormones as will be discussed below.

Estrogen signaling

Estrogen was traditionally thought to be a female reproductive hormone. We now know that it encompasses many more physiological roles in both the male and the female to maintain a homeostatic environment. Testosterone is converted to estradiol by the aromatase enzyme, thus controlling local estradiol production as reviewed in [87, 88]. Estradiol is a lipophilic ligand that passively diffuses through the cell membrane as well as the nuclear membranes of cells. In classic estrogen signaling, estradiol diffuses through the nuclear membrane, binds and activates the estrogen receptor (ER) by eliciting a conformational

change that removes its chaperone heat shock protein 70 (HSP70) as reviewed in [9, 10, 89]. This allows ER to homodimerize, and the dimer can then bind DNA directly at an estrogen response element (ERE) or by tethering through other transcription factors at an ERE half site, AP1 site, or SP1 [8, 11, 90-93]. These sites can be found near the proximal promoter or up 150 Kb away from the promoter, making them difficult to identify [8, 90]. Following ER binding to DNA, co-activators, co-repressors, and/or chromatin modifiers are recruited to transactivate or repress the gene. The receptor is then polyubiquinated and subsequently degraded by the 26S proteosome [94, 95]. ER turnover is important for efficient estrogen signaling [96, 97].

In some cases, estradiol binds the estrogen receptor in or near the plasma membrane to elicit rapid signaling events through phosphorylation cascades where activation can be observed in seconds not hours. In addition to the classical receptors in the membrane mediating some of these rapid effects, other receptors specific to the plasma membrane have been identified. One such candidate receptor is GPR30, a 7-transmembrane G-protein coupled receptor that binds estradiol with a lower affinity than classic ER, but can elicit non-genomic rapid signaling events [98].

There are two ERs encoded by two different genes: ERα [99, 100] and ERβ [101]. These receptors have high sequence conservation in the DNA binding domain and ligand binding domain, but are distinct in their activation domains: AF1, located in the N-terminal region, and AF2, adjacent to the ligand binding domain [102]. Activation of AF1 can occur in the absence of ligand [103-

105] whereas AF2 can only be activated by ligand binding [106, 107]. The predominant ligand for both receptors is 17β-estradiol [108], although selective estrogen receptor modulators can also bind and have receptor-specific effects, as reviewed in [10]. If both ERα and ERβ are expressed in the same tissue, they can form heterodimers [102].

ERα and ERβ have distinct expression profiles and differ in their level of expression. ERα appears to be the predominant receptor in classical estrogen signaling, particularly in the uterus, where ERβ has very low expression in this tissue [108]. Both isoforms are expressed in adipose tissue with ERα being the predominant receptor [109-111]. However, only ERα is found in pre-adipocytes [112]. Both male and female ERα KO mice are obese and display metabolic defects [113]. ERβ KO mice have normal body weight and adipose tissue mass until after sexual maturity, when they begin to accumulate adipose mass [114, 115]. The ratio of ERα and ERβ may be important in the physiological response [112, 116].

Estrogen signaling in adipose tissue

In a pre-menaupausal woman, estradiol is primarily produced in the ovaries, whereas in postmenopausal women it is primarily produced in adipose tissue [87]. The identification of ERα and ERβ in adipose tissue [110, 117] implied that estradiol has a physiological role there. It is now well documented that estrogen signaling regulates adipose metabolism at several levels. Estrogen regulates the size of adipocytes, the number of adipocytes, the deposition of fat,

and the site-specific metabolism of adipose tissue, as reviewed in [84, 86, 118, 119].

Estrogen limits the number of adipocytes by blocking adipogenesis [120]. ERα is present in pre-adipocytes and regulates several genes involved in proliferation. *In vitro* studies demonstrated that pre-adipocytes require a growth arrest and exit from the cell cycle to differentiate as reviewed in [64, 68]. Generally estrogen is pro-proliferative and while it may increase the number of pre-adipocytes [121], it inhibits their ability to differentiate. This is probably a consequence of the regulation of several cell cycle factors including upregulation of cyclin dependent kinase inhibitors such as p21 and p27 [118].

Estrogen decreases the size of adipocytes by decreasing lipogenesis and increasing lipolysis. Estrogen signaling regulates lipogenesis in part through downregulation of the LPL gene via binding at an AP-1 like site in its promoter [122]. As mentioned above, LPL is the rate-limiting enzyme that hydrolyzes triglycerides to allow their uptake into adipocytes. Females have lower LPL levels and thereby store less fat from food consumption in adipocytes. Estrogen also increases fatty acid oxidation in muscle tissue by upregulating key factors such as PPARδ [123] and UCP 2 and 3 [119]. Furthermore, estrogen signaling increases AMP-activated Kinase (AMPK) signaling, preventing de-novo lipogenesis [123].

Estrogen signaling regulates the location of fat deposition [124]. Premenopausal women store more lipid in subcutaneous fat compared to men, who store lipid in visceral fat as reviewed in [86]. This is a consequence of

decreased lipolysis in subcutaneous fat and increased lipolysis in visceral fat in women. In addition, free fatty acid uptake is much higher in subcutaneous fat in women [84]. This depot-specific regulation is in part due to the ratio of α-2 adrenoreceptors to β-3 adrenoreceptors. Estradiol upregulates α-2 adrenoreceptors via ERα signaling in subcutaneous fat, preventing the breakdown of lipids [125]. Meanwhile, in visceral fat estradiol increases β-adrenergic signaling, stimulating lipolysis [125]. As a result, females preferentially store and maintain fat subcutaneously.

In addition to affecting both lipogenesis and lipolysis in the adipocyte, estrogen reduces adipose accumulation through the central nervous system (CNS) by decreasing food consumption and increasing energy expenditure. Removal of ERα in the hypothalamus of mice resulted in obesity and metabolic defects [126]. These mice displayed increased food consumption and decreased energy expenditure through both physical activity and thermogenesis [126]. Estrogen is thought to achieve this by regulating key neuropeptides in the brain and interacting with the leptin signaling pathway [84, 86, 127], as discussed below.

In addition to attenuating obesity, estrogen also promotes metabolic homeostasis by improving insulin sensitivity and regulating adipokine production. While women have less skeletal muscle and more fat, they are still less likely to develop insulin resistance [128]. Insulin is a sensor that maintains glucose homeostasis in the body. Insulin primarily regulates serum glucose levels and catabolism. In the presence of high serum glucose, pancreatic β-cells produce

and secrete insulin. Insulin binds receptors on the surface of peripheral tissues and adipocytes that initiate several signaling cascades that result in glucose storage and uptake as reviewed in [84]. Levels of insulin secretion are positively correlated with visceral adipocity [84, 86]. Visceral adipose tissue is not sensitive to insulin signaling [129, 130] and as a consequence contributes to metabolic syndrome [133]. When tissues exhibit reduced sensitivity to insulin, insulin can no longer maintain homeostatic glucose levels. Estradiol signaling indirectly maintains insulin sensitivity by increasing glucose uptake [134] and suppressing hepatic glucose production [128] as well as protecting pancreatic β-cell function [135].

Adipocytes secrete hormones and cytokines, often termed adipokines, that function to maintain metabolic homeostasis. In an environment with increased inflammation and cellular stress such as obesity, these proteins can contribute to metabolic disorders [136, 137]. Obesity is characterized as a state or chronic inflammation and macrophage infiltration [138, 139]. As a result the secretion of pro-inflammatory cytokines from adipocytes is increased. In addition, chronically elevated levels of these cytokines can impair their function and result in a resistant state. Estrogen modulates the levels of several adipokines at the transcriptional level and thus is often correlated with the circulating levels [84, 127, 128, 140] (Table 1). However, there is conflicting data that is probably the result of tissue, model, and context-specific regulation. The function of adipokines and their regulation by estrogen may be altered in an obese state.

Table 1. The regulation of adipokine expression by estrogen. Estrogen regulates a number of cytokines and is correlated to their circulating levels. This table identifies some of the observations regarding the regulation of adipokines by estrogen in mice.

Adipokine	Obesity	Function	Change in adipokine levels with estrogen
Adiponectin	↓	sensitizes insulin signalling, stimulates fatty acid oxidation	↓ mRNA [195] ↑ mRNA [196],
IL-6	↑	lipid and glucose metabolism, improves insulin sensitivity, B & T-cell activation, immune response, osteoclastogenesis	↓ mRNA [131; 132]
Leptin	↑	supress appetite, increases erengy expenditure	↑ mRNA [140], ↑ circulation
Resistin	↑	inhibits adipogenesis, increases insulin resistance	↓ mRNA [123; 199; 200] ↓ protein [199] ↓ circulation [123]
TNFα	↑	increases lipolysis, impaires insulin signalling	↓ mRNA [194]

The most studied and possibly the most important adiopkine is leptin. Leptin serves as an important energy sensor in the body. It is secreted from the adipocyte and maintains homeostasis by decreasing energy consumption and increasing energy expenditure through regulation of neuropeptides in the hypothalamus [141]. Estrogen signaling increases leptin expression [142] and improves leptin sensitivity [143]. In a fed state, leptin levels are high and in a fasting state, leptin expression is decreased [141]. Chronic high levels of leptin can be detrimental, as a hyperleptinemic state causes leptin resistance [144-146]. Furthermore, leptin increases insulin levels, consequently hyperleptinemia can lead to insulin resistance [146].

In summary, estrogen opposes obesity through a decrease in both the number and the size of adipocytes. This is accomplished through a decrease in feeding, reduction in lipid uptake, a reduction in *de novo* lipogenesis as well as an increase in fatty acid oxidation in adipose and peripheral tissues. In addition it is protective against metabolic defects by improving insulin sensitivity, improving leptin sensitivity, promoting adipose deposition to subcutaneous tissue, and decreasing inflammatory cytokines.

Conclusion

While it is clear that estrogen regulates energy homeostasis at several levels, little is known regarding the mechanisms by which it accomplishes all of these functions. Activated estrogen receptor has been shown to directly interact

with an ERE on very few genes [8, 9, 90, 92]. Genome wide ChIP-ChIP, while arguably limited by the cell type, shows that ER does not directly bind to all the genes whose expression is altered in the presence of estradiol [8]. Estrogen also regulates some events such as hormone sensitive lipase -mediated lipolysis through non-genomic mechanisms [81, 125]. Yet this only accounts for a few of estrogen's actions in metabolism. Several metabolic outcomes of estrogen are likely through indirect mechanisms that are yet to be identified. To understand sexual dimorphism in energy homeostasis and determine proper treatment of metabolic disorders in men versus women, comprehensive knowledge of the factors involved must be identified.

Estrogen upregulates the expression of the transcription factor ZEB1 [4-7], a modulator of mesenchymal tissues [16, 30, 36, 40, 41, 44-46]. This finding combined with genetic evidence from genome-wide scans [52, 147-149] implicates *TCF8* [1] as a likely candidate "anti-obesity" gene in humans. Obesity is a complex disease state that is rarely the result of the gain or loss of one gene, but it is more likely the result of disregulation of several genes involved in energy homeostasis [150]. Identifying such genes will provide a better understanding of the complexity of metabolic disorders and provide new targets for treatment. This thesis demonstrates that *TCF8* is a novel anti-obesity gene and that its resultant protein product ZEB1 mediates some of estrogen's protective effects.

STATEMENT OF RESEARCH

We hypothesized that the TCF8 gene, which encodes the transcription factor ZEB1, is protective against obesity. This was based on genetic evidence linking a region on chromosme 10p11-12 where TCF8 resides to obesity, as well as the knowledge that ZEB1 modulates the development/differentiation of mesenchymal tissues. I have used three major approaches to address whether or not *TCF8* is an anti-obesity gene. I have: 1) identified a polymorphism in the TCF8 gene within a cohort of obese patients who demonstrate linkage between the *TCF8* genomic locus and childhood obesity, 2) shown that mice heterozygous for *TCF8* (*TCF8+/-)* accumulate fat more readily than wild type (WT) controls, and 3) Observed changes in adipogenesis and lipid accumulation in adipogenic cell lines when levels of ZEB1 are manipulated. These data collectively identify a novel mediator of adipose accumulation and lipid metabolism and demonstrate the importance of the ZEB1 transcription factor in adipose biology.

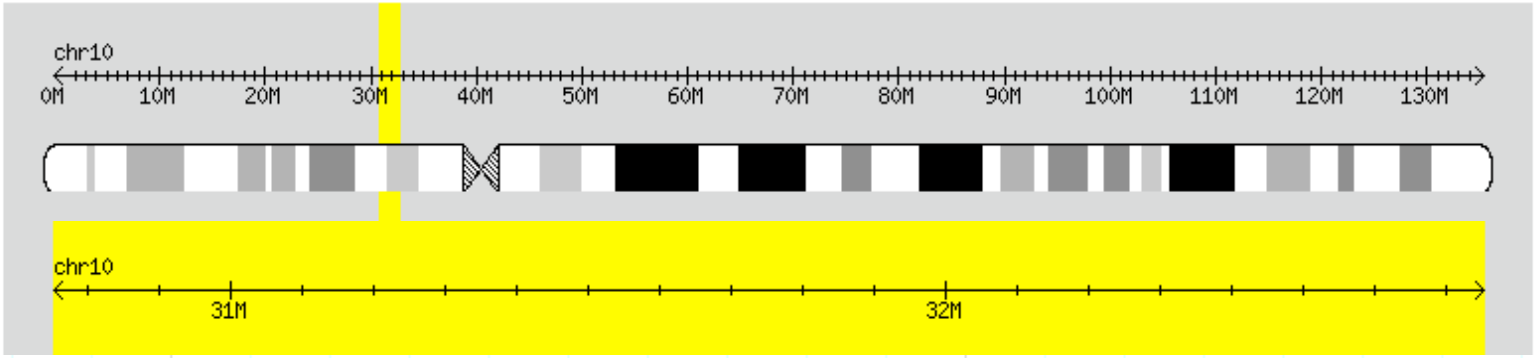
CHAPTER II

Investigating polymorphisms in the *TCF8* gene that link to childhood obesity

INTRODUCTION

Obesity is an international health concern that puts individuals at risk for developing cardiovascular disease, hypertension, type II diabetes, and cancer. While obesity is largely due to environmental factors, it has a sizeable genetic component estimated to be 40-70% [151]. Human obesity is rarely defined by a single gene disorder, as reviewed in [150]. Monogenic contributers to obesity such as the *ob* gene (encoding leptin), leptin receptor, proopiomelanocortin (POMC), and melanocortin 4 receptor (MC4R) only account for a fraction of obese individuals, as reviewed in [152]. Instead, numerous loci are correlated with a predisposition to obesity. This polygenic disease state is complex, with the prediction being that a number of genes are still unknown [153]. To identify genes that may pre-dispose a person to obesity, a number of genome-wide scans on obese individuals and their families have been done. To reduce the influence of environmental background, these studies are often done on children. Of interest to our research, four independent studies using European, American, and African American cohorts linked obesity to a gene located somewhere on chromosome 10p11-12 [147-149, 154]. A causal gene has yet to emerge as the in this region. The *TCF8* gene, which resides at 10p11.2 (Figure 1) attenuates adiposity in mice (Saykally et al. Manuscript in revision Chapter III; Saykally and Sanders manuscript in preparation Chapter IV) and is a likely candidate. *TCF8* encodes the transcription factor Zinc finger E-box Binding homeodomain protein 1 (ZEB1).

Figure 1. Map of genes on chromosome 10p11-12. Seven genes within the 23 cM region of linkage from Hinney et al [148] as reported by the National Center for Biotechnology Information.



NM_005204

MAP3K8: Mitogen-activated protein kinase kinase kinase

NM_030751

TCF8

NM_025209

EPC1: enhancer of polycomb 1

NM_183058

LYZL2: Lysozyme-like 2

NM_018287

ARHGAP12: Rho GTPase activating protein 12

NM_182755

ZNF438: Zinc finger protein 438

NM_004521

KIF58: Kinesin family member 5B

ZEB1 is highly expressed in mesenchymal tissues, including adipose tissue [5, 37]. It binds DNA at E-box consensus sites and positively or negatively regulates gene expression [24, 29]. Through the regulation of a number of genes, ZEB1 impedes the differentiation of bone [17, 36, 43, 44], muscle [30, 45], and chondrocyte lineages [16, 47, 48], suggesting that it may also attenuate adipose development.

I hypothesized that mutation(s) in *TCF8* gene result in a loss of function mutation that predisposes individuals to obesity. Using familial DNA obtained from the Hinney group in Germany [148], we show that the *TCF8* gene is linked to childhood obesity. Due to linkage disequilibrium in this region, linkage does not confirm that *TCF8* is causal. Direct sequencing of human genomic DNA of obese patients in this cohort identified polymorphisms that may disrupt the function of the ZEB1 protein.

MATERIALS AND METHODS

Subjects. Obese families were recruited in German hospitals and DNA collected as described in [148]. Probands were severely obese children or adolescents with a body mass index (BMI) in the 95th percentile and had an obese sibling whose BMI was in the 90^{th} percentile. The probands ranged from 7.59 to 22.05 years of age [148]. This study was designed for sibling pairs linkage analysis [155-157], and it adhered to all state, national, and UMN privacy protocols.

Fine mapping linkage. Using 15 known single nucleotide polymorphisms (SNPs) throughout the *TCF8* genomic locus, we genotyped patients using iPLEX SEQUENOM. This technique determines the allele at a specific SNP using mass spectrometry. Sequenom was performed per the manufacturers instructions (www.sequenom.com). In short, primers with a 5' tag were designed to amplify the SNP from the patient DNA (Table 1). HotStarTaq, Qiagen (Germany) was used to amplify the region around the SNP. ExoSAP (Exonuclease 1 and Shrimp alkaline phosphatase) (USB) digestion was performed to degrade any primers or extra nucleotides. A second set of extension primers was designed to bind specifically to the first set of primers and anneal to the base pair 5' of the SNP (Table 2). A one base pair extension reaction incorporates the SNP into the final product. The mass of this product was determined using MALDI-TOF Mass Spectrometry, allowing the identity of the SNP to be determined. These reactions were carried out by the Biomedical Genomics Center at the University of Minnesota.

Statistical analysis. A multiple affected offspring approach was used for analysis as described in Hinney *et al* [148]. In brief, this is a family-based association analysis where the alleles transmitted versus the alleles not transmitted to the affected offspring are compared. This course of action is similar to a matched case-control approach with the advantage of using internal controls and comparing them to population stratification [157]. Multipoint

logarithim of the odds (LOD) for linkage between obesity and the SNP marker was calculated using GeneHunter.

Table 1: Primers used to amplify SNPs within TCF8 locus. List of natural occurring SNPs and corresponding tagged primers used to amplify the SNP to genotype patient DNA.

SNP	TagPrimer
rs172683 (2)	ACGTTGGATGGTCCACAGTAGATTGGAAAC
rs17295696 (2)	ACGTTGGATGGGACACTGGAGATAGGTTTC
rs11008506 (2)	ACGTTGGATGCAGGCATTCCAGAATGCAAG
rs161260 (2)	ACGTTGGATGTGCAAATGCATGAGGATCAG
rs2839664 (2)	ACGTTGGATGCCCTATAGTAGAGCAGGTTC
rs12783820 (2)	ACGTTGGATGGGTTAATGGGGATCCTTCAG
rs11008510_1618092 (2)	ACGTTGGATGTGTTCTGTAAGTGTCTCACA
rs11008508 (2)	ACGTTGGATGTAGGGCAGGGAAAATGGAAG
rs696206 (2)	ACGTTGGATGCCAGTTCATTGCCTATGTAG
rs17295689 (2)	ACGTTGGATGACACATGCATCTAGGAGAAC
rs161259 (2)	ACGTTGGATGATATCTTCTGACAAGGTGAG
rs7476833_4285779 (2)	ACGTTGGATGGAAAGAACTCAAATGTTACC
rs2839657 (2)	ACGTTGGATGCGCACTGGGAAAATAACCTC
rs11008500 (2)	ACGTTGGATGGCAGACTTTACTTATGAAGG
rs9299657 (2)	ACGTTGGATGAATCAGCTTCCCTAGATCCC
rs172683 (1)	ACGTTGGATGTACCCTAGGCAGAGCTTTTC
rs17295696 (1)	ACGTTGGATGCTGTTAAAACCCAGCTTAGG
rs11008506 (1)	ACGTTGGATGACTAGGAAGGGTATAGCCTC
rs161260 (1)	ACGTTGGATGGCCGCTTATAGATAACTCAAG
rs2839664 (1)	ACGTTGGATGCAGTTGTGTTTCTTTCCCTC
rs12783820 (1)	ACGTTGGATGATCTTACAAAAGCACTGC
rs11008510_1618092 (1)	ACGTTGGATGCAGATCATGTAGAAAGACAC
rs11008508 (1)	ACGTTGGATGGCCGCTGCACCTGAAATTTA
rs696206 (1)	ACGTTGGATGCAGGCATACACATTAGAAAGG
rs17295689 (1)	ACGTTGGATGTGGTATTTACTAACTGGCCC
rs161259 (1)	ACGTTGGATGTGCAGCTCTTCTGGAATGTG
rs7476833_4285779 (1)	ACGTTGGATGGTGTATCATTTGTTCCTTTC
rs2839657 (1)	ACGTTGGATGTTCCCAGCGTATACTGCTTG
rs11008500 (1)	ACGTTGGATGGTGTTGAGGAAAGTATTTGG
rs9299657 (1)	ACGTTGGATGCTGACAATCTGCACAGAAAAG

Table 2: SEQUENOM extension primers. List of primers designed to the tagged amplification primers for the corresponding SNP. Underlined SNPs show linkage with childhood obesity.

SNP	Extension Primer
rs172683_ext	GAACCAAACAAAGCCAA
rs17295696_ext	GGGAGGCAATGTCTAAGC
rs11008506_ext	CAGAATGCAAGACTCCCTG
rs161260_ext	TAATTTACCTCTTCCCAAAC
rs2839664_ext	AGTAGAGCAGGTTCCCATAG
rs12783820_ext	GAGGAGGAGTTGATAGAATG
rs11008510_1618092_ext	CTGTAAGTGTCTCACATCCATT
rs11008508_ext	GGAAGATGAGCCTAGAATAGTA
rs696206_ext	GTAGACAGTCTTGTTTTCTGCAA
rs17295689_ext	CTTAAAAACTGTACTTTAAACCAC
rs161259_ext	AATGGTTGACCATAGAGAAAACCG
rs7476833_4285779_ext	AAAGAACTCAAATGTTACCACTATA
rs2839657_ext	CTCCCACTTTCTTCCAAGGATCACTG
rs11008500_ext	AGACTTTACTTATGAAGGAAATATTA
rs9299657_ext	AAGGGGGTATTGAAAATGCTAGGAGA

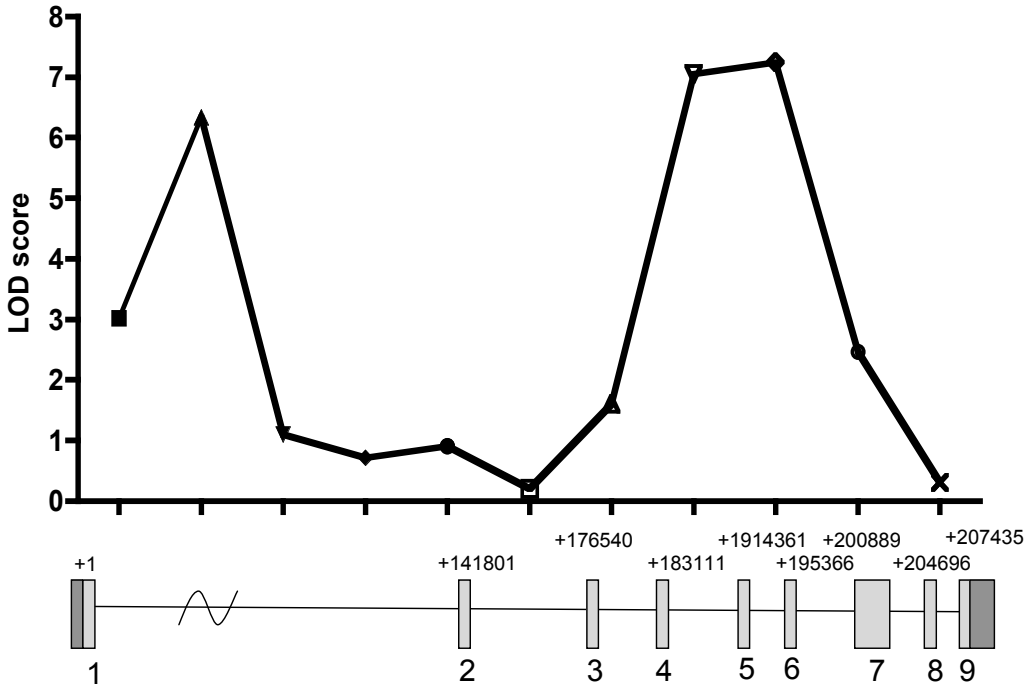
Sequencing. Exons in the *TCF8* gene were amplified from patient genomic DNA using iProof High Fidelity Master Mix (BIORAD #172-5310, Hercules, CA) and primers from [52]. Excess primers and nucleotides were removed by incubation with ExoSAP (USB #78201 78201, Cleveland, OH) at 37 °C for 15 min followed by enzyme inactivation for 15 min at 80°C. Sequencing was performed by the University of Minnesota Biomedical Genomics Center facility with ABI BigDye Version 3.1 (Applied Biosystems) read with a 3730xl. Sequence alignment was done using Biology Workbench (http://workbench.sdsc.edu) and screened for polymorphisms manually.

RESULTS

Sequenom analysis further confirms TCF8 linkage to obesity

Linkage of obesity with chromosome 10p11-12 is a broad region that encompasses several genes. The total peak of linkage from Hinney *et al* was 23 cM. The highest logarithim of the odds (LOD) score was 2.45 at a marker SNP in the *TCF8* gene [148]. The 2 cM surrounding this marker SNP encompasses 7 genes (Figure 1). In effort to fine map the linkage of the TCF8 gene, we performed iPLEX SEQUENOM [158-160] analysis on the patient population used in Hinney *et al*. Extreme obese sibling pairs and their nuclear families were used for this study. Presumably each family member is exposed to similar environmental conditions.

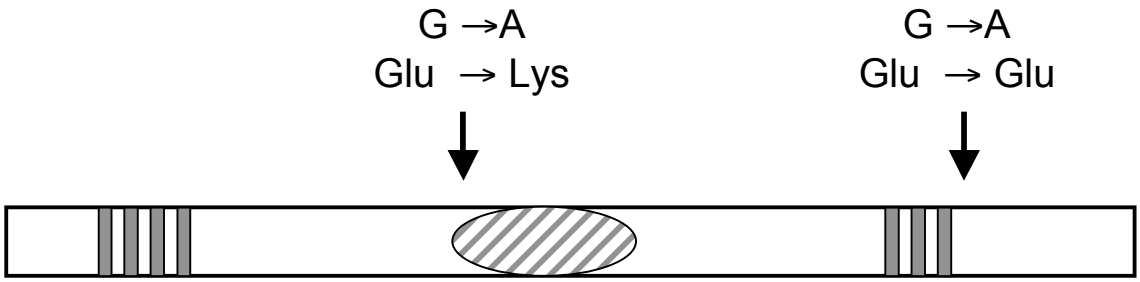
Figure 2. Logarithim of the odds for linkage between the SNP and childhood obesity. iPLEX SEQUENOM identification of the SNP maximum likelihood binomial logarithim of the odds score reported along the TCF8 gene.


LOD score
8
7
6
5
4
3
2
1
0
+1
+141801
+176540
+183111
+1914361
+195366
+200889
+204696
+207435
1
2
3
4
5
6
7
8
9

***TCF8* gene**

We used 15 tagged SNPs that provide 95% coverage of the *TCF-8* genomic locus (including introns, exons, and untranslated regions) (Table 1). LOD was calculated for linkage between the trait and the SNP marker. Analysis revealed two regions of significant linkage to childhood obesity (Figure 2). We saw linkage with the 5' region of the gene at two marker SNPs with a LOD score of 3.02 and 6.33. We also found linkage at two marker SNPs in the 3' region of the gene both with a LOD score over 7.0. Unfortunately, due to the proximity to the centromere, the *TCF8* genomic region has fewer recombination events than genes located closer to the telomeres and thus may be in linkage disequilibrium with several other genes. Consequently, this does not rule out that a different gene within the same haplotype is causal. Nonetheless, these data provide further evidence that the *TCF8* gene maybe linked to childhood obesity.

Figure 3. Polymorphisms within the TCF8 genes of obese patients. The coding region of the *TCF8* gene was sequenced in 20 obese patients. Two polymorphisms were detected G→A bp 1740, and G→A bp 3030. A polymorphism at bp 1740 that results in a glutamic acid to lysine amino acid change within the homeodomain was observed in 50% of the patients sequenced. A presumably silent polymorphism occurred at bp 3030


G →A
Glu → Lys
G →A
Glu → Glu

DISCUSSION & FUTURE DIRECTIONS

Knowledge of all the crucial genes that predispose a person to obesity will improve our understanding of what contributes to obesity and what treatments are available for individuals facing obesity. Monogenic obesity accounts for a miniscule number of obese cases in humans [152]. Most obesity is the result of a complex polygenic disorder that predisposes a person to obesity [150]. Genome-wide linkage studies found linkage to obesity on chromosome 10p 11-12 [147-149, 154]. A marker SNP in the *TCF8* locus showed the highest LOD score, and our data showing that ZEB1 attenuates obesity in mice (Chapter III and IV) made *TCF8* a good candidate "anti-obesity" gene in humans.

To further examine the *TCF8* locus, I fine-mapped it using 15 SNP markers and found two regions of linkage (Figure 2). However, due to the centromeric location of the gene, recombination events are infrequent resulting in high linkage disequilibrium. Consequently, *TCF8* is transmitted, and thus linked, with several other genes. This result supports the contention that *TCF8* is an anti-obesity gene, but it is not confirmative. Sequencing of the coding regions was performed on a subset of the patients, and two polymorphisms were identified. The polymorphism in exon 7 was found in over 50% of the patients sequenced (Figure 3). This polymorphism was not a natural variant recorded with the National Center for Biotechnology Information (NCBI). This polymorphism results in a lysine residue instead of a glutamic acid. Both of these amino acids have similar charge and mass, suggesting that this polymorphism would not disrupt the structure. However, there is too little known

regarding the proteins that interact with ZEB1 and how the homeodomain is involved to determine whether this polymorphism could have merit. This polymorphism could disrupt interactions with specific proteins changing the dynamic on promoters of a subset of genes.

These data are an important contribution to our understanding of the ZEB1 protein and its role in human obesity. However, confirmation of the polymorphism itself and functional analysis must be done to determine whether this mutation has any deleterious effects. A more comprehensive sequencing study should be performed, including confirmation of the polymorphism, sequencing of the reverse strand to ensure that all mutations are identified, and sequencing of regulatory regions. Based on what is known from mice, a decrease in the expression of ZEB1 could contribute to obesity (Chapters III, and IV)). In addition, while this polymorphism could have a functional consequence, it is also likely that another mutation may exist in the regulatory regions. The flanking and proximal promoter 5' region, the first intron, and the 3' UTR should be sequenced to determine whether a polymorphism(s) exists that would reduce the expression levels of ZEB1 as we have shown that reduction of ZEB1 levels in mice results in increased adipose accumulation.

Functional analysis is necessary to assess whether the polymorphism in exon 7 affects the function of ZEB1. However, this is easier said than done considering the limited information that is available in regards to the homeodomain function. Nothing is known about its interactions, if any, with other proteins and whether tissue- and cell-specific context is relevant. Modeling of this

mutation should be done *in vitro*, and reporter assays using known ZEB1 target genes used. Due to the caveats just listed, a number of downstream targets should be tested in a number of cell lines. While an adipocyte cell line may be the best context to test a mutation, it may also have a high background of endogenous ZEB1 expression. A more appropriate start may be to use a cell line where endogenous ZEB1 is present but in a lower concentration. Because ZEB1 is known to attenuate differentiation of other mesenchymal tissues, an experiment should be done to test whether this mutation has any effect on the differentiation of adipocytes.

In summary, a polymorphism was identified in a gene that shows linkage to childhood obesity. While these data are extremely preliminary, they support the concept that *TCF8* as a putative "anti-obesity" gene. Further studies must be done to both confirm the sequence analysis and determine whether this polymorphism has functional relevance.

ACKNOWLEDGEMENTS

This work was done in collaboration with Anke Hinney and Susan Friedel of the Department of Psychiatry University of Duisburg-Essen, Germany. They provided the human patient DNA samples, without which this work could not have been done.

CHAPTER III

The ZEB1 Transcription Factor Is a Novel Repressor of Adiposity in Female Mice

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Running Head:

ZEB1 opposes fat accumulation

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SUMMARY

Background: Four genome-wide association studies mapped an 'obesity' gene to chromosome 10p11-12. As the ZEB1 (zinc finger E-box binding protein 1) transcription factor is located in that region and as it influences the differentiation of various mesodermal lineages, we hypothesized that ZEB1 might also modulate adiposity. The goal of these studies was to test that hypothesis in mice.

Methodology/Principal Findings: To ascertain whether ZEB1 correlated to increased fat accumulation, female C57BL/6 mice were fed a regular chow diet (RCD) *ad libitum* or a 25% calorie-restricted diet from 10-73 weeks of age. ZEB1 mRNA levels in parametrial fat were 6- to 10-fold higher in the obese mice. To determine directly whether ZEB1 affects adiposity, wild type (WT) mice and mice heterozygous for *TCF8* (*TCF8*+/-) were fed a RCD or a high fat diet (HFD, 60% calories from fat). By two months of age on a HFD and by three months on a RCD, *TCF8+/-* mice were heavier than WT controls, which was attributed by EchoMRI to increased fat mass (at 3 months on a HFD: 0.517 $\pm$ 0.081 total fat/lean mass versus 0.313 $\pm$ 0.036; at 3 months on a RCD: 0.175 $\pm$ 0.013 vs 0.124 $\pm$ 0.012). This increase in fat was sufficient to cause impaired glucose uptake and increased circulating leptin levels. No differences were observed in food uptake or physical activity, suggesting that the genotypes differ in some aspect of their metabolic activity.

Conclusion: These results show for the first time that the ZEB1 transcription factor regulates the accumulation of adipose tissue. Furthermore, they

corroborate the genome-wide association studies that mapped an 'obesity' gene at chromosome 10p11-12.

INTRODUCTION

Obesity and its related metabolic disorders have become an international health concern, especially because of their alarming increase in the young [161]. Excessive white adipose tissue puts individuals at risk for medical conditions such as Type II diabetes, cardiovascular disease, and cancer. Therefore, considerable interest exists in defining the molecular pathways that regulate the development of adipocytes and their ability to store lipids in hopes of elucidating new preventative measures and treatment options.

Adipocytes primarily derive from multipotent mesenchymal stem cells (MSCs) that reside in bone marrow and the stroma of adipose tissue [162]. The conversion of MSCs to preadipocytes activates an extensive transcriptional cascade that regulates terminal differentiation (for recent reviews, see [163, 164]). While several transcription factors such as PPARγ (nuclear peroxisome proliferator-activated receptor gamma) and the C/EBPα family are key components of this cascade, other modulatory transcription factors continue to be discovered [165]. This report identifies one such transcription factor, the ZEB1 (zinc finger E-box homeodomain binding protein 1) transcription factor.

As much as 70% of obesity can be attributed to polygenetic traits [166], and numerous genome-wide linkage studies have attempted to define loci that segregate with a predisposition to obesity [147, 166-169]. To increase the

likelihood that these association scans are detecting genetic rather than environmental conditions, they are often done on children. Four studies [147-149, 154], two using children and young adolescents, are of particular interest as they found linkage in a region of chromosome 10 (10p11-12) that harbors *TCF8*, the gene that encodes the ZEB1 transcription factor. So far, no viable candidate obesity/anti-obesity gene in this region has emerged. We hypothesize that mutations or polymorphisms in *TCF8* contribute to childhood/adolescent obesity.

A number of lines of correlative evidence support the hypothesis that ZEB1 plays a role in adipogenesis and/or lipogenesis. Expression of ZEB1 is high in mesenchymal tissues, including adipose tissue [37]. Moreover, ZEB1 modulates the differentiation of the myogenic, osteogenic, and chondrogenic mesenchymal lineages [24, 36, 47, 170, 171] and can even direct which lineage differentiates from the multipotent C2C12 mesenchymal cell line [17]. Thus, it is reasonable to propose that ZEB1 can also affect the differentiation of the adipogenic lineage. Additionally, ZEB1 mRNA expression increases following adipocyte differentiation in cell culture [162], and it is higher in obese women compared to those of normal weight [172], however, nothing has been done as yet to investigate the significance of those observations. The goal of our studies was to determine whether ZEB1 affects adipose accumulation in mice.

ZEB1 (also called dEF1, Nil-2-a, AREB6) is a large transcription factor of 1117 amino acids in humans that is conserved from worm [27, 173] to man (for review, see [24]). ZEB1 binds to DNA via two zinc finger clusters at its N- and C-termini, and it recognizes target genes through a modified E-box sequence (5'-

CACCT(G)). Mechanistically, little is known about how ZEB1 regulates gene expression, but it can either repress or activate target genes [4, 38]. The regulation of ZEB1 is also understudied. We were the first to report that it is induced by estrogen [4], but it is also induced by progesterone [6], suggesting that ZEB1 plays an important role in female physiology. Interestingly, high expression of ZEB1 becomes independent of estrogen in endometrial and ovarian carcinomas [5]. Other regulators of ZEB1 include NF-kB and the TGF-β family [24]. Because *TCF8* null mice are perinatal lethal [38], even less is known about its target genes or normal physiological roles, especially in the adult. ZEB1 is predominantly known for its ability to modulate epithelial to mesenchymal transitions (EMT) in development and cancer by downregulating E-cadherin and other genes encoding cell adhesion and basement membrane proteins [56, 57, 174]. While the effects of ZEB1 on EMT have been studied extensively, its role in the development and differentiation of tissues is complex and less well understood (for review, see [24]). However, ZEB1 is at the intersection of multiple mesenchymal developmental pathways [24, 36, 47, 170, 171], raising the possibility that it also affects adipogenesis.

The experiments in this study were undertaken to directly investigate whether or not ZEB1 modulates adipogenesis and/or lipogenesis *in vivo* using female mice, which were chosen because estrogen induces ZEB1 [4-7]. As considerable data indicate that estrogen protects against weight gain [84, 86], it seems plausible that ZEB1 might mediate estrogen's effects on adiposity. The results with wild type (WT) mice demonstrate that ZEB1 mRNA levels in adipose

tissue increase dramatically with weight gain and plateau as the rate of weight gain decreases. To directly test whether ZEB1 affects this weight gain, mice heterozygous for *TCF8* (*TCF8*+/-) were fed a regular chow or high fat chow diet for up to 5 months, and the effects on body weight, fat mass, activity level, food intake, and various metabolic parameters were determined. The resulting data indicate that haploinsufficiency of ZEB1 is sufficient to elicit significant fat accumulation, regardless of diet. These data are the first to show that ZEB1 plays a protective role against obesity. They are also the first to support the genome-wide association studies that mapped a human 'obesity' gene to chromosome 10p11-12 [147-149, 154].

MATERIAL AND METHODS

Animals and genotyping. All experiments were done in accordance with the University of Minnesota Research Subjects Protection Program in adherence with federal, state, and local regulations. The IACUC protocol number is 0609A91913. All mice were maintained in a University of Minnesota specific pathogen-free animal facility, where light and temperature were regulated. *TCF8* null mice were generated by Dr. Yujiro Higashi and colleagues (Osaka University, Osaka, Japan) as was described previously [36, 38], and heterozygous (*TCF8*+/-) males were provided by Dr. Jennifer Richer, University of Colorado, Aurora, CO. The colony was generated and maintained on a C57BL/6 background. Female breeder mice were purchased from Charles River Biological Laboratories (Wilmington, MA). Genomic DNA was isolated from ear

snips, and polymerase chain reactions (PCR) were performed to determine genotype. To generate products, Choice-Taq (#CB4050-2 Denville Scientific, Metuchen, NJ) with its accompanying 10X buffer and 25 mM dNTPs were used. PCR reactions were done for 35 cycles consisting of 95ºC for 30 sec, 61ºC for 30 sec, and 72ºC for 1 min. Products were amplified using the allele-specific primers designed by Jennifer Richer (University of Colorado) listed below. The *TCF8* forward and reverse primers were used to detect wild type (WT) animals (193 bp product), and the *TCF8* forward and b-gal reverse primers were used to detect heterozygous animals (537 bp product).

TCF8 Forward: 5'-AGCACTATTCTCCGCTACTCCAC-3'

TCF8 Reverse: 5'-ACCGCACCTGGTTTACGACACTC -3'

β-gal Reverse: 5'-AACCGTGCATCTGCCAGTTTGAG-3'

Animal diet and body composition. The caloric-restricted mice used for Figure 1 were treated as described previously [175]. Briefly, C57BL/6 female mice 10 weeks of age were fed a purified regular chow diet (RCD) (AIN-93M, Harlan Teklad, Madison, WI) *ad libitum* or were fed a restricted amount of a diet modified to provide 75% of the calories consumed by the *ad libitum* group [176]. Thus, only the absolute amount of carbohydrate differed between the two dietary regimens. Body weights of the mice were recorded weekly until sacrifice at specific time points as indicated, with the longest being 73 weeks of age. Parametrial fat was harvested at the indicated times (Figure 1), snap frozen in liquid nitrogen, and stored at –80 ºC.

For the remaining experiments, female *TCF8*+/- and WT controls were placed for the indicated times on either a RCD (#2018, Harlan Teklad Global Diets, Indianapolis, IN) or a high fat diet (HFD, 60% of calories from fat, #F3282, Bio-Serv, Frenchtown, NJ) following weaning at 21 days of age. *TCF8+/-* and WT mice were age matched with 8-12 mice per group. Body weights were recorded once a week from 5 weeks of age until sacrifice. Mice were sacrificed at 2, 3, 4, and 5 months for tissue collection; consequently, as the experiment progressed, fewer mice were available to record body weights. Fat pads were harvested and weighed, snap frozen in liquid nitrogen, and stored at -80 °C. Whole body composition was determined by use of nuclear magnetic resonance imaging (MRI) technology produced by Echo Medical Systems LTD (Houston, TX). Mice were placed in a tube and analyzed on the accumulation 4 setting, which is specific for mice, to determine total fat mass, lean mass, body fluid, and water weight.

Real-time PCR analysis. Total RNA was harvested from the parametrial fat of female mice using the RNeasy Lipid Tissue Mini Kit, (Qiagen #74804, Germany). cDNA synthesis was performed using 2 μg total RNA as the template, oligo$(dT)_{13}$ (Integrated DNA Technologies, Coralville, IA), and AMV reverse transcriptase (Roche #10109118001, Germany). ZEB1 mRNA was measured on a BioRad iCycler (#170-8740, Hercules, CA) using iQ SYBR Green Supermix (BioRad #1708885) and normalized to β-actin mRNA. Cycling was performed per the

manufacturer's instructions with annealing temperatures of 61 °C and 61.8 °C for ZEB1 and β-actin, respectively. Primers used to amplify were as follows:

ZEB1 Forward: 5'-CGAGTCAGATGCAGAAAATGAGCAA-3'

ZEB1 Reverse: 5'-ACCCAGACTGCGTCACATGTCTT-3'

β-actin Forward: 5'-CAAAAGCCACCCCCACTCCTAAGA-3'

β-actin Reverse: 5'-GCCCTGGCTGCCTCAACACCTC-3'

Protein expression. ZEB1 protein levels were measured by western blot. Parametrial adipose tissue (100 mg) was homogenized in detergent-free lysis buffer with Mini Complete Protease Inhibitor (Roche, Indianapolis, Indiana). To remove lipid, samples were spun at 10,000 x g for 15 min at 4°C. Solubilized material was pipetted into a new tube and incubated on ice for 30 min with lysis buffer that contained detergents. Samples were spun at 10,000 x g for 15 min at 4°C and supernatant quantified using BioRad d/c assay. Protein (30 mg) was electrophoresed on a 6% polyacrylamide gel and transferred to PVDF membrane. Immunobloting was done using α-ZEB1 1:200 (#H-102, Santa Cruz, Santa Cruz, CA) and α-GAPDH 1:1000 (# MAB 374 Chemicon, Billerica, MA). Secondary anti mouse (Santa Cruz) and anti rabbit (Santa Cruz) peroxidase antibodies were used respectively. Proteins were detected using Pierce Super Signal West Pico Chemiluminescent Substrate (#34080 Rockford, IL).

Glucose tolerance tests. Age-matched mice were fasted for 6 hr prior to testing. The blood glucose baseline was determined using Roche ACCU-CHEK Aviva

(Germany, #04528280001) blood glucose monitor and strips. Mice were injected with an intraperitoneal bolus of glucose dissolved in 0.9% saline. The amount of glucose injected (0.5 - 3 mg/kg) was dependent upon body weight and is indicated in Figure Legend 6. Blood glucose was monitored every 15 min for the first hr and every 30 min for the second hr or until the glucose levels returned to baseline levels.

Food intake. Mice were housed individually following weaning at 21 days. Caloric intake was measured at 10 weeks of age. Cages were adapted with a wire mesh raised 1/8 inch off the bottom, allowing food crumbs to fall through for collection and weighing. One hundred g of regular chow was placed in each cage and after 3 days the remaining food and crumbs were weighed, and food consumption was calculated.

Activity measurements. The activities of WT and *TCF8+/-* mice were determined as described previously [177]. Briefly, 3-month-old mice were housed singly in mock chambers to acclimatize to their surroundings for 24 h before being placed in chambers that record both horizontal and vertical movement based on laser breaks. Prior and during the experiment, mice were fed regular chow.

ELISA assays. Blood was collected via retro-orbital bleed at the time of euthanasia and was allowed to clot at room temperature for 30 min. Serum was

collected following centrifugation at 6000 rpm for 20 min at 4°C and stored at -80°C. Circulating adipocyte hormones were measured using ELISA kits (Invitrogen/Biosource, Carlsbad, CA, leptin #KMC2282, adiponectin #KMP0041). Serum was diluted 1:5 for leptin, and 1:20,000 for adiponectin. Concentrations of hormones were measured by colorimetric change at absorbance of 450 nm on a Perkin Elmer 1420 Multilabel Count Victor 3 plate reader (Perkin Elmer, Waltham, MA).

Statistics. Data were analyzed using the statistics package GraphPad Prism version 4 (San Diego, CA). Data are represented as means +/- standard error of the means (SEM). Unless otherwise noted, Student's *t*-test was used to compare two groups. Two-way analysis of variance (ANOVA) was used when two independent variables are involved and is noted in the figure legend. Areas under the curves were calculated for the glucose tolerance tests (Figure 6). A p-value of <0.05 was used to determine significance. Specific p-values are provided in the figure legends.

RESULTS

ZEB1 mRNA expression increases concomitantly with weight in WT female mice.

Based on genome-wide association studies that mapped a gene linked to obesity in the region of chromosome 10 where *TCF8* is located (10p11.2) [147-149, 154] and on the roles of its resultant protein, ZEB1, in mesenchymal tissue differentiation [24, 36, 47, 170, 171], we speculated that ZEB1 contributes to the transcriptional regulatory cascades that modulate adipogenesis and/or lipogenesis. As a pilot study to address this question, the expression of ZEB1 was initially monitored in WT female mice. While cyclical fluctuations in circulating estrogen and progesterone are often considered an undesirable complication associated with using females, we reasoned that this was unlikely to be a significant problem because of the duration of our experiments (2 – 18 months) and because these hormones might even prove beneficial. As *TCF8* is induced about 10-fold by either estrogen or progesterone [4, 6], we predicted that more dramatic effects might be expected in female mice than in the males, which should have much lower levels of ZEB1. This issue is of particular importance for the subsequent experiments in this paper, which use mice that retain one allele of *TCF8* because null mice are perinatal lethal [38]. Although haploinsufficiency of ZEB1 in humans and mice is sufficient to cause posterior polymorphous corneal dystrophy [52-54], no other significant phenotypes have been reported. It was not clear whether metabolic consequences could be detected in the

heterozygotes. Thus, the prediction was that any effects of ZEB1 haploinsufficiency would be more apparent in females.

To determine whether ZEB1 levels vary with adiposity, ZEB1 mRNA expression was measured in the parametrial fat of WT female mice fed a RCD (regular chow diet) *ad libitum* or a calorie-restricted diet (75% of the calories consumed by the *ad libitum* group) from 10 to 73 weeks of age (Figure 1). The only difference in the diets was a reduction in the amount of carbohydrate in the restricted diet. As expected, the body weights of the *ad libitum* group almost doubled by 40 weeks, then plateaued for the duration of the experiment (Figure 1A). In contrast, the calorie-restricted mice exhibited no significant change in body weight. Real time PCR was done on parametrial tissues harvested from mice at 14, 25, 37, 55, and 73 weeks of age to measure ZEB1 and β-actin mRNA levels (Figure 1B). ZEB1 expression was the same with both dietary regimens at 14 weeks, but it increased dramatically with body weight in the *ad libitum* group and was significantly higher than those mice fed a calorie restricted diet by 37 weeks of age, although the trend was there by 25 weeks (Figure 1B). The same results were observed when the data were also corrected for body weight (data not shown). Thus, an increase in the total body mass correlated with an increase in ZEB1 mRNA expression in parametrial fat, implying that ZEB1 has a specific role in adipose tissue.

Figure 1. ZEB1 mRNA expression increases concomitantly with weight in WT female mice. Mice were fed *ad libitum* or a diet restricted to 75% of the calories of the *ad libitum* group (calorie restricted). **(A)** Body weights (g) were recorded weekly as indicated, *ad libitum* (■), calorie restricted (▲). Significance was determined using 2-way ANOVA as different mice were used at each time point,* denotes $p<0.02$, ** denotes $p<0.0005$. **(B)** Corresponding ZEB1 mRNA expression in parametrial fat was determined by quantitative SYBR real time PCR. ZEB1 mRNA was expressed relative to β-actin mRNA, *ad libitum* (gray bars), calorie restricted (black bars). Significance was calculated using Student's *t*-test. ∗ denotes $p<0.05$, ** denotes $p<0.005$ vs control. (n = 3 - 7 mice/group) **(C)** Western blot confirming that ZEB1 protein expression increases in response to increased body weight. GAPDH was used as a loading control.


Body weight (g)
Ad libitum
Calorie restricted
Age (months)
B
ZEB1/ B-actin
Ad Libitum
Caloric Restricted
Age (months)
C
ZEB1
GAPDH
2.5 6.3 9.3 13.8 18.3

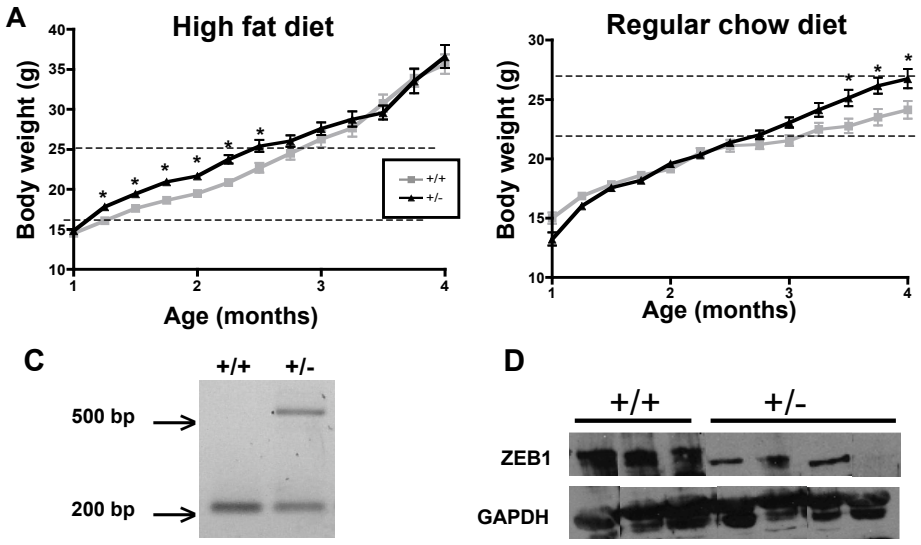
Female mice missing one TCF8 allele gain excess weight more rapidly on a high fat diet than do WT controls.

To directly determine whether ZEB1 affects adipose accumulation, female mice lacking one *TCF8* allele (*TCF8*+/-) as determined by pcr (Figure 2C) and western blot (Figure 2D), were placed on a high fat diet (HFD, 60% of the calories from fat) or a RCD at weaning (21 days of age). *TCF8*+/- mice fed a HFD had significantly increased body mass by five weeks of age compared to WT mice fed HFD (Figure 2A). This increase in weight was maintained until 10 weeks of age, when the rate of weight gain started to decrease, and there was no difference in body weight between age-matched groups for the remainder of the experiment. We suspect that this is because sufficient fat mass has accumulated by 3 months to overcome any modulatory effects of ZEB1 (see Figure 3). These data indicate that ZEB1, directly or indirectly, attenuates the early increase in body mass, during the time when the mice would be considered adolescent, when on a high fat diet.

On normal chow, the mice take longer to accumulate sufficient body weight to be able to detect significant differences between the genotypes (Figure 2B), and, as expected, they do not gain as much total weight as those on a HFD (the y-axes differ between Figure 2A and 2B). *TCF8+/-* mice maintained the same weight as WT controls on regular chow until 14 weeks of age, after which the ZEB1 heterozygous mice became significantly heavier. This trend continued until the termination of the study at 18 weeks of age. Note that the effects of

ZEB1 haploinsufficiency with either diet occurred during a limited period of weight gain, when total body weights were increasing from about 20 to 27 grams and was thus not related to age of the animals as much as to actual body weight. The mechanistic reason for this restricted interval when ZEB1 haploinsufficiency had a measurable consequence is unknown. However, it is likely that once the mice on the high fat diet reach about 27 grams that both genotypes had sufficient fat to overcome the modulatory effects of ZEB1. Similarly, for mice fed a regular diet, too little fat was present below weights of ~20 grams for ZEB1 to have a modulatory effect. Nonetheless, these experiments indicate that there is a substantial interval where total body mass was higher in the ZEB1 heterozygotes than in age-matched WT controls

Figure 2. Female mice missing one *TCF8* allele gain weight more readily. Mice were weaned to **(A)** a diet high in fat (60%) or **(B)** regular chow diet, and body weights (g) of female *TCF8 +/-* (▲) or WT (■) mice were recorded weekly as indicated. N=8-34 mice per group, with the number of mice decreasing due to sacrifices at 2 and 3 months. **(A)** Significance calculated by Student's *t*-test between age-matched groups, using the Bonferroni post-test correction set at $p<0.005$. Significance is denoted by *. **(B)** Significance calculated by Student *t*-test between age-matched groups. All mice from 12-18 weeks have $p<0.05$. However, when corrected for Bonferroni's post-test at $p=0.003$ only those from 14 - 16 weeks are significant. **(C)** An example of the genotyping that was done to identify *TCF8+/-* and WT mice. The band at ~500 bp is from the β-galatisidase gene, which was inserted in one of the *TCF8* alleles. The band at ~200bp represents *TCF8*. **(D)** ZEB1 protein levels in parametrial adipose tissue of *TCF8+/-* and WT female mice at 3 months of age. GAPDH was used as a loading control.


A
High fat diet
Body weight (g)
Age (months)
+/+
+/-
Regular chow diet
Body weight (g)
Age (months)
C
+/+
+/-
500 bp
200 bp
D
+/+
+/-
ZEB1
GAPDH

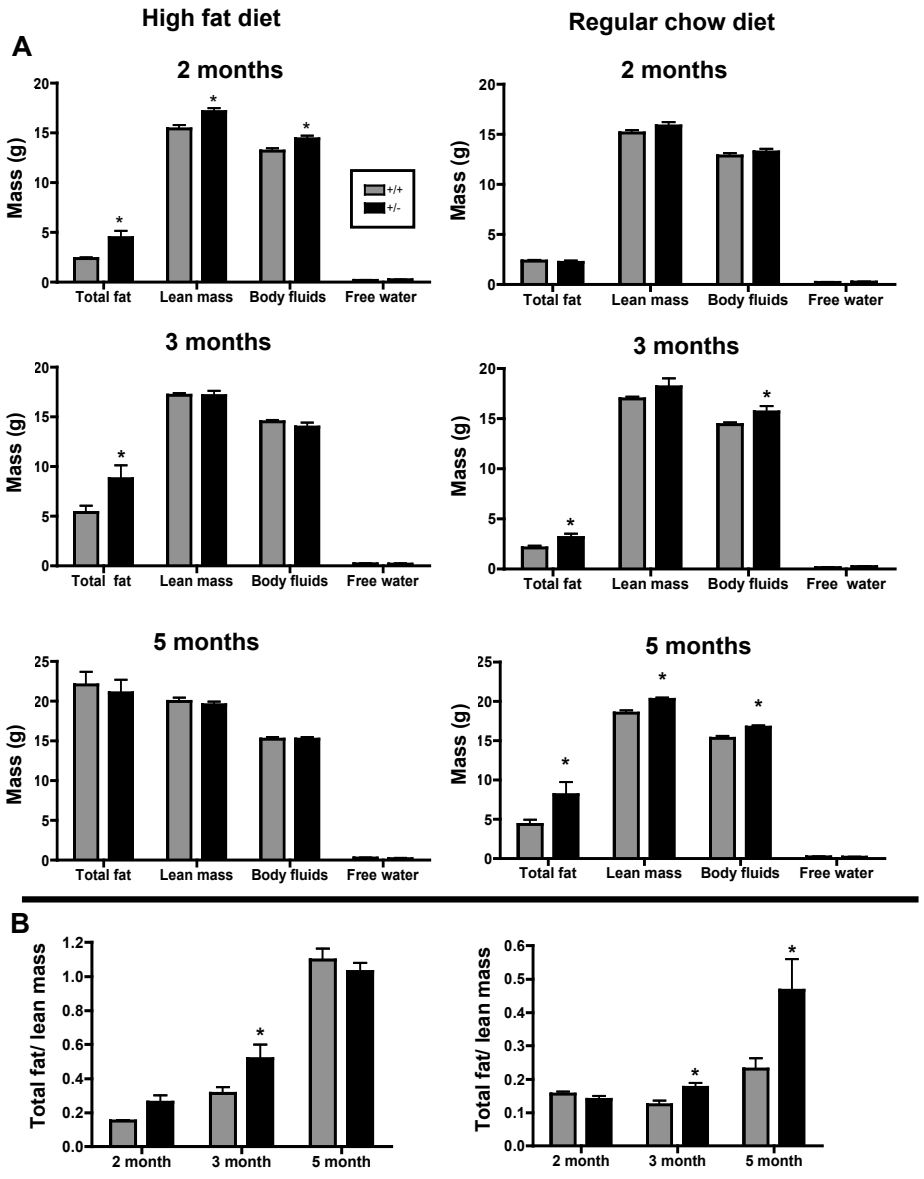
Increased body weight in TCF8+/- female mice is due to an increase in adipose mass.

To ascertain whether the increase in body weight exhibited by the *TCF8*+/- mice can be ascribed to increased adipose mass or to other contributors such as lean muscle mass, Echo MRI was used to measure total fat mass, lean mass, free water, and total body fluids on mice fed either the HFD or RCD (Figure 3). There was no difference in free water between *TCF8*+/- mice and WT controls at any age on either diet. Interestingly, on HFD, *TCF8*+/- mice exhibited increased total fat mass by 2 months of age, which was maintained at 3 months (Figure 3A, left panel). By 5 months, both groups had a high fat mass, and no difference was observed with genotype. However, an increase in lean mass was also observed in *TCF8*+/- mice at 2 months of age, suggesting that these young mice are heavier overall and that both lean mass and fat mass contributed to the difference in total body fluids and overall body weight at this early age. These mice were not significantly longer that the WT mice as determined by measurement from nose to tail (data not shown). To determine whether the increase in body weight at 2 months is primarily a result of increased adipose tissue or increased lean mass, the ratio of fat mass to lean mass was calculated (Figure 3B, lowest left panel). Although there was a trend for a higher total fat to lean mass ratio in the mice fed a high fat diet at 2 months, only 4 mice were available for that time point and statistical significance was not achieved. In

contrast, the increased body weights of the heterozygous mice at 3 months can be ascribed entirely to increased fat mass. These data as a whole indicate that the increase in body weight was due primarily to an increase in fat mass, not just to an overall increase in the size of the mice.

Whole body composition by EchoMRI was also done on *TCF8+/-* and WT female mice fed regular chow (Figure 3A, right panel). In agreement with the body weight data (Figure 2B), *TCF8*+/- mice had no difference in adipose mass at 2 months of age (Figure 3A, right panel). However, at both 3 and 5 months the *TCF8*+/- mice had increased total body fat compared to WT mice. Similarly, the ratio of fat mass to lean mass also increased in *TCF8+/-* mice at 3 and 5 months of age (Figure 3B, lowest right panel). Taken together, these data suggest that ZEB-1 specifically reduces adipose mass in female mice regardless of the diet consumed.

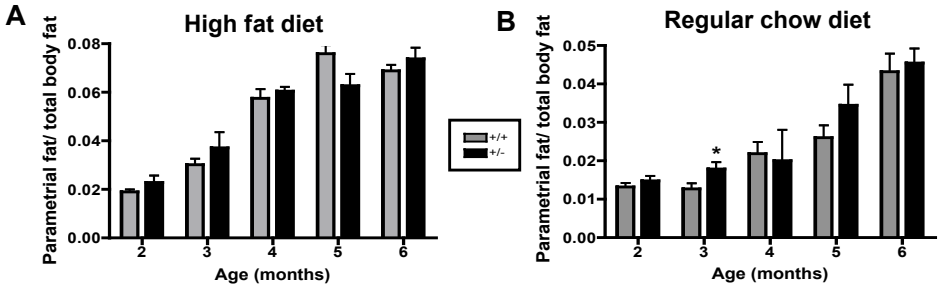
Figure 3. Female *TCF8+/-* mice fed a high fat or regular chow diet have increased adipose mass. Echo MRI analysis of **(A)** whole body composition at 2, 3, and 5 months of age **(B)** fat/lean mass ratio of *TCF8+/-* (black bars) or WT (gray bars) of mice fed a high fat diet (left panel) or regular chow diet (right panel). Note the difference in the y-axes for **(B)**. Student's *t*-test was used to test for significant differences in body composition between genotypes at each age. *$p<0.05$, n = 4-8 mice per group. Only 4 mice were used for the 2 month-old group on the high fat diet.


High fat diet
Regular chow diet
A
2 months
3 months
5 months
Mass (g)
Total fat
Lean mass
Body fluids
Free water
+/+
+/-
B
Total fat/ lean mass
2 month
3 month
5 month

TCF8+/- mice do not store the increased fat mass preferentially in the parametrial fat pads.

Differential gene expression occurs in the various adipose depots [178], and ZEB1 expression is higher in visceral fat than in subcutaneous fat [172]. ZEB1 is also highly expressed in the human uterus [5, 6] and is estrogen-responsive in multiple tissues [4-7]. For these reasons, we hypothesized that ZEB1 may have a greater impact on the parametrial (gonadal) fat pads than on the other adipose depots. Parametrial fat pads were harvested monthly (2 – 6 months of age) from *TCF8*+/- female mice and WT controls fed either a HFD or RCD (Figure 4). No significant differences were observed with regard to genotype or diet except at 3 months, where the *TCF8+/-* mice fed regular chow had significantly higher parametrial fat pad weights compared to WT controls. However, these data *in toto* suggest that ZEB1 does not act locally to affect fat deposition but instead exerts its effects more globally.

Figure 4. Mass of parametrial fat pads does not differ with genotypes. The mass of parametrial fat pads for *TCF8+/-* (black bars) and WT controls (gray bars) ages 2 - 6 months for mice fed **(A)** a high fat diet or **(B)** regular chow was determined by weighing. Note differences in the y-axes. Data are represented as a ratio of parametrial fat weight to total body weight. Statistics were done using Student's *t*-test. * $p<0.05$, n = 5-12.


A
High fat diet
Parametrial fat/ total body fat
0.08
0.06
0.04
0.02
0.00
2
3
4
5
6
Age (months)
+/+
+/-
B
Regular chow diet
Parametrial fat/ total body fat
0.05
0.04
0.03
0.02
0.01
0.00
*
2
3
4
5
6
Age (months)

Increased body fat in TCF8+/- mice is not due to increased food consumption.

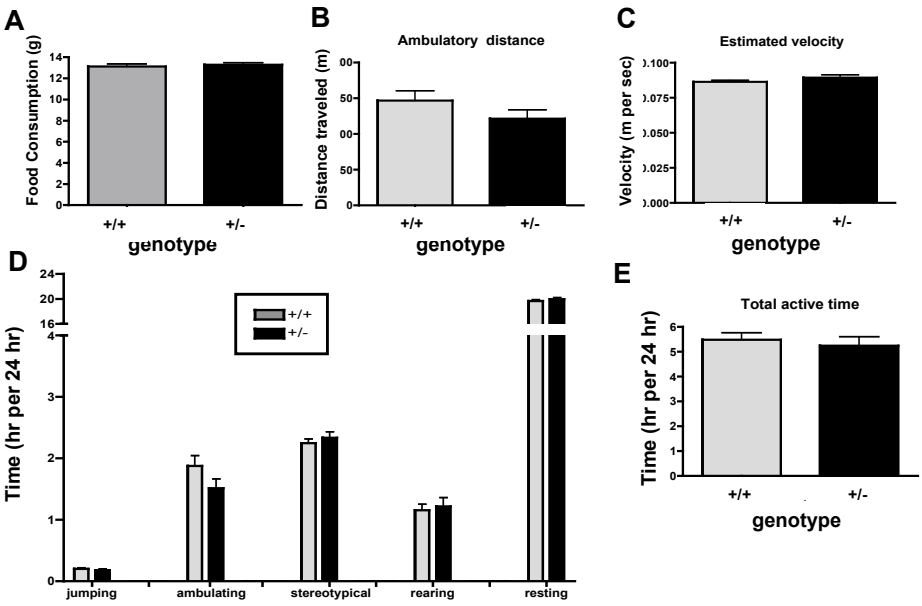
One explanation for the increased adipose tissue accumulation by the *TCF8*+/- mice on both the high and low fat diets is that they might consume more calories. To assess this, the weight of regular chow consumed by 10-week old *TCF8+/-* and WT female mice over a period of three light and dark cycles was measured (Figure 5A). No significant differences in food intake were observed in any of the three repetitions. The body weights of 10-week-old mice fed regular chow are not statistically different between the genotypes (Figure 2B and data not shown), so metabolic changes as a result of increased body weight do not compromise this assessment. These data indicate that ZEB1 has no impact on caloric consumption.

Increased adiposity of female TCF8+/- mice is not due to decreased physical activity.

As the *TCF8+/-* mice do not consume more calories, another explanation for their enhanced adiposity is that they might be more sedentary. Evidence in *Drosophila* suggests that total loss of the ZEB1 ortholog causes impaired development of neuromuscular junctions [179, 180]. This raises the possibility that the *TCF8*+/- mice may have minor muscle or motor neuron developmental deficiencies, which could restrict their mobility and thus reduce caloric expenditure. In addition, female mice primarily alter energy expenditure rather than energy intake to regulate body weight [84], so physical activity was

extensively characterized. Three-month-old *TCF8+/-* and WT mice fed regular chow were monitored in an activity chamber for 24 h (Figures 5B-E). Laser breaks recorded movement both horizontally and vertically. Activity was quantified for the actual ambulatory distance (Figure 5B) and for the duration or time spent performing an activity (Figure 5D). The velocity at which ambulation took place was estimated by calculating m/sec (Figure 5C). No differences were observed in any type of activity or overall time spent being active (Figure 5E, p=0.3395). Thus, these data imply that significant metabolic differences must exist between the two genotypes that contribute to differences in fat accumulation. Metabolic chamber measuring of VO_2 would more directly answer this question, but chambers for mice are not available at the University of Minnesota.

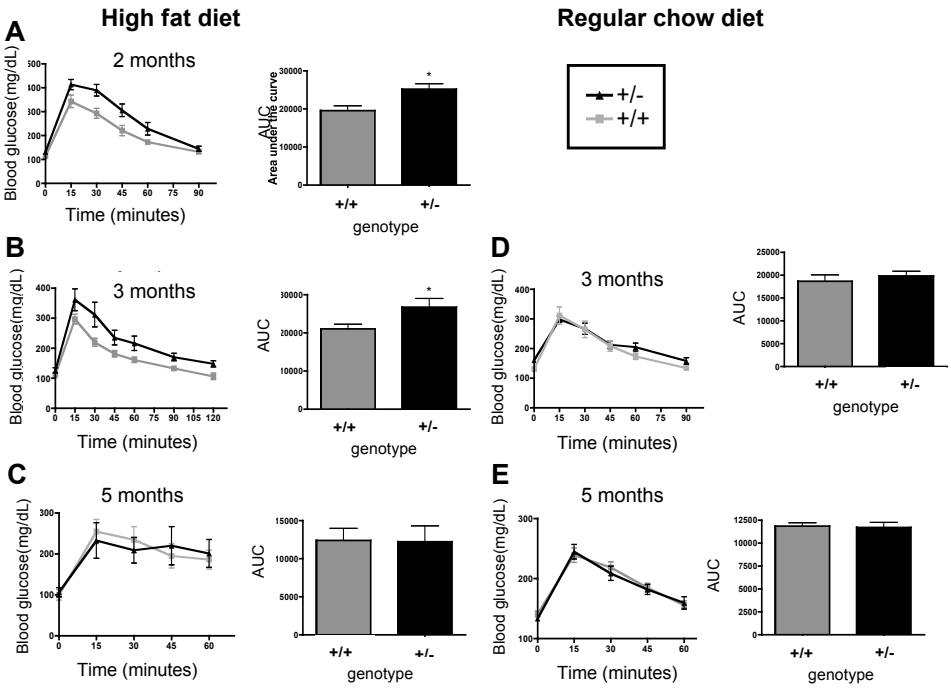
Figure 5. Increased fat accumulation is not the result of increased food consumption or decreased physical activity. (A) Food intake was measured for 72 h in 10-week old *TCF8+/-* and WT mice fed regular chow. The data were analyzed using Student's *t*-test. p=0.6353, n = 10 mice per group. **(B)** The total distance the mice moved. **(C)** The estimated velocity at which the mice moved. **(D)** The duration of each activity or time spent resting. **(E)** Total duration the mice spent performing any activity. *TCF8+/-* mice are depicted by black bars and WT controls by gray bars. Statistical significance was calculated for each activity individually using Student's *t*-test, n= 15-17 mice per group for **B - E**. No significance difference ($p<0.05$) was found for time spent on any activity, resting time, total activity, estimated velocity, or number of times an activity was performed.


A
Food Consumption (g)
14
12
10
8
6
4
2
0
+/+
+/-
genotype
B
Ambulatory distance
Distance traveled (m)
00
50
00
50
0
+/+
+/-
genotype
C
Estimated velocity
Velocity (m per sec)
.100
.075
.050
.025
.000
+/+
+/-
genotype
D
Time (hr per 24 hr)
24
20
16
4
3
2
1
0
+/+
+/-
jumping
ambulating
stereotypical
rearing
resting
E
Total active time
Time (hr per 24 hr)
6
5
4
3
2
1
0
+/+
+/-
genotype

TCF8+/- mice have impaired glucose uptake. Obesity has many metabolic consequences. To investigate whether weight differences due to the loss of one copy of *TCF8* are sufficient to disrupt metabolic homeostasis, glucose tolerance tests were performed on *TCF8+/-* and WT controls fed either a HFD or RCD (Figure 6). Mice were injected with a bolus of glucose that ranged from 0.5 - 3 mg/kg depending upon their body weight. By 2 months of age, *TCF8+/-* mice fed a HFD did not process glucose as well as WT controls (Figure 6A). The same was observed with 3-month-old mice (Figure 6B), but by 5 months there was no difference in glucose uptake (Figure 6C). This is not surprising as the groups have the same body weight and fat mass by then (Figure 3).

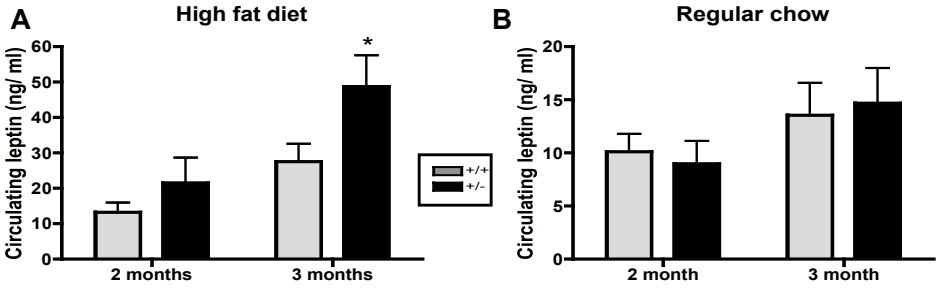
On a RCD, there was no difference in glucose uptake between *TCF8*+/- mice and WT controls at any age (Figures 6D and E). While there is significantly more adipose tissue in the heterozygous mice (Figure 3), the actual mass is not enough to confer decreased glucose clearance. These data imply that *TCF8+/-* mice fed a HFD have impaired glucose uptake because they have increased adipose tissue rather than reduced ZEB1 levels.

Figure 6. Female *TCF8 +/-* exhibit impaired glucose uptake. Mice were fed a high fat diet **(A - C)** or regular chow **(D, E)** until they were 2, 3, or 5 months of age. Blood glucose was measured at the indicated times following injection of glucose at 2 mg/kg **(A, B, E),** 0.5 mg/kg **(C)**, or 3 mg/kg **(D)**. Area under the curve was calculated and graphed as histograms. **A**: p=0.0225, **B**: p=0.049, n = 5-8 mice per group, *TCF8 +/-* (▲) or WT (▒) mice.


High fat diet
Regular chow diet
A
2 months
Blood glucose(mg/dL)
Time (minutes)
AUC
Area under the curve
genotype
+/+
+/-
B
3 months
C
5 months
D
3 months
E
5 months

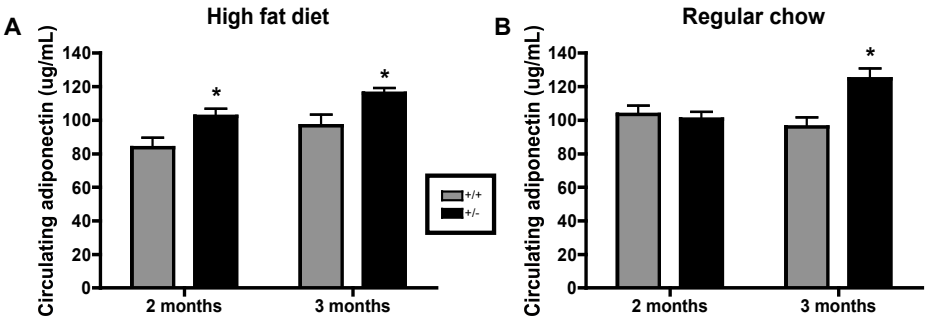
Circulating leptin levels are increased in TCF8+/- mice. Adipose tissue is an important endocrine organ that secretes specific hormones and cytokines, often called adipokines, that regulate various processes throughout the body including metabolism, inflammation, and neuroendocrine signaling, to name a few. The adipocyte hormone leptin negatively regulates satiety through its binding to receptors in the central nervous system [181], and circulating leptin levels positively correlate with fat mass [141]. Adipose mass is significantly increased in *TCF8+/-* mice and one would expect an increase in circulating leptin levels. To investigate whether or not leptin levels are elevated in *TCF8*+/- mice compared to WT, serum leptin was measured in animals fed a HFD or RCD (Figure 7). No difference was observed between genotypes in 2-month-old mice fed a HFD, presumably because total adipose mass was too small (Figure 7A). However, by 3 months of age, when adipose mass has more than doubled for both genotypes (Figure 3), the *TCF8+/-* mice had significantly higher circulating leptin than WT mice (Figure 7A). No differences were observed in mice fed regular chow at either age (Figure 7B), again presumably because of low fat mass. On regular chow, 3-month-old mice have a comparable fat accumulation to 2-month-old mice on a high fat diet (Figure 3). Nonetheless, when adipose tissue mass is sufficient to trigger leptin synthesis and release, *TCF8*+/- mice have higher serum leptin levels, as expected.

Figure 7. Female *TCF8* +/- mice have increased circulating leptin levels. Serum leptin was measured by ELISA for *TCF8*+/- (black bars) and WT (gray bars) mice fed a **(A)** high fat diet or **(B)** regular chow diet until 2 or 3 months of age. Student's *t*-test was used to compare differences in genotype. Note the differences in the y-axes. * p=0.0467. **A**: *n* = 5-9, **B**: *n* = 7-8 mice per group.


A
High fat diet
Circulating leptin (ng/ ml)
60
50
40
30
20
10
0
*
2 months
3 months
+/+
+/-
B
Regular chow
Circulating leptin (ng/ ml)
20
15
10
5
0
2 month
3 month

Circulating adiponectin levels are increased in TCF8+/- mice. The adipocyte hormone adiponectin is the most abundant adipokine in the blood stream [182]. Many questions remain regarding its metabolic functions, but most publications suggest adiponectin has favorable health attributes including regulation of insulin sensitivity, fatty acid oxidation, healing of vascular injury, and reduction of inflammation (for reviews see, [183, 184]). In most studies, adiponectin has a negative correlation with fat mass [182]. Thus, one would expect a decrease in circulating adiponectin to accompany the increase in adipose tissue observed in the ZEB1 heterozygous mice. To determine whether adiponectin levels were reduced in these mice, total serum adiponectin levels were determined from mice at 2 and 3 months of age on both dietary regimens (Figure 8). Surprisingly, the circulating levels of adiponectin increased in all of the *TCF8 +/-* mice that exhibited increased adiposity. By two months of age, *TCF8 +/-* mice fed a HFD had significantly higher circulating adiponectin levels (Figure 8A). Even *TCF8 +/-* mice fed regular chow had higher circulating adiponectin at 3 months of age (Figure 8B), a time when they show increased fat accumulation (Figure 3A). This suggests that the ZEB1 transcription factor not only reduces fat accumulation but also represses circulating adiponectin levels, directly or indirectly.

Figure 8. Female *TCF8*+/- mice have increased circulating total adiponectin. Total serum adiponectin was measured by ELISA for *TCF8 +/-* (black bars) and WT control (gray bars) mice. **(A)** Mice were fed a high fat diet. Student t-test was used to compare differences in genotype at each age. *p=0.0288 at 2 months and p=0.0349 at 3 months of age with n=7-8. **(B)** Mice were fed regular chow. Student's *t*-test was used for analysis. *p=0.0211 with n=5-8.


A
High fat diet
Circulating adiponectin (ug/mL)
140
120
100
80
60
40
20
0
*
*
2 months
3 months
+/+
+/-
B
Regular chow
Circulating adiponectin (ug/mL)
140
120
100
80
60
40
20
0
*
2 months
3 months

DISCUSSION

Based on genome wide association studies that link obesity to the region on chromosome 10 that encompasses the *TCF8* gene [147-149, 154] and on the ability of ZEB1 to impact the differentiation of other mesenchymal cell lineages [24, 36, 47, 170, 171], we hypothesized that ZEB1 opposes obesity. Using *TCF8+/-* mice, we demonstrate herein that ZEB1 haploinsufficiency elicits excessive adipose tissue accumulation in female mice (Figures 2 – 3). This increased adiposity occurred on both regular chow and high fat diets (Figure 3), although significant differences in body weight were delayed by one month on the regular diet. These data suggest that ZEB1 is an important modulator of adipose tissue mass, not just under circumstances of high caloric pressure but also with a normal diet. Because ZEB1 is induced by estrogen [4-7], we anticipated that *TCF8+/-* mice might have increased gonadal (parametrial) fat pads, but this is not the case (Figure 4). This suggests that circulating estrogen levels are sufficient to induce ZEB1 in all fat depots so that proximity to the site of estrogen synthesis is irrelevant, that the effects of ZEB1 on adiposity are estrogen-independent, or that ZEB1 has a more global effect on fat deposition. These increases in adiposity were profound enough to elicit metabolic defects, including a decrease in glucose uptake (Figure 6), an increase in plasma leptin when fat depots are sufficiently large (Figure 7), and an increase in plasma adiponectin (Figure 8). These data show for the first time that ZEB1 plays a role in intermediary metabolism and that it opposes fat accumulation.

The increase in total serum adiponectin in the *TCF8*+/- mice was unexpected (Figure 8). Most reports demonstrate an inverse relationship between adiponectin levels and adiposity, as reviewed in [181, 183], but most of the data with biologically relevant adiponectin are based on lowering adiponectin levels rather than elevating them. In addition, the majority of these studies have been done in male mice, and sexual dimorphism in serum adiponectin levels exists in both humans and rodents [182, 185]. Furthermore, estrogen represses serum adiponectin levels, [185], which may be mediated by ZEB1, so the increase in adiponectin levels in the *TCF8*+/- mice makes sense. However, the paradox is that this increase only occurs in the mice with significantly increased fat depots, i.e., it does not occur in 2-month-old mice fed a RCD (Figure 8B). This indicates that ZEB1 haploinsufficiency is having additional as yet undetermined effects on adiponectin levels that relate to excessive fat accumulation. Even the reported effects of estrogen on adiponectin levels are paradoxical as estrogen represses adiponectin, yet serum levels of adiponectin in females are higher than in males [185]. Furthermore, adiponectin levels are not elevated in obese CD-1 nude mice compared to controls [186]. Thus, our understanding of the regulation of adiponectin expression and its role in metabolism is still incomplete, making it more difficult to predict why it is elevated in the *TCF8*+/- mice. One explanation is that it increases to “counteract harm” in a defense-type mechanism, much as has been proposed for its elevation in Type I diabetes, anorexia nervosa, and chronic renal failure [187]. Additional experimentation will be needed to ascertain

whether ZEB1 directly regulates adiponectin expression and how this is modulated by fat accumulation.

In order to begin to delineate the potential mechanisms by which ZEB1 represses obesity, food intake and activity were measured. No differences were observed in food consumption between *TCF8*+/- and WT mice or in the total amount or duration of physical activity of the two strains (Figure 5) indicuates that ZEB1 has an as yet undiscovered metabolic effect. Experiments are in progress to identify gene targets for ZEB1 in adipose tissue to get a better understanding of the mechanisms whereby it suppresses fat accumulation. Of course, the effects of ZEB1 may not be directly in adipose tissue. Certainly most of the genes associated with monogenetic or syndromic obesity described thus far are expressed in the brain [188]. Thus, ZEB1 might be exerting its primary effects in non-adipose tissues. However, the large increase in ZEB1 expression that occurs as the mice become obese (Figure 1B) implies at least some role for ZEB1 in adipose tissue. Experiments are in progress to assess whether ZEB1 is acting in adipose and/or peripheral tissues.

Another paradox that exists with these data relates to the observations that ZEB1 mRNA increases in mice as they gain adipose mass (Figure 1B), yet ZEB1 haploinsufficiency leads to increased fat accumulation (Figures 2 – 3). High expression of ZEB1 has also been reported in cell culture during adipogenesis [162] and in obese women compared to those of normal weight [172]. Thus, ZEB1 opposes fat accumulation yet is highly expressed in fat. One plausible explanation is that ZEB1 increases as a negative feedback mechanism

in order to repress additional fat accumulation, much as proposed above for adiponectin. It is also possible that ZEB1 has multiple roles in modulating adiposity. Many of these questions will need to be resolved using tissue-specific *TCF8* knockouts.

Transcriptional cascades regulate the differentiation of mesenchymal stem cells into mature adipocytes [64, 69]. While the major players have already been identified, modulators such as ZEB1 are just beginning to be elucidated. As most genetic causes of obesity are polygenic [166], a complete understanding of the proteins involved will be necessary to determine when a cell commits to the adipocyte lineage, when it differentiates into an adipocyte, and how lipids are stored within adipocytes. The data herein demonstrate for the first time that the ZEB1 transcription factor plays a significant role in opposing obesity, at least in females. They also provide the first evidence that *TCF8* is the gene on chromosome 10 that is linked to childhood obesity [147-149, 154]. The goal now is to determine how ZEB1 fits into the transcriptional pathways that modulate fat metabolism.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

Conceived and designed Figure 1A: SD MPC. Conceived and designed all other figures: JNS MMS. Performed the experiment in Figure 1A: SD. Performed all the remaining experiments: JNS. Analyzed the data in Figure 1A: SD. Analyzed the data in the remaining figures: JNS MMS. Wrote the paper: JNS MMS. Edited the paper: JNS SD MPC MMS.

CHAPTER IV

The ZEB1 transcription factor mediates the effects of estrogen in attenuating adiposity in mice

Manuscript in preparation

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Running Head:

Estrogen reduces adiposity via ZEB1

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SUMMARY

In humans, the propensity to accumulate fat in response to environmental factors such as excessive food intake or sedentary lifestyle is more likely the consequence of polymorphisms in many genes rather than deleterious mutations in single genes. Identification of the genes that modulate energy homeostasis will facilitate our understanding of obesity and its pathogenesis. Recently, the *TCF8* gene was identified as one such modulator. It encodes the zinc finger homeobox E-box binding protein 1 (ZEB1), which is protective against adipose accumulation. ZEB1 expression is induced by estradiol, raising the possibility that it mediates some of estrogen's effects on adiposity. Estrogen signaling attenuates adipose accumulation by decreasing both the size and number of adipocytes. To determine whether ZEB1 is sexually dimorphic in its regulation of adiposity and whether it mediates estrogen's metabolic effects, male and anti-estrogen (Faslodex) treated female mice heterozygous for *TCF8* (*TCF8+/-*) were fed a high fat diet. Body weight, body composition, adipocyte size, and several metabolic parameters were monitored. Interestingly, the female but not the male *TCF8+/-* mice accumulate excess fat. This gender difference can be ascribed to estrogen as Faslodex treatment abolishes the differences between the genotypes. However, under some conditions, ZEB1 may modulate adiposity in males as its expression significantly increases in both gonadal and subcutaneous fat following injection with estradiol. Also, *TCF8+/-* mice exhibit increased adipocyte size regardless of gender. While the mechanisms whereby ZEB1

reduces adipose accumulation in female mice are unclear, we demonstrate that it is an important factor in their metabolic homeostasis.

INTRODUCTION

Men and women differ in the mechanisms by which they accumulate, store, and utilize fat, largely as a consequence of the ovarian sex steroid estrogen [84, 86, 118, 119, 123, 125]. Considerable evidence indicates that estrogen is protective against obesity and metabolic disorders. This is accomplished through many effects on metabolism [84, 86, 118, 119, 123, 125], including a reduction in both the size and number of adipocytes [189, 113]. Both estrogen receptor-α (ERα) and ERβ are present in white adipose tissue [117], emphasizing a direct role for estrogen signaling in this tissue. ERα null mice have more than double the normal fat mass [113]. Similarly, reducing 17β-estradiol levels by eliminating the aromatase enzyme results in increased abdominal adiposity in mice [190]. Thus, estrogen plays an important role in opposing fat accumulation.

While estrogen affects many aspects of metabolism [84, 86, 118, 119, 123, 125], a comprehensive understanding of its mechanism of action is lacking. Recently, we demonstrated that the zinc finger E-box binding homeobox protein 1 (ZEB1, δEF1, AREB6, zfhx1a) is novel modulator of adipose accumulation (Saykally *et al*. manuscript in revision). ZEB1 is a large, highly conserved transcription factor that contains several functional motifs including 7 zinc fingers that recognize E-boxes in target genes, a central homeodomain that appears to

facilitate protein-protein interactions, a C-terminal transcriptional activation domain, and recognition domains for co-repressors [32, 33, 35]. ZEB1 can both activate and repress transcription (as reviewed in [24]), however, the molecular basis for this is poorly understood. The ZEB1 protein is encoded by the *TCF8* gene, located at human chromosome 10p11.2. The loss of one allele of *TCF8* gene is sufficient to increase body weight and fat accumulation in female mice, resulting in significant changes in serum adipokine levels and in glucose homeostasis (Saykally *et al*. manuscript in revision).

Although relatively little is known about the regulation of ZEB1, it is induced by estrogen in chick oviduct [4], mouse pituitary [7], human ovary [5] and uterus [6]. Because our previous study demonstrated that ZEB1 insufficiency causes increased fat accumulation in female mice, goals of this study were to determine whether ZEB1 also modulates fat accumulation in male mice and whether ZEB1 mediates at least some of the effects of estrogen in reducing adiposity. To address these questions, male and female mice heterozygous for *TCF8* (*TCF8+/-*) were placed on a high fat diet and various metabolic and physical parameters including glucose homeostasis, adipokine levels, fat accumulation, and adipocyte size and number were monitored. (*TCF8* null mice are perinatal lethal [36]). We found that the increase in adipose tissue in *TCF8+/-* mice can be ascribed to an increase in adipocyte size, although this is not the result of increased lipoprotein lipase (LPL) activity or decreased hormone sensitive lipase activity. Interestingly, when WT female mice were treated with the specific ER antagonist Faslodex, their body weights, fat mass, glucose

uptake, and many of their adipokines resembled those of the ZEB1 haploinsufficient female mice as well as the male mice. These results demonstrate that at least some of the inhibitory effects of estrogen on fat accumulation are mediated by the ZEB1 transcription factor.

MATERIALS AND METHODS

Animals. All mice were maintained in the pathogen-free facility at the University of Minnesota. Protocols were approved and are in compliance with all local, state and federal guidelines. The IACUC protocol number is 0609A91913. *TCF8* null mice were generated by Dr. Yujiro Higashi and colleagues (Osaka University, Osaka, Japan) as was described previously [36], and heterozygous (*TCF8*+/-) males were provided by Dr. Jennifer Richer, University of Colorado, Aurora, CO. The colony was generated and maintained on a C57BL/6 background. Genotyping was done as reported previously (Saykally *et al*., manuscript in revision). Faslodex-treated mice were injected ip with 10 mg/ kg of Fulvestrant (ICI 182,780)[191] every 10 days. Pilot studies indicated that this treatment was sufficient to reduce uterine weights (data not shown) to the extent previously reported with ICI 182,780 [192, 193].

Animal diet and body composition. *TCF8*+/- and WT controls were fed either regular chow (#2018, Harlan Teklad Global Diets, Indianapolis, IN) or a diet high in fat (60% of calories from fat, #F3282, Bio-Serv, Frenchtown, NJ) *ad libitum* for the indicated months of age following weaning at 3 weeks of age. Whole body

composition was determined by use of nuclear magnetic resonance imaging (MRI) technology produced by Echo Medical Systems (Houston, TX) just prior to euthanasia and tissue harvest.

Real-time PCR analysis (qPCR). Total RNA was harvested from gonadal fat using the RNeasy Lipid Tissue Mini Kit, (Qiagen #74804, Germany). cDNA synthesis was performed with Transcriptor Reverse Transcriptase (Roche #10109118001, Germany). Gene expression was measured on a BioRad iCycler (#170-8740, Hercules, CA) using iQ SYBR Green Supermix (BioRad #1708885) and normalized to 36B4 mRNA, which is not affected by estrogen [194]. Primers used to amplify were as follows:

		Tm (°C)
ZEB1:	Forward 5'-CGAGTCAGATGCAGAAAATGAGCA-3'	60
	Reverse 5'-ACCCAGACTGCGTCACATGTCTT-3'	
Adiponectin:	Forward 5'-AGAGAAGGGAGAGAAAGGAGATGC-3'	58
	Reverse 5'-TGAGCGATACACATAAGC GGC-3'	
Resistin:	Forward 5'-GTACCCACGGGATGAAGAACC-3'	58
	Reverse 5'-GCAGAGCCACAGGAGCAG-3'	
Leptin:	Forward 5'-CAGGGAGGAAAATGTGCTGGAG-3'	56
	Reverse 5'-CCGACTGCGTGTGTGAAATGTC-3'	
TNFα:	Forward 5'-ACGCTCTTCTGTCTACTGAACTTCG-3'	59
	Reverse 5'-ATAGCAAATCGGCTGACGGTGTGG-3'	
PPARy:	Forward 5'-CCAGAGCATGGTGCCTTCGCT-3'	57
	Reverse 5'-CAGCAACCATTGGGTCAGCTC-3'	

SDF1α	Forward 5'-ATGAACGCCAAGGTCGTG-3'	55
	Reverse 5'-GGTACAGGGCATGGATGAAT-3'	
SCD1:	Forward 5'-CCTACGACAAGAACATTCAATCCC-3'	60
	Reverse 5'-CAGGAACTCAGAAGCCCAAAGC-3'	
FAS:	Forward 5'- GGCATCATTGGGCACTCCTT-3'	55
	Reverse 5'- ACCAACAGCTGCCATGGATC-3'	
ACC1:	Forward 5'-CGGACCTTTGAAGATTTTGTCAGG-3'	57
	Reverse 5'-GCTTTATTCTGCTGGGTGAACTCTC-3'	
36B4:	Forward 5'-GACCTCCTTCTTCCAGGCTTT-3'	60
	Reverse 5'- GACCTCCTTCTTCCAGGC TTTG-3'	
HSL:	Forward 5'-CACCCATAGTCAAGAACCCCTTC-3'	58
	Reverse 5'-TCTACCACTTTCAGCGTCACCG-3'	
LPL:	Forward 5'-AGGGCTCTGCCTGAG TTGTA-3'	54
	Reverse 5'-AGAAATTTCGAAGGCCTGGT-3'	
Perilipin A:	Forward 5'-TGC TGG ATG GAG ACC TC-3'	55
	Reverse 5'-ACC GGC TCC ATG CTC CA-3'	

Glucose tolerance tests. Glucose tolerance tests were performed as previously described (Saykally *et al*., manuscript in revision). In brief, mice were fasted for 6 hours. The baseline blood glucose levels were recorded prior to ip injection of 0.5 to 2 mg/kg, as indicated, of glucose dissolved in 0.9% saline. Blood glucose was monitored every 15 min for 90 min using Roche Accucheck strips and meter.

ELISA assays. Blood was collected via retro-orbital bleed at the time of euthanasia and serum was collected as previously described (Saykally *et al.,* manuscript in revision). Circulating adipocyte hormones were measured using ELISA kits (Invitrogen/Biosource, Carlsbad, CA, leptin #KMC2282, adiponectin #KMP0041) (ALPCO, resistin #44-RESMS-E01). Serum was diluted 1:5 for leptin, 1:20,000 for adiponectin, and 1:3 for resistin. Concentrations of hormones were measured by colorimetric change at absorbance of 450 nm on a Perkin Elmer 1420 Multilabel Count Victor 3 plate reader (Perkin Elmer, Waltham, MA).

LPL activity was measured per the manufacturer's instructions. In brief, 100 mg of gonadal adipose tissue was homogenized in detergent-free lysis buffer. To remove lipid, lysates were spun at 10,000 x g at 4 °C for 10 min. The samples were incubated with detergent containing lysis buffer for 30 minutes on ice, spun at 10,000 x g at 4 °C for 10 min, and supernatant collected in a fresh tube. Total protein was determined using Bio-Rad D/C assay and 10 μg incubated with LPL substrate for 30 minutes at 37°C. Fluorescence was measured at 370 nm excitation/ 450 nm emission nm on a Perkin Elmer 1420 Multilabel Count Victor 3 plate reader (Perkin Elmer, Waltham, MA).

Adipocyte size and number. Gonadal adipose tissue was removed from the mice and placed in 10% neutral buffered formalin for 48 h. The tissues were stored in 70% ethanol until used for hematoxylin and eosin staining and slide preparation by the Comparative Pathology Shared Resource Core Facility, Masonic Cancer Center, University of Minnesota. Photographs of the slides were

taken at the Biomedical Image Processing Laboratory, University of Minnesota, using an Axiovert 2 Upright Zeiss microscope at 40x magnification. Several fields were taken for each animal. Adipocyte cross-sectional area was obtained from perimeter tracings of adipocytes on digital images of histological sections using Image J software (Sun Microsystems, Palo Alto, CA), 3 fields per animal with a total of greater than 100 cells counted per group.

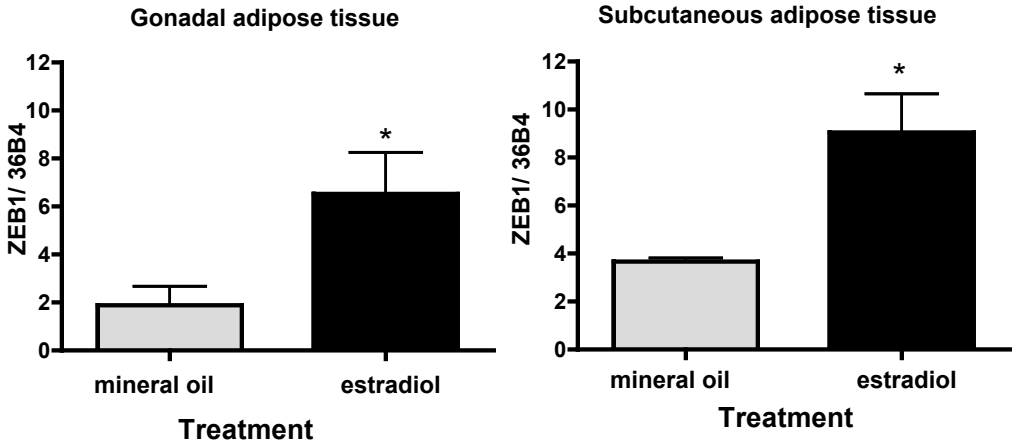
Statistics. Data were analyzed using the statistics package GraphPad Prism version 4 (San Diego, CA). Data are represented as means +/- SEM. Unless otherwise noted, Student's *t*-test was used to compare two groups. A p-value of <0.05 was used to assign significance. Specific p-values are provided in the figure legends.

RESULTS

Estrogen increases ZEB1 mRNA expression in male adipose tissue

Estrogen increases ZEB1 expression in a number of tissues. This was first observed in chicken oviduct [4], but induction by estrogen was later demonstrated in human ovary [5], uterus [6], and mouse pituitary [7]. While the biological role of ZEB1 in these tissues remains unclear, ZEB1 attenuates adipose tissue accumulation (Saykally *et al*., manuscript in revision). Because estrogen decreases both the size and number of adipocytes, as reviewed in [118], and because ERα is present in adipose tissue [117], it is plausible that estrogen regulates ZEB1 expression in fat. To determine whether this is the case, male C57BL/6 mice were injected ip with 5 ng of 17β-estradiol. Twenty-four hours later, ZEB1 mRNA expression was measured in both gonadal and subcutaneous adipose tissues using qPCR (Fig. 1). ZEB1 mRNA expression increased in both fat pads, indicating that estrogen directly or indirectly increases ZEB1 expression in adipose tissue. This lends support to the contention that ZEB1 acts directly in adipose tissue to oppose fat accumulation.

FIGURE. 1. Estrogen induces ZEB1 expression in adipose tissue. WT male C57BL/6 mice were injected ip with 5 ng/kg 17β-estradiol (black bars) or vehicle (gray bars). Twenty four hours later, gonadal (left panel) and subcutaneous (right panel) fat pads were harvested and RNA isolated. ZEB1 mRNA levels were determined by qPCR and were normalized to 36B4 mRNA, an mRNA that is not affected by estrogen [194]. Means ± SEM are plotted. n = 4-7, * $p<0.05$.


Gonadal adipose tissue
ZEB1/ 36B4
12
10
8
6
4
2
0
*
mineral oil
estradiol
Treatment
Subcutaneous adipose tissue
ZEB1/ 36B4
12
10
8
6
4
2
0
*
mineral oil
estradiol
Treatment

Activated ER is required to elicit the effects of ZEB1 on body weight and fat accumulation

We previously observed that the functional loss of one copy of *TCF8* is sufficient to cause a significant increase in body weight in female mice 4 - 12 weeks of age (Saykally *et al.,* manuscript in revision). To determine whether these effects of ZEB1 haploinsufficiency are gender-specific and triggered by estrogen, the body weights of WT and *TCF8+/-* male mice fed a high fat diet (HFD) (Figure 2A) or regular chow diet (RCD) (Figure 2B) were monitored from 4 to 16 weeks of age. The RCD diet was included because male C57BL/6 mice gain weight so rapidly that it seemed possible that the modest modulatory effects of ZEB1 might be overwhelmed by the rapid fat accumulation. Body weights did not differ between WT and *TCF8+/-* male mice on either diet, even at early ages, supporting the idea that the effects of ZEB1 are gender- or estrogen-specific.

To test this more directly, female mice on a HFD were injected with the anti-estrogen ICI 182,780 in the form of Faslodex (Fulvestrant). This is a slow release compound, so the mice were only injected every 10 days from 5 until 20 weeks of age. Pilot studies (data not shown) indicated that this regimen was sufficient to reduce uterine weights by ~40%, as anticipated with anti-estrogen treatment [192, 193]. As expected, *TCF8* +/- mice injected with mineral oil had increased body weight over WT mice (Fig. 2C). The differences between genotypes were not as dramatic as previously observed (Saykally *et al*., manuscript in revision), presumably because the stress of the repeated injections reduced overall weight gain. Nonetheless, no differences in body weight were

observed between *TCF8*+/- and WT mice treated with Faslodex (Fig. 2D), indicating that estrogen signaling is required for ZEB1 to oppose fat accumulation.

The increase in body weights in female *TCF8*+/- mice compared to WT mice was attributed to an increase in adipose mass as determined by nuclear magnetic resonance imaging (EchoMRI) (Saykally *et al.,* manuscript in revision). To determine whether the inhibitory effects of ZEB1 on fat accumulation were elicited by estrogen, whole body composition was measured by EchoMRI for both genotypes in male mice and in Faslodex-treated female mice (Fig. 3). As expected based on body weights, fat mass did not differ in the male mice at 3 months of age on either a HFD or a RCD (Fig. 3A and 3B). Furthermore, WT female mice treated with Faslodex accumulated the same amount of fat as did the ZEB1 haploinsufficient mice at both 3 and 5 months of age (Fig. 3C and 3D), again consistent with body weight measurements. For reasons that are unclear, the lean body mass differed significantly between genotypes in the female mice at 3 months of age. However, this did not cause a significant difference between the WT and *TCF8+/-* mice in the fat to lean mass ratio (Fig. 3E). Thus, these data support the contention that ZEB1 mediates the protective effects of estrogen on fat accumulation.

FIGURE. 2. Activated ER is required to elicit the effects of ZEB1 on body weight. The body weights of WT and *TCF+/-* male mice fed a HFD (A) or a RCD (B) for 4 – 16 weeks were monitored weekly (n = 20-30 mice per group). The body weights of WT and *TCF+/-* female mice injected with mineral oil (n=4-8 mice per group) or Faslodex (n-25-30 mice per group) every 10 days (D) were monitored weekly. Gray lines = WT, Black lines = *TCF8+/-.*

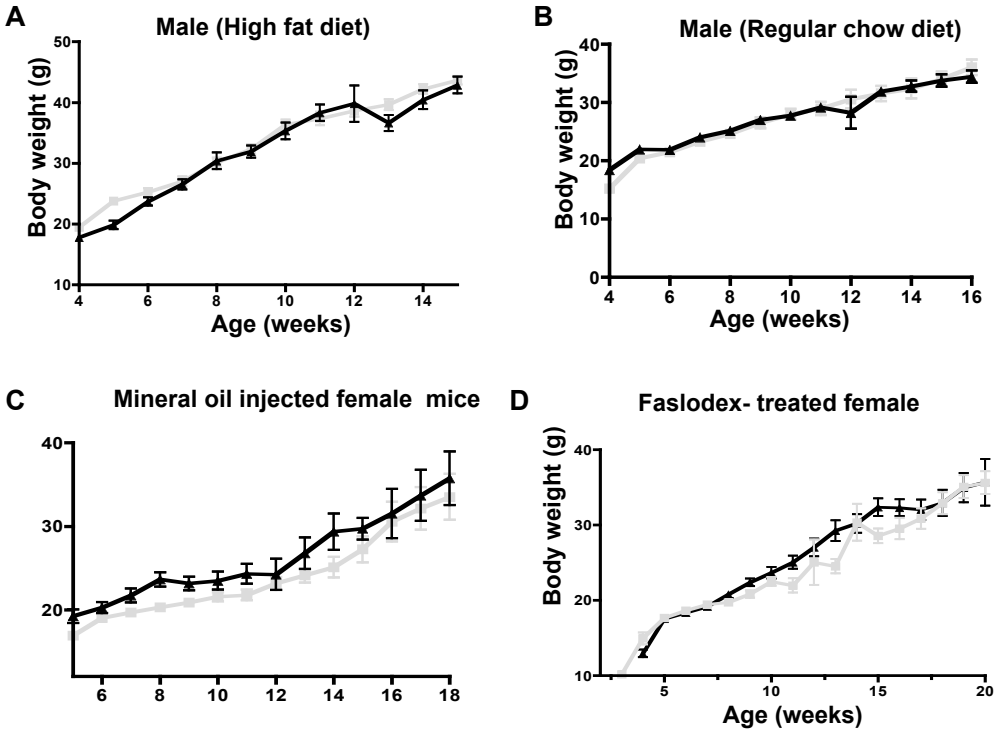
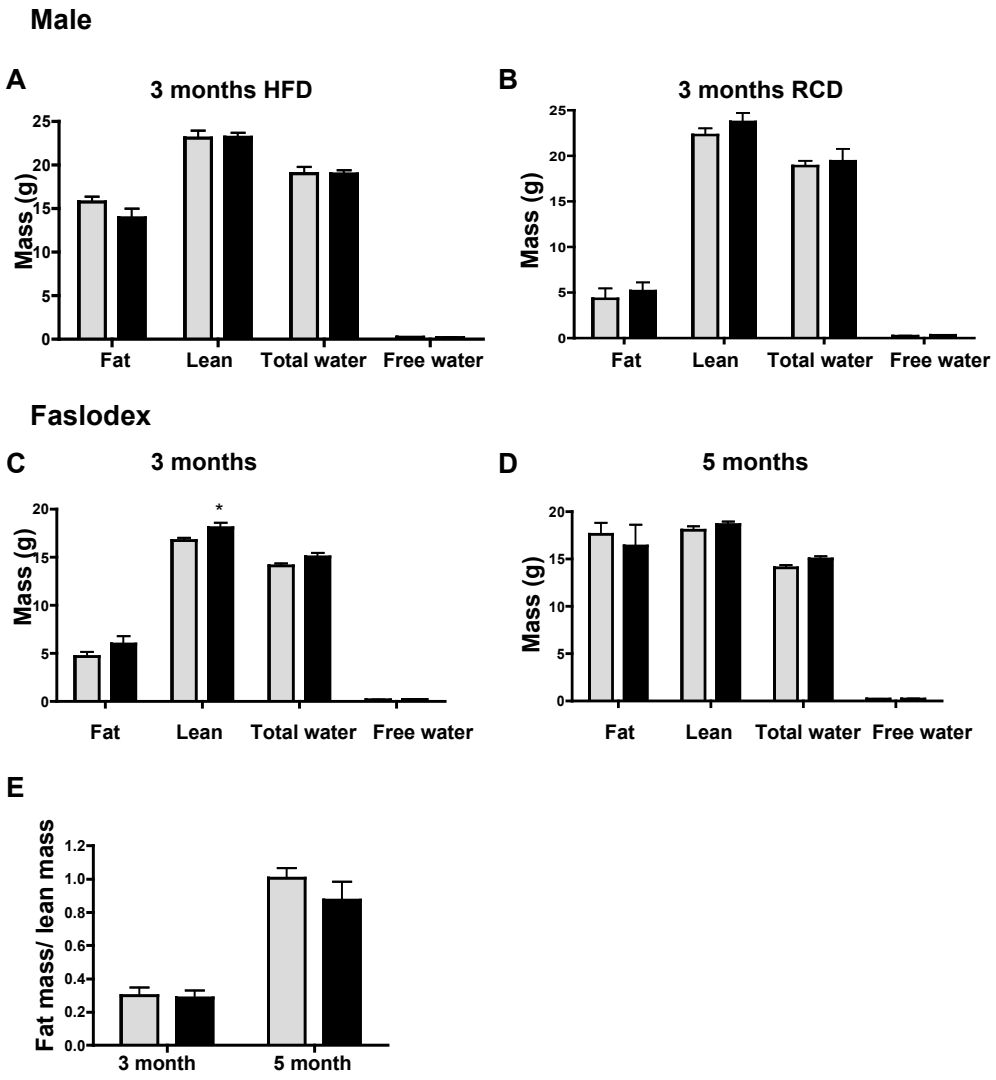

A
Male (High fat diet)
Body weight (g)
Age (weeks)
B
Male (Regular chow diet)
Body weight (g)
Age (weeks)
C
Mineral oil injected female mice
D
Faslodex- treated female
Body weight (g)
Age (weeks)

FIGURE 3. Activated ER is required to elicit the effects of ZEB1 on fat accumulation. Whole body composition (fat mass, lean body mass, total water, and free water) was determined in WT (gray bars) and *TCF8+/-* (black bars) male and female mice by EchoMRI. (A) Male mice on a HFD, (B) Male mice on a RCD, (C) Faslodex-treated female mice at 3 months of age, (D) Faslodex-treated female mice at 5 months of age, and (E) the fat mass to lean mass ratio in the female mice. n =6 -15 mice per group, * = $p<0.05$


Male
A
3 months HFD
Mass (g)
25
20
15
10
5
0
Fat
Lean
Total water
Free water
B
3 months RCD
Mass (g)
25
20
15
10
5
0
Fat
Lean
Total water
Free water
Faslodex
C
3 months
*
Mass (g)
20
15
10
5
0
Fat
Lean
Total water
Free water
D
5 months
Mass (g)
20
15
10
5
0
Fat
Lean
Total water
Free water
E
Fat mass/ lean mass
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3 month
5 month

Weight gain elicits an increase in ZEB1 expression in WT female mice

Although ZEB1 clearly affects fat accumulation in female mice, it appears to do so for a limited period when the mice are between about 20 and 28 grams body weight, regardless of whether they are on a HFD or RCD (Saykally *et al*., manuscript in revision). As the mice are at those weights at different ages with the two diets, this is unlikely to be a consequence of age. One plausible explanation is that ZEB1 expression correlates with fat accumulation (Saykally *et al*., manuscript in revision) [172]. To examine this issue more closely, ZEB1 mRNA levels were monitored by qPCR in the gonadal fat of female and male WT and *TCF8+/-* mice over time (Fig. 4). The ZEB1 mRNA levels were normalized to 36B4, an mRNA whose expression is not affected by estrogen [194]. While ZEB1 expression in *TCF8+/-* in female mice is about half that of WT mice at 2 and 3 months of age, as expected, that difference begins to diminish by 4 months of age when ZEB1 mRNA expression in the two genotypes is not significantly different (Fig. 4A).

Although male and female mice have about equal numbers of ER in adipose tissue and male mice also have some circulating estradiol [112], no effects of ZEB1 haploinsufficiency on adiposity were observed in males (Figs. 2 and 3). This may be because the C57BL/6 mice at 3 months of age already have 3 times the fat mass as comparable females even though their lean mass is only about 20% higher (Fig. 3). To ascertain whether male mice have increased expression of ZEB1, which could compromise the phenotypic response, ZEB1 mRNA was measured in the gonadal fat pads of 2- and 3-month old mice fed a

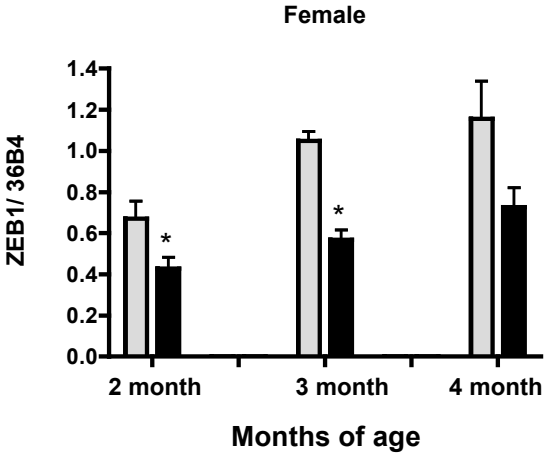
HFD (Fig. 4B). The relative expression of ZEB1 even at 2 months of age is as high as that of female mice at 4 months, which may explain why no significant differences were observed in body weight or fat accumulation in males with the two genotypes.

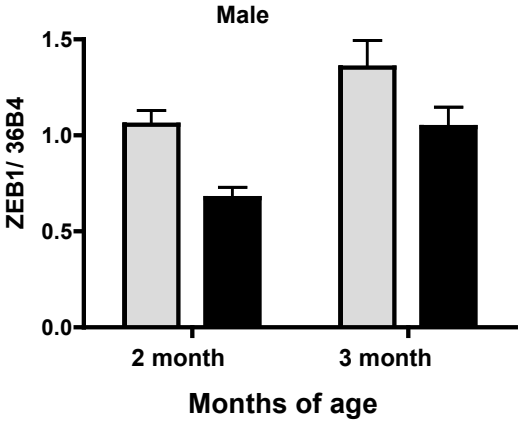
ZEB1 does not affect gonadal fat deposition

Health risks associated with obesity vary depending on the location of the adipose tissue. Men store fat predominately in the visceral region, which predisposes them to a greater risk for metabolic disorders, while pre-menopausal women store fat subcutaneously (for review, see [86]. This is largely due to estradiol signaling, which decreases visceral fat deposition and increases subcutaneous deposition [84, 86, 118, 119, 123]. We have previously shown there was no statistical difference in parametrial fat accumulation in female mice between the genotypes, although there was a trend for the *TCF8*+/- mice to have more parametrial (visceral) fat (Saykally *et al*., manuscript in revision). To extend those studies, the gonadal fat mass was determined in WT and *TCF8+/-* male mice and Faslodex-treated female mice (Fig. 5). The prediction is that if ZEB1 mediates the effect of estrogen on fat depot selection, then WT male mice might have more subcutaneous fat and less gonadal fat mass than would the *TCF8*+/- mice, whereas the Faslodex-treated females would not significantly differ from WT. As anticipated, no genotypic difference was observed in Faslodex-treated female mice at 3 or 5 months of age in (Fig 5A). This was also true of the male

mice on a HFD at 2 and 3 months, but a significant difference emerged at 4 months of

FIGURE. 4. Weight gain elicits an increase in ZEB1 expression in WT female mice. Gonadal fat pads were collected from WT (gray bars) and *TCF8+/-* (black bars) female (A) and male (B) mice at the ages in months. ZEB1 mRNA levels were measured by qPCR and normalized to human acidic ribosomal phosphoprotein PO (36B4) mRNA. n = 5-9, * $p<0.05$


Female
ZEB1/ 36B4
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
2 month
3 month
4 month
Months of age


Male
ZEB1/ 36B4
1.5
1.0
0.5
0.0
2 month
3 month
Months of age

age (Fig. 5B). Interestingly, there was also a significant increase in gonadal fat accumulation in *TCF8+/-* mice at 2 months of age on a RCD, when the male mice had considerably less total body fat (Fig. 5C). These data imply that ZEB1 might also affect adiposity or adipose distribution in males. However, ZEB1 does not appear to have a major affect on body fat distribution in females.

Glucose homeostasis in WT mice without estrogen signaling resembles that of *TCF8+/-* mice

Our previous study demonstrated that glucose uptake is impaired in 2- and 3-month old female *TCF8+/-* mice fed a HFD (Saykally *et al.*, manuscript in revision). To determine whether the effects of ZEB1 insufficiency on glucose homeostasis are dependent upon estrogen, male mice and female mice treated with Faslodex were subjected to a standard glucose tolerance test (Fig. 6). Although the *TCF+/-* mice appear to have slightly better glucose clearance than WT mice of either gender, no significant differences between WT and *TCF8+/-* animals in blood glucose uptake were observed in either the male mice at 3 months of age (Fig. 6A) or the Faslodex-treated females at 3 or 5 months of age (Fig. 6B and 6C, respectively). These results support the contention that the effects of estrogen on glucose homeostasis are mediated at least in part directly or indirectly via ZEB1.

FIGURE. 5. Gonadal fat pad mass is not affected by ZEB1 haploinsufficiency. Gonadal fat pads from *TCF8+/-* (black bars) or WT (gray bars) Faslodex-treated female mice on a HFD (A) and male mice on a HFD (B) or RCD (C) were isolated at the indicated times, ages in months and weighed. n = 7-10 mice per group, * $p<0.05$

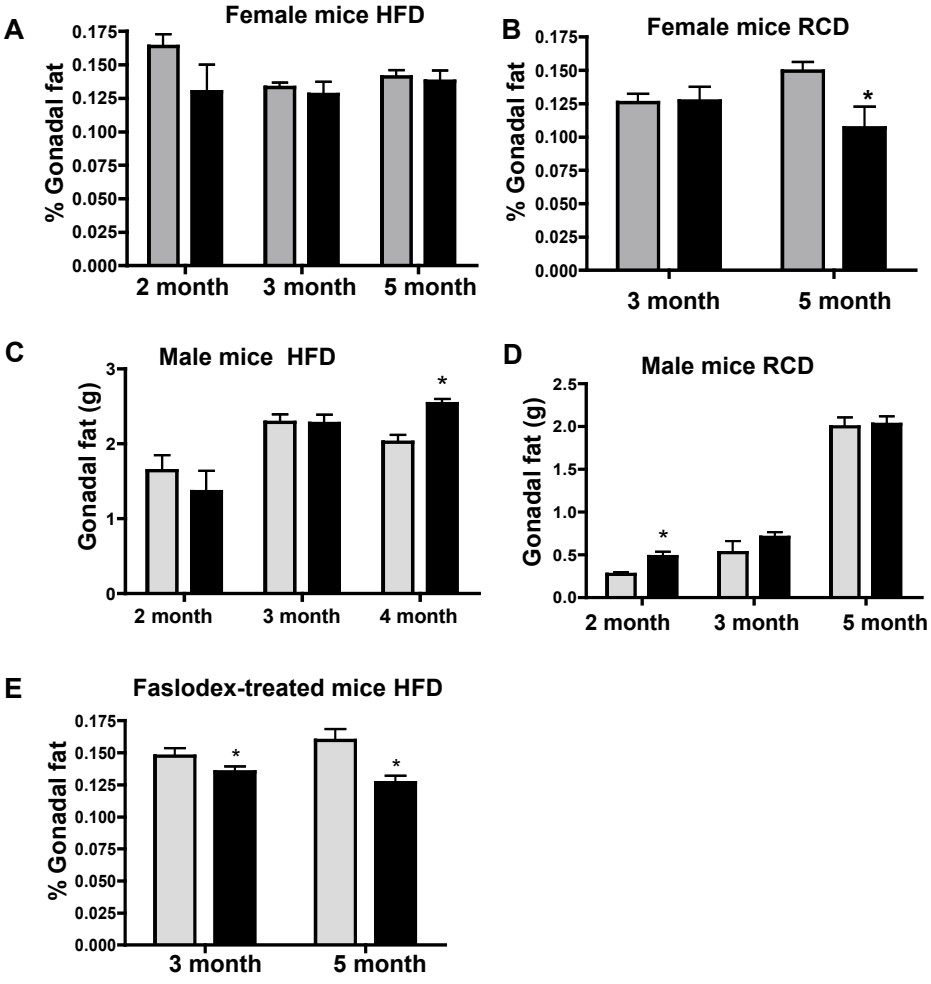
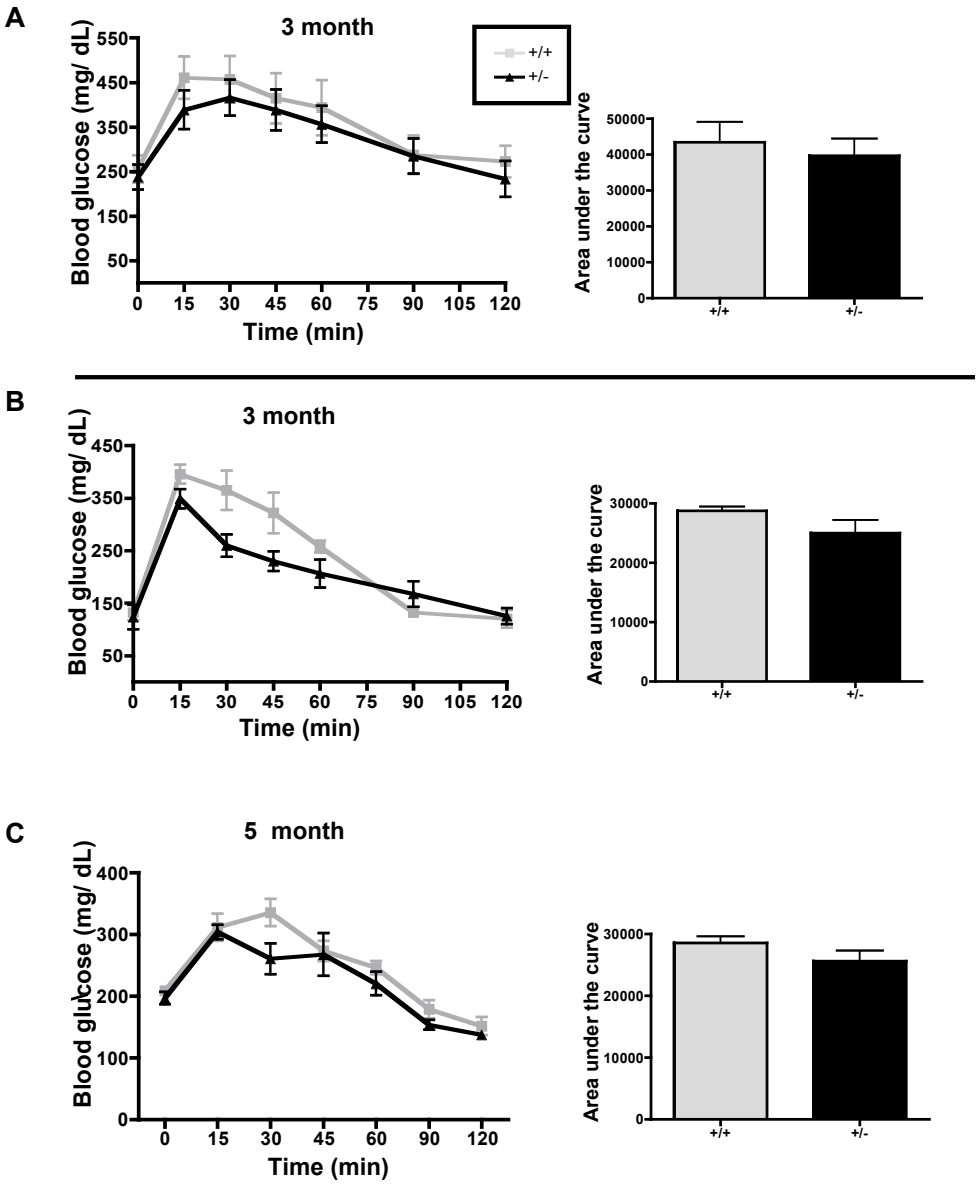

A
Female mice HFD
% Gonadal fat
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
2 month
3 month
5 month
B
Female mice RCD
% Gonadal fat
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
*
3 month
5 month
C
Male mice HFD
Gonadal fat (g)
3
2
1
0
*
2 month
3 month
4 month
D
Male mice RCD
Gonadal fat (g)
2.5
2.0
1.5
1.0
0.5
0.0
*
2 month
3 month
5 month
E
Faslodex-treated mice HFD
% Gonadal fat
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
*
*
3 month
5 month

FIGURE. 6. Glucose homeostasis in WT female mice lacking estrogen signaling is comparable to that of the *TCF8+/-* mice. WT and *TCF8+/-* male mice at 3 months of age **(A)** and Faslodex-treated female mice at 3 months **(B)** or 5 months of age were subjected to a standard glucose tolerance test (2 mg/kg glucose). Blood glucose levels were determined and are plotted over time (left panels) or as area under the curve (right panels). No significant differences existed between genotypes for area under the curve. n= 4-8 mice per group, * $p<0.05$


A
3 month
+/+
+/-
Blood glucose (mg/ dL)
550
450
350
250
150
50
0 15 30 45 60 75 90 105 120
Time (min)
Area under the curve
50000
40000
30000
20000
10000
0
+/+
+/-
B
3 month
Blood glucose (mg/ dL)
450
350
250
150
50
0 15 30 45 60 75 90 105 120
Time (min)
Area under the curve
30000
20000
10000
0
+/+
+/-
C
5 month
Blood glucose (mg/ dL)
400
300
200
100
0
0 15 30 45 60 90 120
Time (min)
Area under the curve
30000
20000
10000
0
+/+
+/-

Resistin increases in *TCF8+/-* female mice

Cytokines and hormones, collectively often called adipokines, secreted from adipocytes are actively involved in modulating energy homeostasis and adipose accumulation. Although the metabolic consequences of these adipokines remain poorly understood and their roles in mice sometimes differ from those in humans [181], they are often monitored as a measure of metabolic status. Because ZEB1 is a transcription factor, we measured the mRNA levels of four of the most commonly monitored adipokines in gonadal fat from male, female, and Faslodex-treated female WT and *TCF8+/-* mice at 3 months of age that were fed a HFD (Fig. 7).

TNFα is an inflammatory cytokine that reduces insulin sensitivity and typically increases with obesity [195]. No significant differences were observed in TNFα mRNA levels between genotypes with any of the groups (Fig. 7A). However, the male mice have significantly more TNFα mRNA, presumably as a consequence of their extensive adipose tissue depots and low levels of estrogen signaling. Although estrogen typically represses TNFα expression [196], no difference was observed between the Faslodex-treated and untreated female mice. This may suggest that body weight has more impact on TNFα expression than estrogen signaling.

The regulation of adiponectin by estrogen is somewhat controversial, with some reports indicating that estrogen represses adiponectin levels in mice [197] and others indicating that it induces it [198], These differences are presumably due to differences in experimental conditions and mouse models. As ZEB1 is

both an activator and repressor of target gene transcription, these observations raise the possibility that it mediates both of these opposing actions of estrogen. However, adiponectin mRNA did not significantly differ between genotypes in either gender, although there was a trend for higher adiponectin levels in the male and Faslodex-treated *TCF8+/-* mice (Fig. 7B). Adiponectin mRNA decreased in the Faslodex-treated WT female mice compared to the untreated WT females, indicating that estrogen induces adiponectin in this model. This effect does not appear to be mediated by ZEB1 as adiponectin levels are not significantly lower in the *TCF8+/-* mice nor are they affected by Faslodex treatment. In contrast to the mRNA data, circulating adiponectin levels increase in both genotypes with Faslodex treatment. In fact, the levels in Faslodex-treated mice are very similar to those in males. Thus, mRNA expression is induced by estrogen and protein expression is repressed. This may explain some of the discrepancies in the literature. In any event, the effects of estrogen on adiponectin levels do not appear to be mediated by ZEB1.

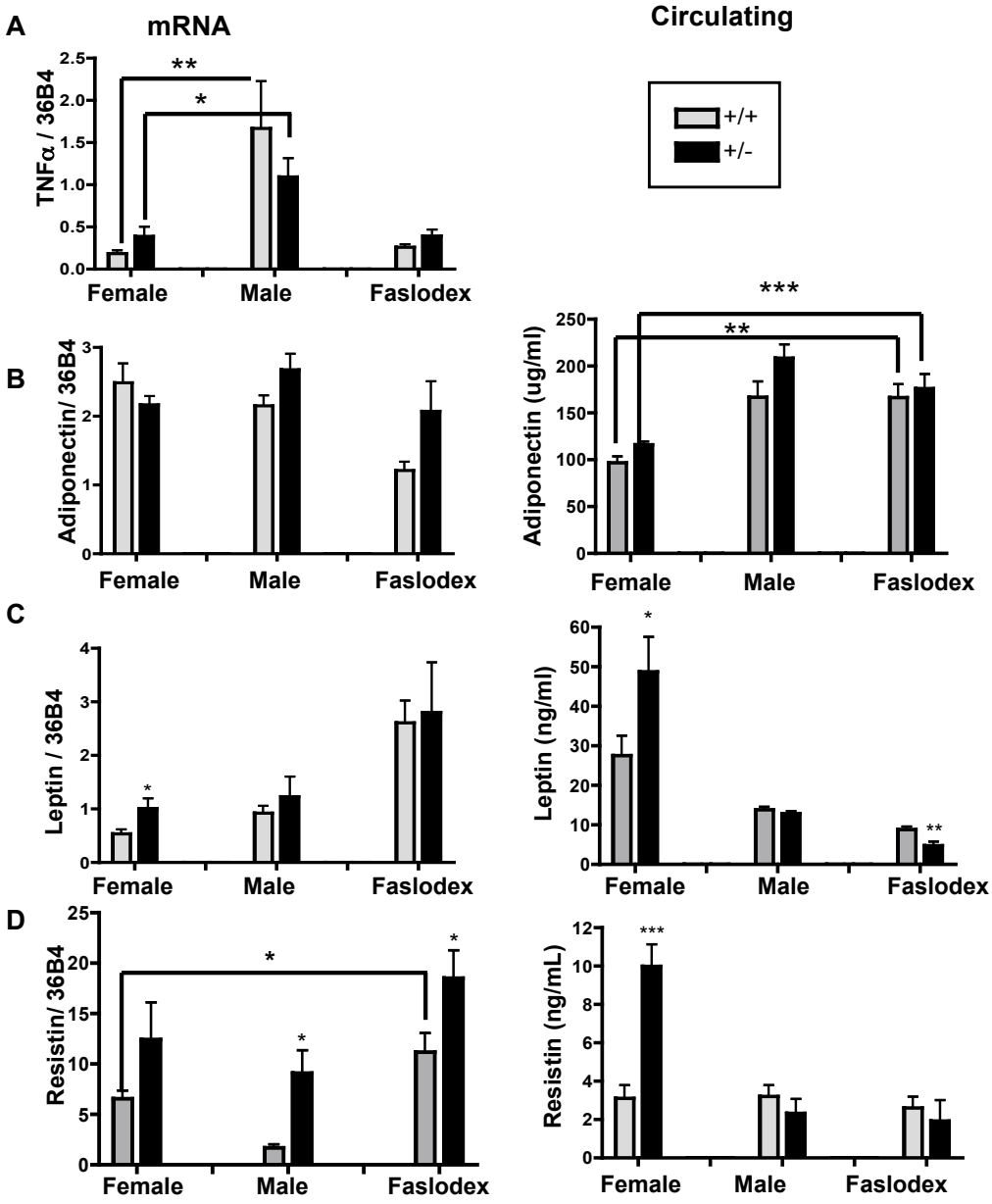
Leptin is secreted from adipocytes but exerts its effects in the hypothalamus [141, 203]. It functions to decrease feeding and increase energy expenditure and is positively correlated with obesity. Therefore, one would expect leptin mRNA and circulating leptin to be elevated in *TCF8+/-* female mice compared to WT, which they are (Fig. 7D). Because leptin is also directly upregulated by estrogen [204] we would expect to see a decrease in Faslodex-treated animals. Interestingly, Faslodex-treated mice of either genotype had increased leptin mRNA levels. This may be a consequence of leptin resistance.

The loss of estrogen signaling decreases leptin sensitivity [143, 205, 206] and as a consequence Faslodex-treated mice may be compensating for the reduced affinity by increasing leptin mRNA levels. In contrast to the mRNA data, circulating leptin levels are not induced in the Faslodex-treated animals, this difference is presumably because we are measuring leptin expression in visceral fat, and serum leptin levels are primarily secreted from subcutaneous adipose tissue [84]. As expected female mice have higher circulating leptin [84, 86, 119], and *TCF8*+/- female mice have significantly more circulating leptin, which is consistent with their increased adiposity. Overall, these data suggest that removal of estrogen signaling by Faslodex treatment has unexpected effects on leptin mRNA but not on protein expression.

Resistin mRNA levels in rodent adipose tissue positively correlate with fat mass [199]. As with adiponectin, the effects of estrogen on resistin expression in rodents are somewhat controversial, with some reports suggesting it is induced [200] and others suggesting it is repressed [201, 202]. In our experiments, resistin mRNA increase in *TCF8+/-* female mice treated with Faslodex (Fig. 6C, left panel). Furthermore, both the male and Faslodex-treated female *TCF8+/-* mice have significantly higher levels of resistin mRNA even though their fat mass does not differ from their WT counterparts. *TCF8+/-* mice show increased resistin mRNA expression regardless of gender, suggesting that ZEB1 negatively regulates resistin gene expression in an estrogen-independent manner. Interestingly, there is little correlation between the resistin mRNA expression and the circulating protein. While ZEB1 appears to repress resistin mRNA

expression, circulating resistin is dramatically increased in female *TCF8+/-* mice (Fig. 6C, right panel). Because this increase is only observed in female mice with intact estrogen signaling, ZEB1 apparently inhibits circulating resistin levels in an estrogen-dependant manner.

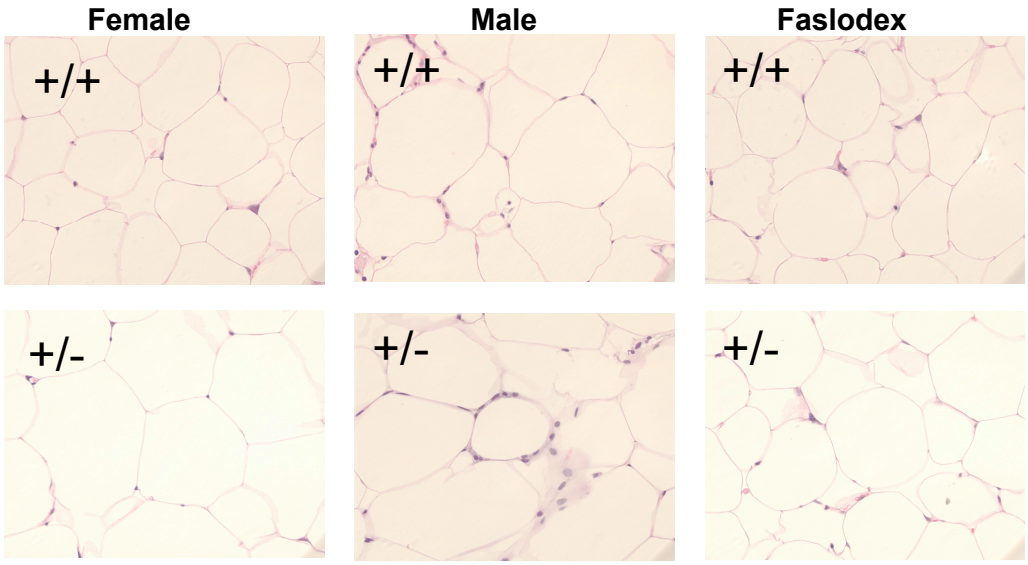
FIGURE. 7. ZEB1 represses resistin expression. Gonadal fat pads were isolated from WT (grey bars) and TCF8+/- (black bars) female, male, and Faslodex-treated female mice fed a HFD at 3 months of age. mRNA was isolated and subjected to qPCR for TNFα (A), adiponectin (B, left panel), leptin (C, left panel), and resistin (D, left panel). Serum adiponectin (B, right panel), leptin (C, right panel), and resistin (D, right panel) serum levels were also measured by ELISA (C, right panel). n = 5-9 mice per group, * $p<0.05$

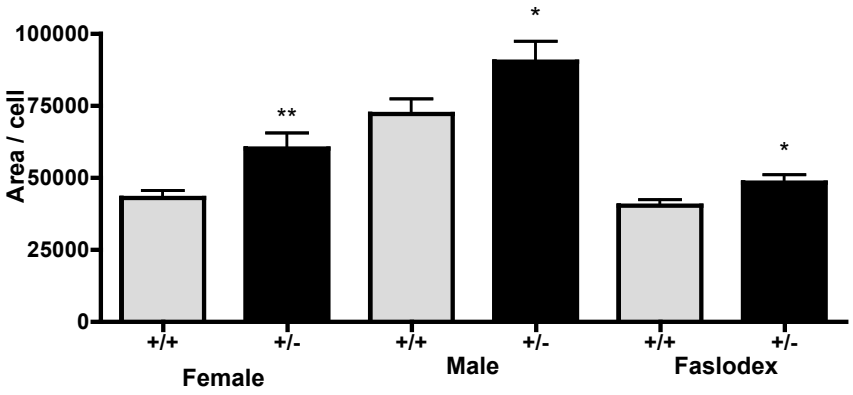

A
mRNA
Circulating
TNFα / 36B4
+/+
+/-
B
Adiponectin/ 36B4
Adiponectin (ug/ml)
C
Leptin / 36B4
Leptin (ng/ml)
D
Resistin/ 36B4
Resistin (ng/mL)
Female
Male
Faslodex

Adipocyte size increases as a result of ZEB1 haploinsufficiency

Estrogen regulates both the size and number of adipocytes as reviewed in [118, 119]. To ascertain whether ZEB1 mediates the effects of estrogen on adipocyte size and/or number, gonadal fat pads from WT and *TCF8+/-* female, male, and Faslodex-treated female mice were stained with hematoxylin and eosin, and the area of the cells measured by digital imaging (Fig. 8). Histological staining (Fig. 8A) as well as quantification of cell area (Fig. 8B) showed that all of the *TCF8*+/- mice had greater cell areas. This result is somewhat surprising as there was no difference in overall body weight or fat mass in either the Faslodex-treated female mice or the male mice (Figs. 2 and 3), although the *TCF8+/-* male mice did have more gonadal fat mass at 4 months on a HFD and at 2 months on a RCD (Fig. 5). This lack of absolute concordance of adipocyte area with total fat mass may relate to the sensitivity of the other measurements, to effects of ZEB1 that are estrogen-independent, to effects of ZEB1 on adipocyte number, or to some yet unidentified confounding metabolic effect. Nonetheless, these data show unequivocally that adipocyte size increases as a consequence of ZEB1 haploinsufficiency and, conversely, that ZEB1 opposes fat deposition in adipocytes.

FIGURE. 8. *TCF8+/-* mice have increased adipocyte size. Gonadal fat pads were isolated at 3 months of age from WT (grey bars) and TCF8+/- (black bars) female, male, and Faslodex-treated female mice fed a HFD. The fat pads were sectioned and subjected to hematoxylin and eosin staining **(A)**. The area per cell was calculated from 9 fields, with a total of >100 cells (B). n=3 mice per group, * $p<0.05$


Female
Male
Faslodex
+/+
+/+
+/+
+/-
+/-
+/-

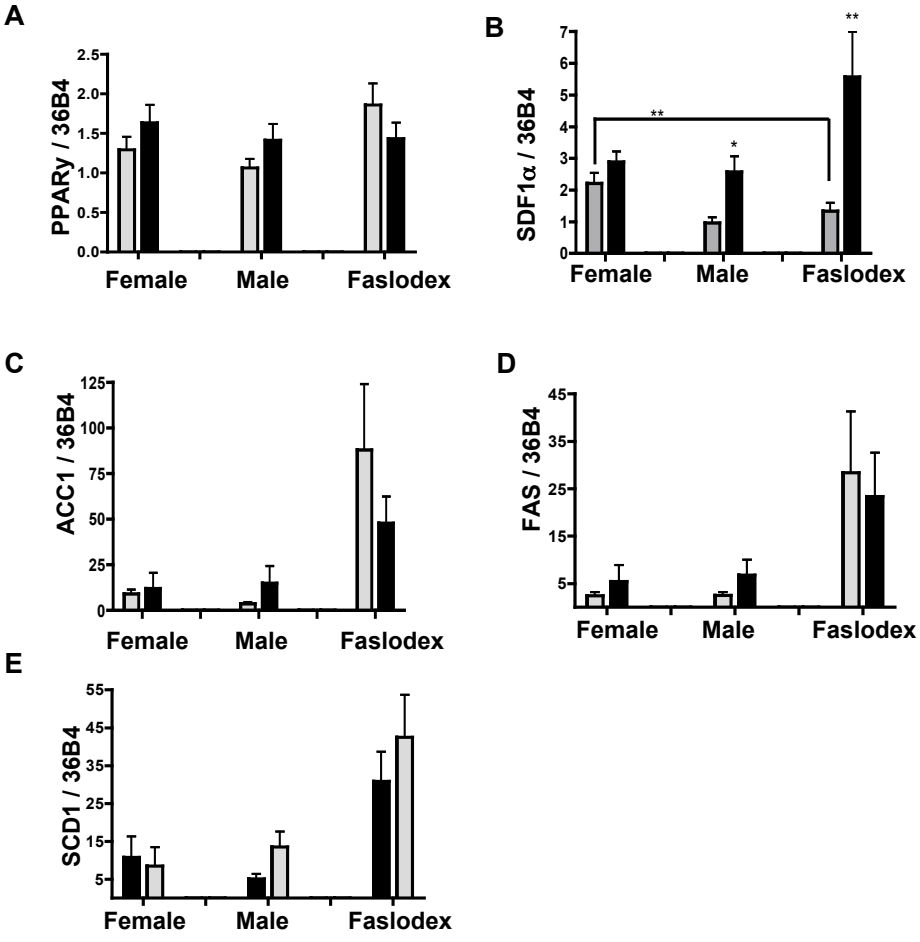

Area / cell
100000
75000
50000
25000
0
**
*
*
+/+
+/-
+/+
+/-
+/+
+/-
Female
Male
Faslodex

ZEB1 does not alter de novo lipogenesis

Adipocyte size is determined by the net result of fatty acid synthesis, uptake, and utilization. Estrogen regulates a number of genes that affect fatty acid metabolism and ultimately adipocyte hyperplasia [84, 119, 122, 123, 125, 207], Thus, it seems plausible that ZEB1 might mediate some of the effects of estrogen on adipocyte size. To assess whether the adipocyte hypertrophy observed in the ZEB1 deficient mice (Fig. 8) might be the result of changes in the expression of one or more of these genes, the mRNA levels for 4 estrogen-regulated genes in fatty acid metabolism were determined in the gonadal fat of WT and *TCF8+/-* female, male, and Faslodex-treated female mice on a HFD (Fig. 9). PPARγ, which is not estrogen-regulated, was used as a negative control (Fig. 9A) and SDF1α (Fig. 9B), which is estrogen-regulated [194], was used as a positive control for verifying the efficacy of the Faslodex treatment. As expected, PPARγ mRNA levels were not affected by either genotype or gender. In contrast, Faslodex treatment significantly decreased SDF1α mRNA levels in WT mice, as expected (Fig. 9B). Interestingly, SDF1α mRNA levels significantly increased in *TCF8+/-* male and Faslodex-treated mice, suggesting that ZEB1 inhibits SDF1α expression independently of estrogen. No significant differences were observed between *TCF8+/-* and WT mice of either gender in the mRNAs that encode acetyl-CoA carboxylase-1 (ACC1), fatty acid synthase (FAS), or stearoyl coA desaturase-1 (SCD1), although Faslodex treatment increased expression of all of these mRNAs, as expected (Figs 8C, 8D, and 8E, respectively). These data indicate that ZEB1 does not mediate the repressive effects of estrogen on the

fatty acid synthesis genes ACC1, FAS, and SCD1 genes, all genes by estrogen. Also, these data suggest that the increase in adipocyte area as a consequence of ZEB1 haploinsufficiency is not the result of increases in fatty acid synthesis. The limitation of this interpretation is that enzyme activities were not measured.

FIGURE. 9. ZEB1 does not regulate key enzymes involved in fatty acid synthesis to control adipocyte size. Gonadal fat pads were isolated at 3 months of age from WT and *TCF8+/-* female, male, and Faslodex-treated female mice fed a HFD. mRNA was isolated and subjected to qPCR for PPARγ (A), SDF1α (B), ACC1(C), FAS (D), and SCD1 (E). n = 5-9 mice per group * $p<0.05$, **$p<0.005$.


A
PPARγ / 36B4
2.5
2.0
1.5
1.0
0.5
0.0
Female
Male
Faslodex
B
SDF1α / 36B4
7
6
5
4
3
2
1
0
**
*
**
Female
Male
Faslodex
C
ACC1 / 36B4
125
100
75
50
25
0
Female
Male
Faslodex
D
FAS / 36B4
45
35
25
15
5
Female
Male
Faslodex
E
SCD1 / 36B4
55
45
35
25
15
5
Female
Male
Faslodex

ZEB1 may limit adipocyte size through down-regulation of perilipin

Because fat accumulation is dependent upon uptake as well as synthesis, the effects of ZEB1 on the enzymes involved in fatty acid uptake into adipocytes were examined. LPL is the rate-limiting enzyme for lipogenesis, and its function is to hydrolyze triglycerides in the capillary wall, releasing lipids for uptake into the adipocyte [78]. Furthermore, estrogen decreases LPL activity [122], so it seemed plausible that ZEB1 might mediate the repressive effect of estrogen on LPL expression. To determine whether ZEB1 limits adipocyte size by regulating LPL, its mRNA levels and protein activity were determined in gonadal fat pads (Fig. 10). LPL expression increased in all *TCF8+/-* mice regardless of gender, as determined by 2 way ANOVA (Fig. 10A). However, LPL activity did not increase in the gonadal fat of the *TCF8+/-* mice (Fig. 10B). In fact, there is a decrease in activity in female *TCF8+/-* mice. This decrease is not a consequence of direct effects on transcription of the LPL gene as mRNA levels were not repressed.

To examine whether the increase in adipocyte area in the *TCF8+/-* mice was a consequence of decreased lipolysis, the expression of hormone sensitive lipase (HSL), a key enzyme necessary for lipolysis [79], was measured (Fig. 11A). As expected, no effects of gender were observed in WT animals as HSL activity is regulated by its phosphorylation by protein kinase A following stimulation by β-adrenergic agonists [79]. Estrogen increases lipolysis by increasing the number of β-adrenergic receptors [84, 119, 123]. In some cases treatment with estrogen also increased HSL protein expression, which may contribute to increased lipolysis [81]. Although there was a trend for HSL mRNA

levels to be elevated in *TCF8+/-* mice, only the males reached significance. These data suggest that ZEB1 does not mediate estrogen's effects on HSL expression, at least at the mRNA level.

HSL-mediated lipolysis can only occur when lipids are accessible for oxidation. Lipids in adipocytes are stored in droplets that are coated by the protein perilipin A, preventing HSL from hydrolyzing the lipids. Removal of perilipin is required for HSL-mediated lipolysis [208], and this serves as another mechanism for regulating lipolysis [208-210]. To examine whether perilipin levels are altered in the *TCF8+/-* mice, its expression was determined by qPCR. An increase in perilipin expression was observed in all *TCF8+/-* mice (Fig. 11B), which may explain the adipocyte hypertrophy seen in these mice (Fig. 8). We do not observe an estrogen-specific change in perilipin expression suggesting that ZEB1 this is not the mechanism through which ZEB1 is mediating estrogens protective effects.

FIGURE. 10. LPL mRNA but not activity is increased in *TCF8+/-* animals. Gonadal fat pads were isolated at 3 months of age from WT and *TCF8+/-* female, male, and Faslodex-treated female mice fed a HFD. (A) mRNA was isolated and subjected to qPCR for LPL and 36B4. (B) Protein was isolated and 10 ng used to measure LPL activity. n=5-9 mice per group, * <0.05.

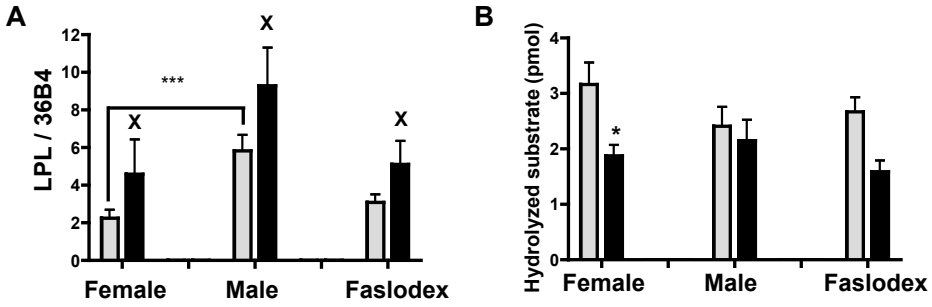
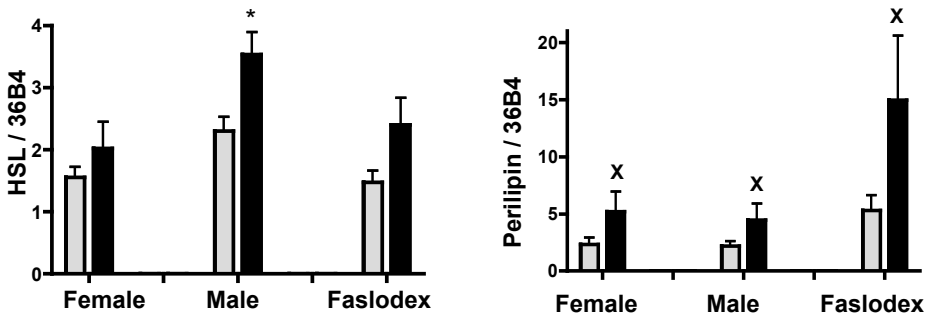

A
LPL / 36B4
12
10
8
6
4
2
0
***
X
X
X
Female
Male
Faslodex
B
Hydrolyzed substrate (pmol)
4
3
2
1
0
*
Female
Male
Faslodex

FIGURE. 11 *TCF8 +/-* mice have increased perilipin expression. Gonadal fat pads were isolated at 3 months of age from WT and TCF8+/- female, male, and Faslodex-treated female mice fed a HFD. mRNA was isolated and subjected to qPCR for **(A)** HSL, **(B)** perilipin, and 36B4. N=5-9 mice per group


HSL / 36B4
4
3
2
1
0
*
Female
Male
Faslodex
Perilipin / 36B4
20
15
10
5
0
X
X
X
Female
Male
Faslodex

DISCUSSION

Haploinsufficiency of the transcription factor ZEB1 increases body weight and adipose mass, through as yet undefined mechanisms (Saykally *et al*. manuscript in revision). The studies herein demonstrate that ZEB1 is mediating at least some of the effects of estrogen in opposing adiposity (Figs. 2 and 3) and in modulating glucose uptake (Fig. 6), providing an explanation for ZEB1's effects on fat accumulation. Our data show that ZEB1 deficiency has no effect in mice lacking estrogen signaling, such as female mice treated with Faslodex or male mice, and that those mice gain excessive amounts of weight and increased fat mass regardless of genotype (Figs 2 and 3). This highlights the role that ZEB1 plays in mediating the effects of estrogen on metabolism.

Although ZEB1 clearly mediates at least some of the anti-obesity effects of estrogen in female mice, some of our data suggest that ZEB1 may attenuate adiposity in males as well. Even though the *TCF8+/-* male mice do not show increased body weight or fat mass, this may be because C57Bl/6 males gain weight so rapidly that ZEB1's modest modulatory effects in the heterozygous context are unable to oppose it. A complicating issue is that ZEB1 mRNA levels increase with adiposity, so that even the *TCF8+/-* males and the older females have significant amounts of ZEB1 in their adipose tissue. At 3 months of age, males have 3-fold the amount of fat as do Faslodex-treated females (Fig. 3, Fig. 5), even though the males are only 20% heavier. At the cellular level, it appears as though ZEB1 modulates lipid accumulation in males as well as females as both show increased adipocyte hypertrophy (Fig. 8). This is in the context of

increased HSL expression in male heterozygous mice (Fig. 11), which would be expected to reduce fatty acid depot size. The potential role of ZEB1 in males will need to be examined more extensively by using adipose tissue-specific *TCF8* knockout mice when the floxed *TCF8* gene becomes available.

Because ZEB1 mediates the effects of estrogen on body weight and fat accumulation, we wanted to assess whether it mediates the effect of estrogen on adipokine expression (Fig. 7). As ZEB1 is a transcription factor, we were most interested in investigating its effects on mRNA expression, although it is ultimately the serum levels of the resultant proteins that are of most metabolic consequence. We chose to measure four of the most commonly monitored adipokines and found no effect of the *TCF8+/-* genotype on TNFα mRNA (Fig. 7A) or on adiponectin mRNA or protein levels (Fig. 7B). In contrast, both leptin mRNA and protein were elevated in *TCF8+/-* female animals, which is expected because they have a higher fat mass than the WT females (Fig. 7C). Surprisingly, Faslodex-treated *TCF8+/-* mice had significantly lower serum levels than the Faslodex-treated WT mice, suggesting that ZEB1 acts independently of estrogen to increase serum leptin levels. This is likely to be indirect as this effect was not present at the mRNA level. Of most interest was the observation that serum resistin levels increased significantly in the female *TCF8+/-* mice. This might be attributed to the fact that resistin increases with obesity [199]. Although this difference was not significant at the mRNA level, this is likely due to the high amount of variation seen in the data from the *TCF8+/-* mice. This contention is supported by the fact that resistin mRNA levels are elevated in both the *TCF8+/-*

males and Faslodex-treated females. Thus, ZEB1 appears to repress resistin gene expression independently of estrogen. This increase in resistin mRNA levels in the *TCF8+/-* males and Faslodex-treated females cannot be attributed to obesity as they are not significantly different in fat mass or body weight from the WT controls. Thus, it does not appear as though ZEB1 mediates any of the effects of estrogen on these cytokines, at least in a physiological context where there are significant levels of ZEB1 still present. Instead, ZEB1 appears to independently repress resistin mRNA levels. It will be of interest to determine whether this is a direct or indirect effect at the level of the resistin gene.

The increase in adiposity in the female *TCF8+/-* mice may be attributed to an increase in lipid accumulation in adipocytes. We found that the *TCF8+/-* mice, regardless of gender, have larger adipocytes (Fig. 8), which suggests that ZEB1 may modulate adiposity even in males. Measurement of the expression of a number of estrogen-regulated proteins involved in *de novo* protein synthesis indicates that ZEB1 is not affecting the transcription of ACC1, FAS, or SCD1 (Fig. 9). It also does not affect the accumulation of LPL mRNA, although there is a trend for more LPL mRNA in the haploinsufficient mice (Fig. 10A). However, this is unlikely to have biological consequences as serum levels of LPL are not elevated. In fact, they are significantly reduced in the *TCF8+/-* female mice (Fig. 10B). Thus, the uptake of fatty acids by adipocytes is unlikely to be enhanced by ZEB1 haploinsufficiency. Another possibility for the increased accumulation of lipids was that their utilization is decreased in the *TCF8+/-* mice. Although HSL levels are not decreased in these mice, perilipin levels are increased (Fig. 11).

Perilipin is positively associated with obesity because it surrounds fatty acid droplets and prevents their hydrolysis by HSL. The increase in perilipin expression in the *TCF8+/-* mice cannot be ascribed to obesity as the male and Faslodex-treated females do not have increased fat mass compared to WT. Thus, the increase in perilipin expression in the ZEB1 deficient mice may contribute to the increase in adipocyte area. This is supported by the fact that perilipin is increased in all of the *TCF8+/-* mice, as is adipocyte hypertrophy (Fig. 8).

The data in this paper demonstrate that the ZEB1 transcription factor mediates many of the effects of estrogen in attenuating obesity, at least in mice. They also show that ZEB1 may have metabolic effects that are independent of estrogen. Initial experiments indicate that ZEB1 insufficiency increases adipocyte size without affecting adipocyte number, but additional experiments will be needed to ascertain whether or not ZEB1, like estrogen, affects adipogenesis. The increase in lipid deposition in adipocytes may be the result, at least in part, of increased perilipin expression. While the experiments herein demonstrate that estrogen establishes a transcriptional cascade that includes ZEB1 to attenuate adiposity, additional experimentation is necessary to delineate ZEB1's roles in energy homeostasis. This will require identifying ZEB1 target genes. Experiments are in progress to gene profile targets of ZEB1 using *TCF8* null mouse embryo fibroblasts that are stimulated to undergo adipogenesis. Nonetheless, ZEB1 is a important transcription factor in preventing adipose accumulation and promoting

glucose homeostasis in female mice, improving our understanding of sexual dimorphism in metabolism.

CHAPTER V

Reduction in ZEB1 expression diminishes adipocyte differentiation *in vitro*

INTRODUCTION

We recently demonstrated that haploinsufficiency of ZEB1 accentuates adipose accumulation in female mice and increases adipocyte size (Saykally et al., manuscript under revision). Whether ZEB1 impacts the number of adipocytes as well as their size was not clear. Through the regulation of a number of genes, ZEB1 impedes the differentiation of mesodermal tissues such as bone [17, 36, 43, 44], muscle [30, 45], and chondrocyte lineages [16, 47, 48], suggesting that it may also attenuate adipogenesis.

Opposing data suggest that ZEB1 might promote adipogenesis. ZEB1 is positively regulated by insulinlike growth factor-1 (IGF-1) in primary human prostate cancer cells [12], raising the possibility that IGF-1 regulates ZEB1 in adipose tissue as well. IGF-1 is a critical factor in adipocyte development *in vitro* [211]. IGF-1 activates intracellular signaling pathways, such as MAP Kinase (MAPK) and Phosphoinositide 3-Kinase (PI3K), to both promote pre-adipocyte proliferation and adipocyte differentiation [212, 213]. While these events are typically thought to be mutually exclusive, to achieve adipocyte differentiation *in vitro* cells must proliferate to reach a confluent state and then exit the cell cycle [214-216]. Treatment with a differentiation cocktail prompts the cells to re-enter the cell cycle and go through two rounds of mitotic clonal expansion, as reviewed in [64, 68, 69, 217, 218]. This initiates a transcriptional cascade that results in upregulation of a number of transcription factors including C/EBPα and the critical transcription factor PPARγ [219]. Thus it is possible that IGF-1 may

induce ZEB1 in pre-adipocytes, and ZEB1 then participates in the subsequent clonal expansion of cells and their subsequent differentiation into adipocytes.

Additional support for ZEB1 as a pro-proliferative transcription factor comes from studies on its role in cancer progression. Because cancer is characterized by the unregulated, rapid proliferation of cells, and because ZEB1 expression is high in a number of cancers [58-63], this supports the hypothesis that ZEB1 promotes cell proliferation. Also, Liu et al. [49] showed that loss of ZEB1 results in cellular senescence. Mouse embryonic fibroblasts (MEFs) generated from the *TCF8* knockout mouse only survived 2 passages compared to 10 passages for the WT MEFs. Furthermore, ZEB1 directly binds and represses the p73 tumor suppressor in muscle [30]. All these data support the contention that ZEB1 promotes cell proliferation, perhaps by regulating cell cycle progression

Because our data in mice indicate that ZEB1 opposes adiposity, we wanted to investigate whether ZEB1 affects adipocyte proliferation and/or differentiation in cell culture. To address this question, I transduced NIH3T3-L1 preadipocytes with lentiviral constructs that were designed to reduce ZEB1 expression. Although I was unable to complete these studies, initial data suggest that low levels of ZEB1 reduce adipocyte differentiation and lipid accumulation.

MATERIALS AND METHODS

Cell culture. 3T3-L1 cells were obtained from ATCC and maintained in DMEM with 10% calf serum and 1% penicillin/streptomycin. To differentiate these pre-adipocytes into mature adipocytes, the cells were grown to confluency, and 48 h later they were treated with differentiation media consisting of DMEM with 10% fetal bovine serum supplemented with bovine insulin, dexamethasone, and isobutylxanthine [68, 69, 218]. Two days following treatment, the medium was refreshed with DMEM +10% FBS and 10% bovine insulin

C3H10T1/2 cells were differentiated as in [71]. Cells were maintained in DMEM with 10% fetal bovine serum and 1% penicillin/ streptomycin. Fifty ng/mL BMP-4 (bone morphogenetic protein-4) (R& D systems, Minneapolis, MN) was added to the cells when they reached ~75% confluency. Once cells were confluent they were treated to differentiate with the same cocktail as the 3T3-L1 cells.

Lipid accumulation was measured using Oil Red O lipophilic stain. In brief, cells were fixed with 10% neutral buffered formalin for 24 h, washed with water and isopropyl alcohol, dried, and stained for 10 min with Oil Red O. Thorough washing removed excess stain and photographic digital images were taken using an Axiovert 2 Upright Zeiss microscope at 20x magnification.

Quantitative PCR (qPCR). RNA was isolated from cells using TriZOL (Invitrogen, Carlsbad, CA). Two μg of total RNA was reverse transcribed using

AMV reverse transcriptase. cDNA was amplified on the BioRad iCycler (#170-8740, Hercules, CA) using iQ SYBR Green Supermix (BioRad #1708885) and normalized to 36B4 mRNA quantified in the same manner. Primers and melting temperatures are given in Table 1.

Constructing ZEB1 knockdown cell lines. Sh#1 (V2LMM_15202) and Sh#2 (V2LMM_18746) were purchased from Open Biosystems (Huntsville, AL). miR200c oligos were annealed at 65 °C for 15 minutes and cloned into the pGIPz (Open Biosystems) vector at the XhoI and EcoR1 sites that are within the flanking 5' miR30 sequences. A Scrambled nucleotide control was cloned from pSHAG (kindly provided by the Reuben Harris lab) into the pGIPz vector. All sequences of inserts are provided in Table 2. Constructs were transfected into HEK293FT cells with VSV, gag/pol, and RT encoding vectors (provided by the U of MN Obesity Center). Green fluorescent protein (GFP) within the pGIPz vector served as a control for transfection efficiency. Medium was refreshed 24 h post transfection. Lentiviral conditioned media were collected 72 h post transfection. Media were spun down to remove cellular debris and run through a 0.45 micron filer, aliquotted, and frozen at -80°C until use. 3T3-L1 cells were transduced using the lentiviral conditioned media and 8 μg/ml polybrene per the manufacturer's instructions. Puromycin was used to select those cells that contained the pGIPz vector. ZEB1 levels were measured by qPCR as endogenous miRs have been shown to reduce ZEB1 mRNA levels in some cells [18-20].

Table 1: Primers used to amplify genes throughout differentiation. List of primers and corresponding melting temperatures used to amplify cDNA throughout pre-adipocyte differentiation.

Gene	real-time PCR primers	Tm °C
ZEB1	Forward 5'-CGAGTCAGATGCAGAAAATGAGCA-3' Reverse 5'-ACCCAGACTGCGTCACATGTCTT-3'	60
PPARy	Forward 5' CCAGAGCATGGTGCCTTCGCT 3' Reverse 5' CAGCAACCATTGGGTCAGCTC 3'	57
Cyclin D1	Forward 5' TGACTGCCGAGAAGTTGTG 3' Reverse 5' ATGGCCAGCGGGAAGAC 3'	55
36B4	Forward 5' GACCTCCTTCTTCCAGGCTTTG 3' Reverse 5' CCACCTTGTCTCCAGTCTTTATCAG 3'	60

Cell proliferation and viability. Cellular proliferation was measured using the CellTiter Aqueous NON-Radioactive Cell Proliferation Assay (Promega, Madison, WI). The assay was used per the manufacturer's instructions. In brief cells were plated in 96 well plates at the numbers noted and allowed to adhere overnight. A MTS solution (MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was incubated with the cells for 2 h. Metabolically active cells convert MTS into formazan, resulting in a colorimetric change, which was measured at absorbance 490 on a Perkin Elmer 1420 Multilabel Count Victor 3 plate reader (Perkin Elmer, Waltham, MA). No corrections were made for cell number or protein, although the same number of cells was added per cell line as indicated.

The xCELLigence System (Roche Diagnostics, Indianapolis, Indiana) was used to measure cell adhesion, which is extrapolated to mean cell number, by electrical impedance. Cells were plated in a 96 well plate with the bottom of the wells coated with gold. An electrical current was passed through each well on the plate, and the electrical impedance recorded. Increased proliferation results in increased electrical impedance because of the increase in the cell surface adhered to the plate. Cells were continuously monitored with readings every 15 min for the first 4 h post treatment and every h for the following 6 days.

Apoptosis was measured using the EnzCHEK Caspase 3 Assay Kit (#R35100, Invitrogen, Carlsbad, CA). Cell lysates were harvested from actively proliferating cultures and incubated in duplicate with the Z-DEVD 110 substrate

in Reaction Buffer for 30 minutes. Caspase 3 enzymes present in the lysate cleave the Z-DEVD substrate, releasing the quenched fluorescence. Fluorescence was measured on a Perkin Elmer 1420 Multilabel Count Victor 3 plate reader (Perkin Elmer, Waltham, MA) at 496/520 nm excitation/ emission. Numbers were normalized to total protein quantified using the BioRad D/C Protein Assay (BioRad, Hercules, CA).

Statistics. The Graphpad Prisim program was used for statistical analysis. Standard error of the mean was calculated, and Student's t-Test used to analyze pair wise comparisons. The resultant p-values are as indicated in the figure legends.

RESULTS

ZEB1 expression increases during adipogenesis

ZEB1 is involved in the development of several tissues, opposing the differentiation of some mesenchymal tissues [16, 30, 36, 44-46], and in promoting the differentiation of others including T-cells [38] and smooth muscle cells [40, 41, 46]]. To explore the role, if any, that ZEB1 has in adipose tissue development endogenous ZEB1 mRNA levels were measured throughout differentiation of NIH3T3-L1 pre-adipocytes into mature adipocytes. The cells were induced to differentiate as described in Methods, and ZEB1 mRNA expression was measured using qPCR on the indicated days relative to treatment with cocktail (Figure 1A). ZEB1 mRNA levels show about a 4-fold increase when they reach confluency (Day 0) compared to levels during proliferation. By Day 2, ZEB1 mRNA levels return to that observed in pre-adipocytes and then increase to maximal levels (about 5-fold) by four days after treatment. The changing ZEB1 mRNA levels suggest that it may have a role in the early and late stages of adipocyte development. This is in agreement with our observations in mice where ZEB1 increases in response to increased adipose accumulation (Chapter 2 Figure 1, Chapter 3 Figure 4). PPARγ and cyclin D mRNAs were also measured to serve as positive controls for adipocyte development (Figure 1 B & C). They gave the expected results, indicating that the cells differentiated properly.

Because 3T3-L1 cells are already at the pre-adipocyte stage their use as a model system does not tell us whether ZEB1 affects commitment to the

adipocyte lineage. Adipocytes are formed from pluripotent mesenchymal cells that reside in the stroma of adipose tissue as well as bone marrow [42]. *In vivo* unknown cues trigger the commitment and differentiation of these cells into the bone, muscle, chondrocyte, or adipose lineages. To determine whether ZEB1 impacts pre-adipocyte commitment, the C3H10T1/2 pluripotent mesenchymal cell line was used as a model for adipogenesis. Treatment of C3H10T1/2 cells with BMP4 commits the cells to the pre-adipocyte lineage [42, 71]. After which they are differentiated into adipocytes with the same differentiation cocktail as used with the 3T3-L1 cells. Endogenous ZEB1 mRNA expression was measured throughout commitment and differentiation at the indicated time points (Figure 2A). A similar trend was observed with the C3H10T1/2 cells as was seen with the 3T3-L1 cells. ZEB1 mRNA levels increased at confluency, decreased with the initiation of differentiation, and then increased with lipid accumulation. Endogenous ZEB1 mRNA levels do not change prior to commitment of the pre-adipocyte (Figure 2B). These data suggest that ZEB1 either does not regulate commitment, or that appropriate level of ZEB1 is present in the mesenchymal precursor cells. PPARγ was measured (Figure 2C) and cells were stained for Oil Red O (data not shown), both of which confirmed a high level of differentiation.

Figure 1. ZEB1 expression changes throughout adipogenesis. 3T3-L1 cells were differentiated from pre-adipocytes into mature adipocytes and RNA harvested on the days indicated. ZEB1(A), PPARy (B), and Cylcin D1(C) mRNA expression were measured by qPCR. All genes were normalized to ribosomal protein 36B4. Cells reached confluency at Day -2 and were treated with a differentiation cocktail on Day 0. * $p < 0.05$

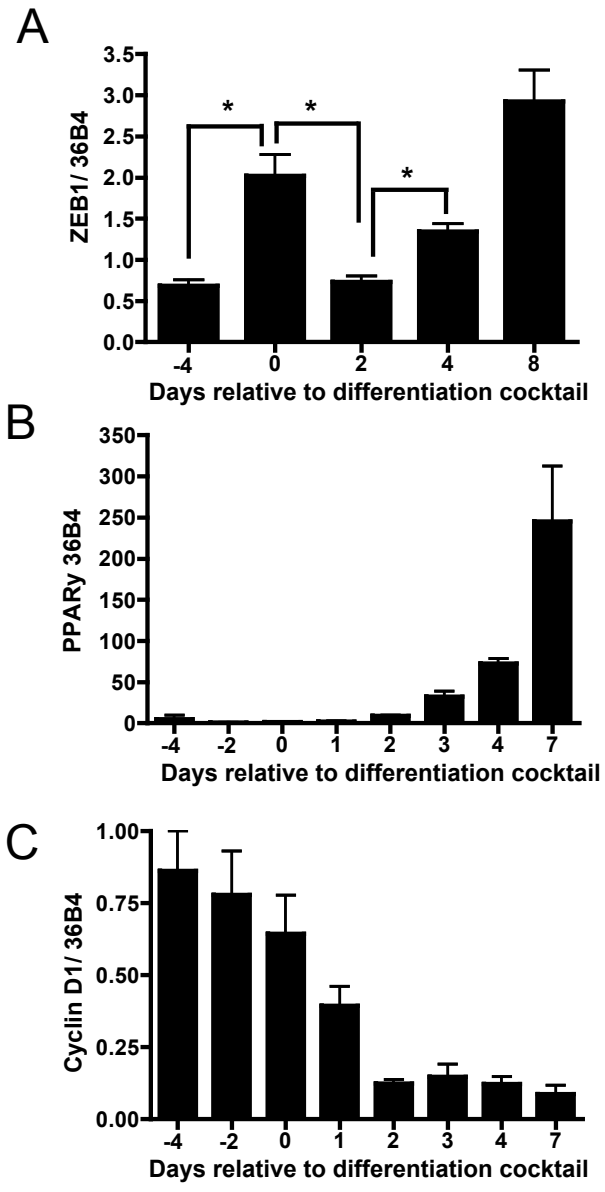
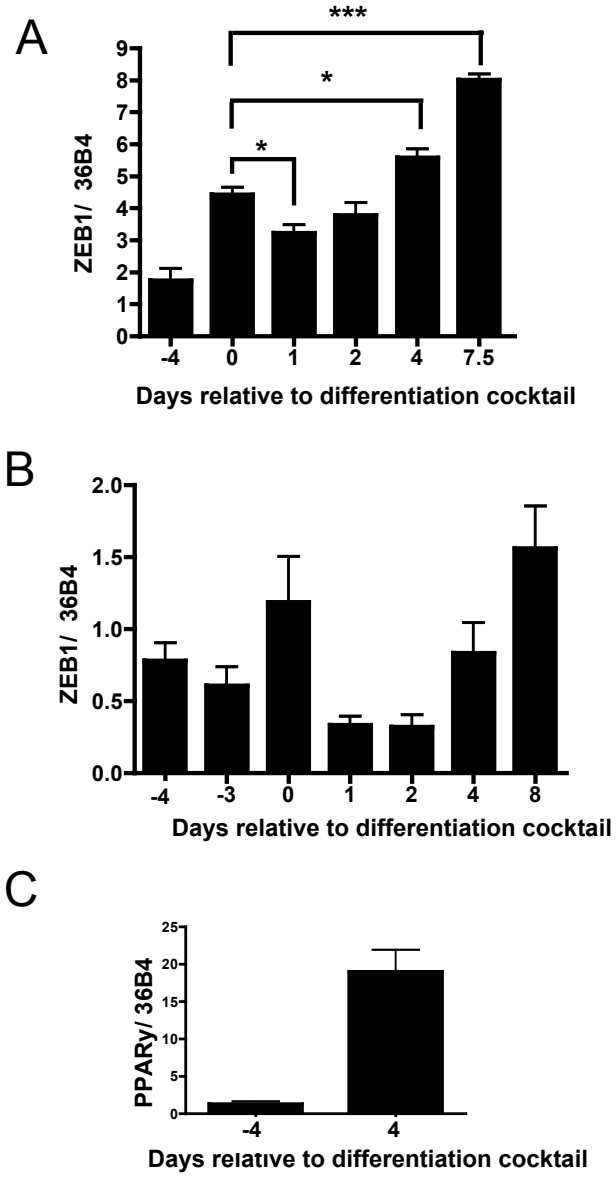

A
ZEB1/ 36B4
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
-4
0
2
4
8
Days relative to differentiation cocktail
B
PPARy 36B4
350
300
250
200
150
100
50
0
-4
-2
0
1
2
3
4
7
Days relative to differentiation cocktail
C
Cyclin D1/ 36B4
1.00
0.75
0.50
0.25
0.00
-4
-2
0
1
2
3
4
7
Days relative to differentiation cocktail

Figure 2. ZEB1 expression does not change during commitment to the pre-adipocyte lineage. C3H10T1/2 pluripotent mesenchymal stem cells were committed to the pre-adipocyte lineage by treatment with BMP4 at Day -4, when they were 75% confluent. Cells reached confluency at Day -2 and were treated with a differentiation cocktail on Day 0. RNA was harvested in triplicate at the days indicated. ZEB1 (A, B) and PPARγ (C) mRNA expression were measured by qPCR. All genes were normalized to ribosomal protein 36B4. * $p < 0.05$


A
***
*
*
ZEB1/ 36B4
0
1
2
3
4
5
6
7
8
9
-4
0
1
2
4
7.5
Days relative to differentiation cocktail
B
ZEB1/ 36B4
0.0
0.5
1.0
1.5
2.0
-4
-3
0
1
2
4
8
Days relative to differentiation cocktail
C
PPARγ/ 36B4
0
5
10
15
20
25
-4
4
Days relative to differentiation cocktail

Sh#2 and miR 200c effectively reduce ZEB1 expression

One interpretation of the increase in ZEB1 expression late in adipogenesis is that it may be important for monitoring or sensing lipid accumulation. To determine whether ZEB1 is important for promoting or inhibiting adipogenesis, ZEB1 levels were reduced in the 3T3-L1 cell line using anti- ZEB1 short hairpin microRNAs (shmiR) inserted into lentiviral constructs. miR200c endogenously regulates ZEB1 in a variety of cell types by binding to the seed sites in the 3' UTR which leads to degradation of the mRNA [18-20]. To exploit this endogenous mechanism for reducing ZEB1 levels, I cloned the miR200c sequence into the pGIPZ vector (Table 2). Two exogenous knockdown shmiR constructs labeled Sh#1 and Sh#2 were also used to make stable ZEB1 knockdown cell lines (Table 2). ZEB1 mRNA expression was measured in each of the stable cell lines to determine the extent of knockdown (Figure 3). Sh#2 was the most efficient construct at knocking down ZEB1 mRNA reducing it by about 90% compared to the Scrambled cell line. miR200c was also effective at knocking down ZEB1 mRNA levels, but only to approximately 50% of control levels. Sh#1 modestly reduced ZEB1 mRNA levels. Thus, two of the thee knockdown cell lines reduced endogenous ZEB1 levels to an extent that additional experiments with them might be useful.

Table 2. Shorthairpin microRNA anti-ZEB1 constructs.

Sh#1

TAATACTGCCGGGTAATGATGGTAGTGAAGCCACAGATGTACCATCATTACCCGGCAGTATTA

Sh#2

GCTGTAGATGGTAACGTAATTAGTGAAGCCACAGATGTAATTACGTTACCATCTACAGC

miR200c

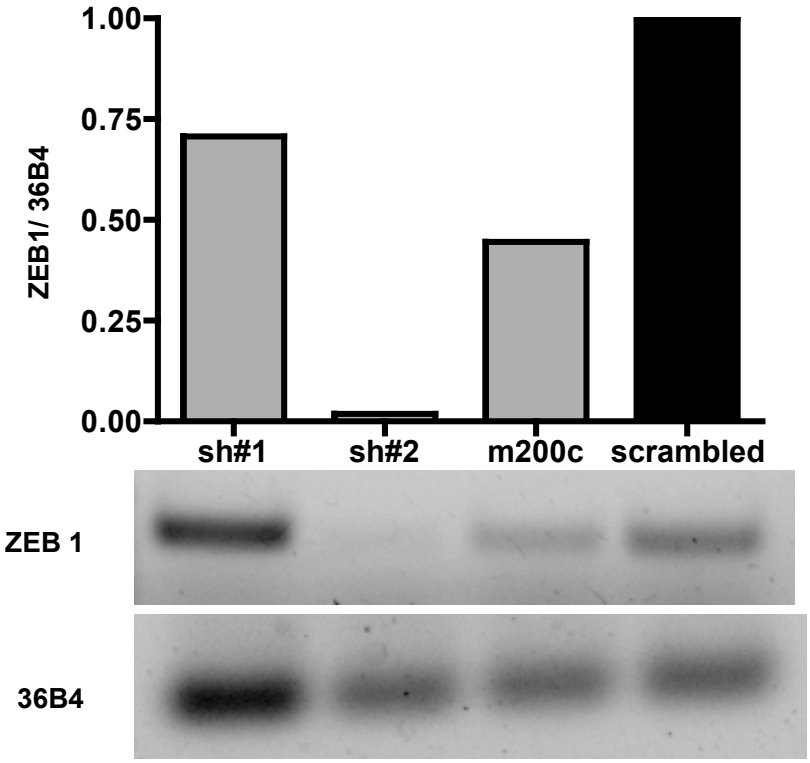
CCATCATTACCCGGCAGTATTATAGTGAAGCCACAGATGTATAATACTGCCGGGTAATGATGG

Scrambled

GAGCGAAACCGCCTCCAATACCGCCAATAGTGAAGCCACAGATGTATTGGCGGTATTGGAGGCG
GTTTCGCTC

Color Codes: mir-30 context sense loop antisense

Figure 3. Sh#2 and miR200c are efficient at reducing ZEB1 expression. Stable cell lines expressing shorthairpin microRNAs against ZEB1 were created and RNA harvested from proliferating cells. ZEB1 expression was measured using qPCR (A), and RT-PCR (B).


1.00
0.75
0.50
0.25
0.00
ZEB1/ 36B4
sh#1
sh#2
m200c
scrambled
ZEB 1
36B4

The loss of ZEB1 expression impairs viability

While culturing these stable lines, a difference in the time it took for the cells to become confluent became apparent. The Sh#1 and Scrambled stable cell lines required passaging (subdividing of the cells into new dishes) much more frequently than did the Sh#2 and miR200c stable lines (data not shown). These differences could be due to increased cell death in the Sh#2 and miR200c lines and/or to increased cell proliferation by the Sh#1 and Scrambled cell lines. To determine whether there were differences in cell proliferation, we performed an MTS proliferation assay. The premise of this assay is that metabolically active cells convert MTS into formazan resulting in a colorimetric change that can be quantified. We observed reduced proliferation in all of the knockdown constructs compared to Scrambled with the Sh#2 and Sh#1 constructs showing the least proliferation (Figure 4). These data imply that cell proliferation may not be related to ZEB1 mRNA levels as we would have expected Sh#1 to have higher MTS activity as it has higher ZEB1 expression. However, this assay is not corrected for cell number and if cell death were occurring the cell population would appear to have reduced proliferation.

To address the question of whether reduced MTS activity is a consequence of decreased cell proliferation or increased cell death, we measured caspase 3 activity as a marker of apoptosis. Caspase 3 is the predominant effector caspase during apoptosis and is activated in response to activation of the caspase cascade by both the extrinsic and the intrinsic pathways [220]. The caspase 3 activity assay shows a significant increase in cell death for

the Sh#2 cell line, whereas no difference was observed in miR200c (Figure 5). Thus, the cell line with the least amount of ZEB1 exhibited the most cell death during a time when the cells were actively proliferating. The reason for the reduced cell death in Sh#1 is unknown, but it may be related to its reduced proliferative rate (Figure 4). This particular cell line may be aberrant in its overall behavior due to something that is independent of ZEB1 levels. Alternatively this caspase 3 experiment was performed only once, albeit in triplicate, so the possibility exists that the results may not be truly representative of the relative amounts of cell death.

Figure 4. Reduced ZEB1 expression diminishes proliferation in 3T3-L1 cells. Equal numbers of cells from each of the stable lines were plated in triplicate and allowed to adhere for 24 hours. Proliferation was measured for the cell lines indirectly through MTS conversion to formazan, which is an indication of metabolically active cells. The mean + the SEM is graphed. * $p < 0.05$

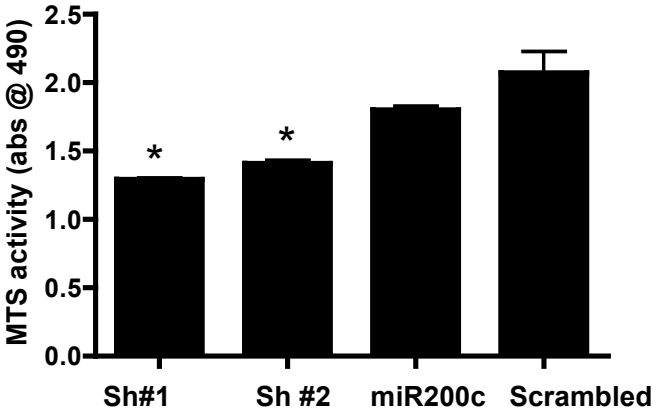
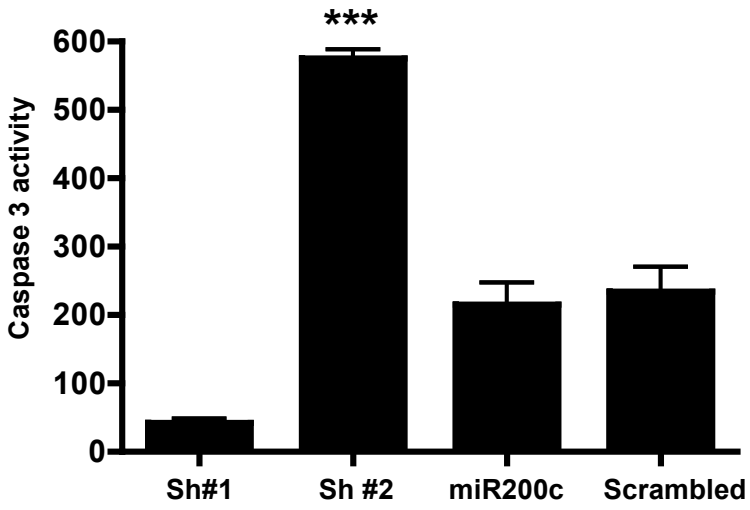

MTS activity (abs @ 490)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Sh#1
Sh #2
miR200c
Scrambled

Figure 5. The Sh#2 cell line has increased apoptosis. Total protein was isolated from the stable cell lines and caspase-3 activity was measured in triplicate. Fluorescence was normalized to total protein concentration. The mean + the SEM is graphed. ***p<0.0005


***
600
500
400
300
200
100
0
Caspase 3 activity
Sh#1
Sh #2
miR200c
Scrambled

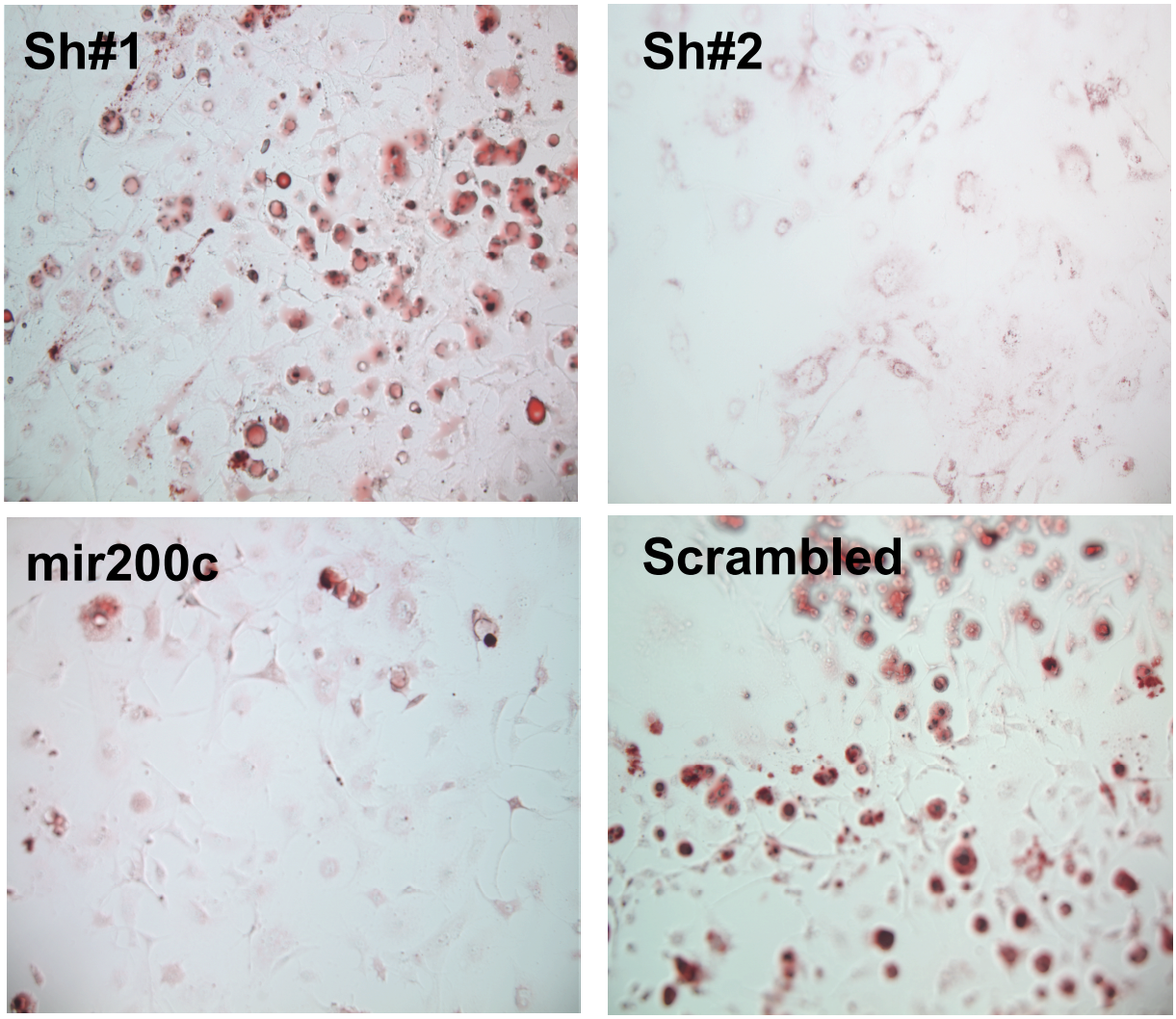
Reduced ZEB1 expression diminishes lipid accumulation

These stable 3T3-L1 cell lines were triggered to differentiate into adipocytes to see whether reduced ZEB1 expression affected adipogenesis (Figure 6). Because of the data from the *TCF8+/-* mice (Chapters II and III), we expected to see either a more rapid onset of lipid accumulation, or more lipid deposition in the cells. Surprisingly, the Sh#2 and miR200c cell lines had reduced differentiation compared to that of the the cell line expressing the scrambled construct as determined by oil red O staining. These results suggest that ZEB1 promotes differentiation of adipocytes, which is in contrast to what we saw with the *TCF8+/-* mice.

In order to get continued measurement of adipogenic differentiation we employed the xCELLigence System. This assay monitors cell growth by continuously measuring electrical impedance. Cells are grown in gold plated plates, which are attached to an instrument that passes electrical current through the plate. Cells adhered to the plate impede the electrical current, which provides a continuous relative measurement of the number of cells adhered. This method is particularly well suited to study adipogenesis as cellular morphology changes significantly throughout differentiation. Pre-adipocytes are fibroblastic with several extensions from the cell body. Two days post treatment to differentiate the cells round up and have a more spherical appearance (Chapter I, Figure 3). As lipid accumulates within the cells they increase in size and take up more surface area on the plate.

Figure 6. Reduced ZEB1 expression diminishes lipid accumulation. The pre-adipocyte 3T3-L1 stable cell lines were induced to differentiate into mature adipocytes. Cells were fixed and stained with oil red O on Day 12 post treatment. Pictures were taken at 20x magnification.

Day 12



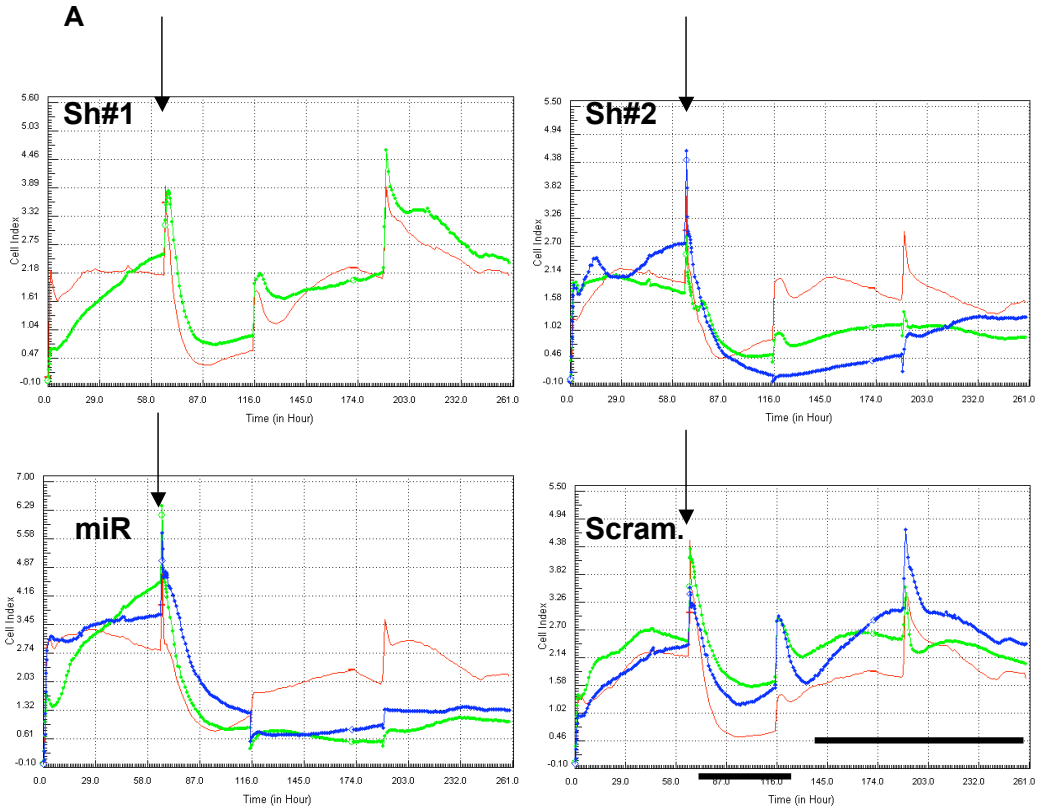
We took advantage of the changes in cellular morphology and the sensitivity of the xCELLigence system to measure the differentiation of the stable cell lines. Three clonal stable cell lines generated from each of the knockdown constructs were used in this experiment (Figure 7A). Cells were plated at two different plating densities of 5,000 or 10,000 cells per well (Figure 7B). This highest density was designed so that all the cell lines would rapidly become confluent. By reducing population doublings we hoped to eliminate the proliferation differences and also to treat the cells at the same time, in theory reducing variability. Cell impedance was measured throughout differentiation for all of the cell lines (Figure 7).

Clonal cell lines expressing the same knockdown construct showed a similar trend. A spike occurred immediately following treatment to differentiate with a subsequent dip as the cells rounded up. Because all of the cell lines showed reduced electrical impedance 2 days following treatment it appears that all the cell lines can respond to the differentiation cocktail. Control wells with no differentiation cocktail did not change morphology (data not shown). All the cell lines appeared to round up, but Sh#1 most resembled that of Scrambled. A morphological change was apparent in Sh#2 following treatment as the cells had a marked reduction in electrical impedance. The lipid accumulation, which is measured by an increase in cell surface area and thus impedance, is reduced in the Sh#2 and the miR200c cell lines compared to Scrambled (Figure 7). A duplicate plate stained with Oil Red O staining showed reduced lipid accumulation in Sh#2 and miR200c cell lines (data not shown). The majority of

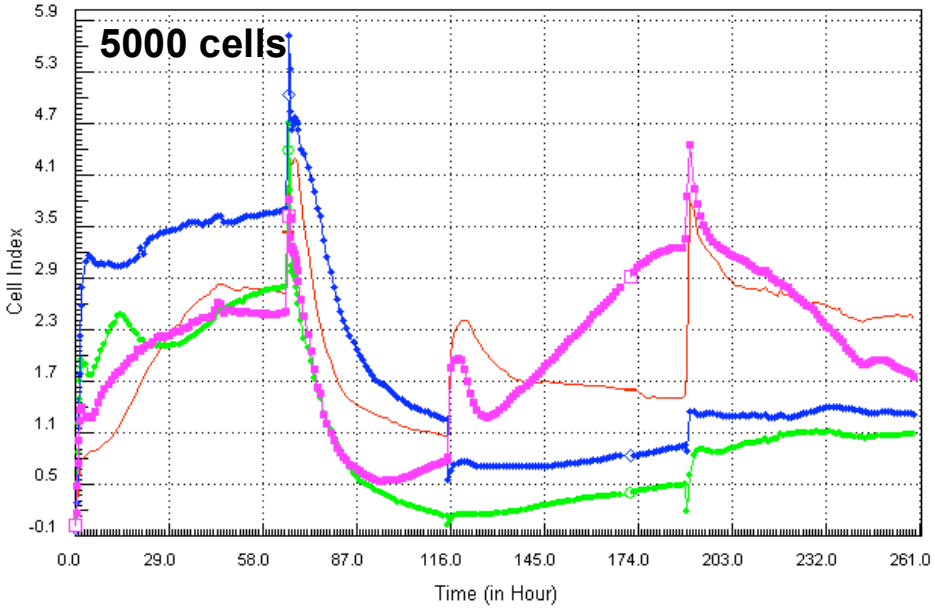
Sh#1 clones do not differ from that of Scrambled. These results indicate that significantly reduced expression of ZEB1 (the Sh#2 and miR200c lines) leads to altered morphological changes that are consistent with the cells not differentiating properly. Interestingly, ZEB1 expression levels during differentiation (Figure 1) parallel the morphological changes (Figure 7A, Scrambled). Whether ZEB1 expression contributes to these morphological changes or whether the changes affect ZEB1's expression remain to be determined.

When comparing the differentiation curves of the stable cell lines on the same graph (Figure 7B), none of the cell lines, with the exception of the Sh#1 line show appropriate morphological changes associated with lipid accumulation at the higher starting cell number. This suggests that the cell density may have been too high to permit an appropriate response to the differentiation cocktail. However, seeding the cells at a density of 5,000/ well gave the expected morphological responses. Interestingly, both the Sh#2 and miR200c lines show little lipid accumulation compared to the other lines with starting cell number of 5000 cells (Figure 6). This supports the contention that ZEB1 expression is necessary for adipogenesis in the 3T3-L1 cell line.

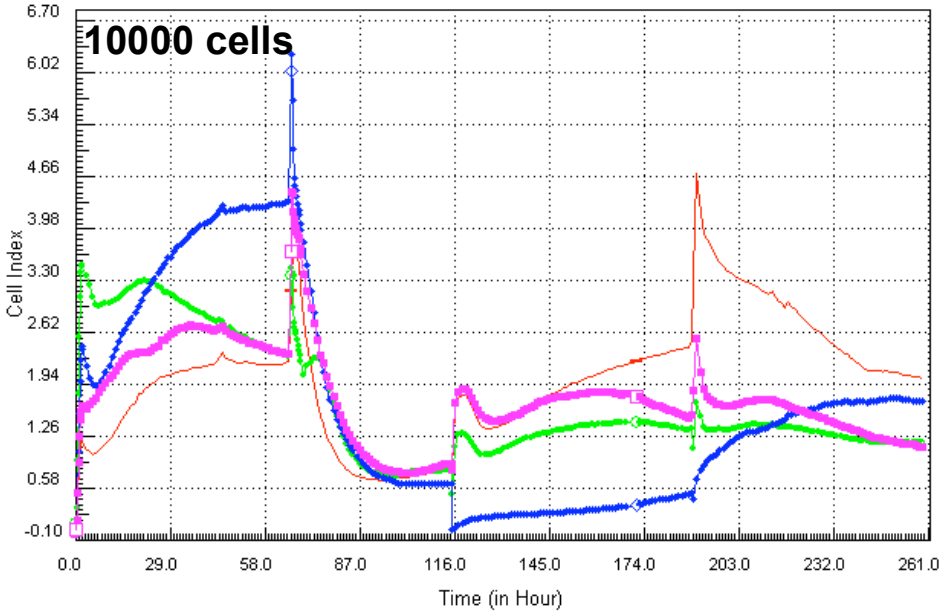
Figure 7. Reduced ZEB1 levels impede differentiation as well as lipid accumulation. The pre-adipocyte 3T3-L1 stable cells lines were induced to differentiate into mature adipocytes and electrical impedance measured every 15 min for the first 4 h post differentiation cocktail, and every h for the next 8 days. The electrical impedance is measured in 3 stable cell lines per construct at two plating densities (A). Electrical impedance for the stable cell lines at different plating densities 5,000 and 10,0000 (B).


A
Sh#1
Sh#2
miR
Scram.
Cell Index
Time (in Hour)
rounding up
Lipid accumulation
= treatment with differentiation cocktail

B



Red: Sh#1
Green: sh #2
Blue: miR
Pink: scrambled



DISCUSSION

The ZEB1 transcription factor opposes the differentiation of several mesenchymal tissues [16, 30, 36, 40, 41, 44-48]. However, loss of this transcription factor also impairs the development of several tissues including that of smooth muscle vascular tissue [46], T-cells, and neuronal crest closure [36]. We show here that although ZEB1 attenuates adipose accumulation in female mice (Chapters 2 and 3), in an *in vitro* system the elimination of ZEB1 results in impaired differentiation of adipocytes (Figure 6 and 7). While it is plausible that the reduced proliferation and viability observed in Sh#2 and miR200c cell lines may have inhibited the mitotic clonal expansion and thus resulted in poor differentiation, data from the xCELLigence system suggests that the cells are rounding up in response to the differentiation cocktail, which is an indication of the initiation of adipogenic differentiation. The caveat of the system is that it only measures electrical impedance and this reduction in impedance could be a result of cell death and not just the rounding up of the cells, especially for the Sh#2 cell lines. It would have been informative to assess this directly by Trypan Blue exclusion or by measurement of caspase-3 activity during all stages of adipogenesis.

A slight reduction in the ability of cells to round up in the cell lines where expression of ZEB1 was significantly reduced (Sh#2. miR200c) as well as a decrease in endogenous ZEB1 expression following differentiation cocktail suggests that ZEB1 is necessary for adipogenesis. However, we found no obvious affect on adipogenesis *in vivo* with the ZEB1 haploinsufficient mice. This

may be because sufficient ZEB1 was present in the haploinsufficient mouse to permit adipogenesis. A moderate level of ZEB1 may be necessary for proliferation of pre-adipocytes to attain contact inhibition and to initiate mitotic clonal expansion. It is also possible that these *in vitro* results may not apply to adipogenesis *in vivo*. While cell cycle arrest and mitotic clonal expansion is necessary *in vitro,* as reviewed in [64, 68, 69, 217, 218], the *in vivo* cues that regulate differentiation are not well understood. Overexpression of ZEB1 in these pre-adipocytes could certainly improve our understanding of the function of ZEB1 in adipose biology. Based on what is known regarding ZEB1 in female mice (Chapters III and IV), one would expect that the overexpression of ZEB1 may also block adipocyte differentiation or at least lipid accumulation.

The ZEB1 knockdown cell lines indicate that ZEB1 is necessary for adipocyte differentiation and lipid accumulation *in vitro* (Figures 4 and 6), however, ZEB1 insufficiency increased lipid accumulation *in vivo* (Chapter III). This suggests a bimodal regulatory role for ZEB1. I hypothesize that it both promotes the differentiation of new adipocytes and blocks lipid accumulation. While these are thought to be mutually exclusive events comparable bimodal regulation is observed by both estrogen [118, 121] and IGF-1 [212, 213]. Estrogen is pro-proliferative and increases the number of pre-adipocytes [118, 121]. It also reduces lipid accumulation in adipocytes through a variety of mechanisms [84, 86, 118, 119, 125]. Because ZEB1 is regulated by estrogen in adipocytes (Chapter III, Figure 1), it may be mediating some of these effects *in vivo*. It is possible ZEB1 acts as a lipid sensor in adipocytes and functions to

reduce excess lipid accumulation and increase new adipocytes, thereby preventing the accumulation of lipid in peripheral tissues. Additional experiments will be required to test whether this hypothesis is true. Nonetheless, it appears as though ZEB1 plays a heretofore unrecognized yet important role in modulating adiposity in adults, especially females. This novel modulator contributes to our understanding on the sexual dimorphism in metabolism improving how we characterize and treat obesity and its metabolic disorders in men versus women.

SUMMARY & FUTURE DIRECTIONS

This thesis identifies the ZEB1 transcription factor as a novel modulator of white adipose tissue. This was demonstrated by using three different approaches: human genetics, a mouse model, and cell culture models. Using a genetic approach, I showed that the gene encoding ZEB1 is linked to childhood obesity. Through genotyping of the *TCF8* locus in a cohort of obese children and their families, I found linkage between *TCF8* and childhood obesity (Chapter II, Figure 2). Due to the close proximity of *TCF8* to the centromere, recombination events are infrequent. As a result, genes in these areas share the same haplotype. Hence, linkage analysis tells us only that childhood obesity is linked to a gene transmitted in the same haplotype as *TCF8.*

To more directly test whether a mutation in ZEB1 contributed to obesity in this cohort, I sequenced the coding region in *TCF8* from a subset of obese patients and found a polymorphism in over 50% of those sequenced. The polymorphism results in a glutamic acid to lysine amino acid change in the central homeodomain of the ZEB1 protein (Chapter II, Figure 3). While this substitution would result in similar charge and mass as the predominant allele, this specific substitution was not identified as a natural variant in the population (as reported at National Center for Biotechnology Information), suggesting it is specific to this obese population. We cannot predict the consequence of this change because while this is a conserved domain, no information is available regarding the function of this homeodomain. In structurally similar proteins, the homeodomain functions to facilitate protein-protein interactions [24], but there

have been no reports for function in ZEB1. Assuming it is important for protein interactions, several experiments modeling the polymorphism should be done to confirm whether it has any effect on the function of ZEB1. The primary function of ZEB1 is to bind to target genes and regulate their transcriptional activity. First, *in vitro* modeling of the point mutation could be made in a ZEB1 expression vector and the capability of ZEB1 to repress or activate known target genes such compared to WT ZEB1 can be assessed. It will likely be necessary to test more than one target gene as we do not know the tissue and context where this altered protein may have a consequence. Because of this, functional analysis may be difficult. Another approach would be to use simulated structural modeling of the altered protein versus the WT protein. If computer modeling suggested the polymorphism may disrupt a structural component, solution NMR could be used to solve the structure as was done for the structure of the WT ZEB1 homeodomain [221]. This would enable us to predict whether the structure of the protein changes as a result of the substitution. Even if no effects on target gene expression are detected, which is possible because this assay is so sensitive, it's possible that the amino acid substitution might affect the stability of the protein, its nuclear localization, its modification, or some other important property.

Without a charge or weight difference between the amino acids it is possible that the polymorphism will have a functional effect. If no functional change in ZEB1 is detected with the amino acid substitution, then it may be the actual quantity of the protein that differs in obese individuals. Our studies in mice indicate that reduced amounts of ZEB1 can lead to adiposity (Chapters III, and

IV). Thus, direct sequencing of the regulatory regions of the *TCF8* gene is just as important as sequencing the coding region. A polymorphism in the 5' proximal promoter region, the first intron, or the 3' UTR could alter the ZEB1 expression, contributing to the propensity to gain weight.

In addition to the genetic approach, a mouse model was also employed to assess whether haploinsufficiency of the *TCF8* gene is sufficient to observe changes in adipose tissue mass. We found that *TCF8+/-* female mice accumulate more fat than WT mice fed either a high fat diet or regular chow diet (Chapter III, Figures 2 and 3). Reducing ZEB1 expression by only 50% was sufficient to increase adiposity with normal calorie consumption suggesting that ZEB1 has a role in normal adipocyte physiology, and not just under extreme conditions of caloric excess. The increased adipose accumulation was sufficient to elicit changes in metabolic homeostasis such as decreased glucose uptake and increased cytokine production (Chapter III, Figures 6 and 7; Chapter IV, Figure 7). Because the increased adiposity was not due to an increase in caloric consumption or a decrease in physical activity (Chapter III Figure 5), we focused our subsequent studies on investigating the potential impact of ZEB1 reduction on intermediary metabolism.

Because the effects were observed with female mice, I wanted to determine whether 1) ZEB1 deficiency had any effects in male mice and 2) ZEB1 is mediating the protective effects of estrogen on weight gain. To assess whether ZEB1 is estrogen-responsive in adipose tissue of males, I injected males with estrogen. ZEB1 mRNA was upregulated in both gonadal and subcutaneous

adipose tissue by 24 hours following injection of estrogen, indicating that not only that males express ZEB1 in adipose tissue but that it is responsive to estrogen. I proceeded to measure body weight, body composition, and metabolic parameters in male and Faslodex-treated female mice. No differences were observed between *TCF8+/-* and WT for body weight, body composition, gonadal fat weight, or glucose tolerance implying that ZEB1 is mediating some of the protective effects of estrogen against weight gain (Chapters III and IV). In addition fasting blood glucose was much higher in both genotypes of Faslodex-treated female mice compared to female mice (Chapter III, Figure 6; Chapter IV, Figure 6). Faslodex-treated mice had 1.5 times more basal blood glucose as compared to female mice of either genotype at 3 months of age. This nearly doubled by five months, with no discernable difference in body weight (Chapter III, Figure 2; Chapter IV, Figure 2). This suggests that ZEB1 may also mediate some of estrogen's protective effects on glucose homeostasis. Additional measurements such as the expression of glucose transporters in muscle and fat, fatty acid oxidation in muscle and fat, insulin tolerance, and lipid deposition in peripheral tissues such as muscle and liver would have provided clues to the extent of this phenotype. While we observed no differences in overall body weight or body composition between *TCF8+/-* and WT mice in male or Faslodex-treated female mice, increased adipocyte hypertrophy was observed in all of the *TCF8+/-* mice regardless of gender (Chapter IV, Figure 8). This suggests that ZEB1 may also regulate adiposity independently of estrogen signaling. Because ZEB1 is a transcription factor, expression of several genes involved in

lipogenesis and lipolysis was measured, focusing on those genes known to be estrogen-regulated. ZEB1 does not appear to alter *de novo* lipogenesis as there was no difference between genotypes for any of the genes measured (Chapter IV, Figure 9). It was not surprising that all mice treated with Faslodex showed an increase in expression of these genes involved in *de novo* lipogenesis as estrogen has the opposite effect [84, 86, 119]. While the increased adipocyte size is not attributable to *de novo* lipogenesis, it may be a consequence of altered lipid flux in the adipocyte and this was addressed by looking at LPL and HSL. While *TCF8+/-* mice had increased LPL mRNA expression, measurement of LPL activity suggests that this is not the mechanism by which ZEB1 regulates adipocyte size. If anything, ZEB1 may normally increase LPL activity, as the activity in female *TCF8+/-* mice was reduced from that of WT (Chapter IV, Figure 10). Because the mRNA levels are in contrast with the enzyme activity, this suggests that the effects of ZEB1 on LPL are indirect. While this does not explain the increased adipocyte size, decreased LPL activity in *TCF8+/-* female mice is in agreement with what we observed *in vitro,* with a reduction in ZEB1 levels attenuating lipid accumulation (Chapter V, Figures 6 and 7). It is difficult to delineate what is the cause and what is the result of increased adiposity. Nonetheless, these changes are suggestive of altered lipogenesis and /or lipolysis in the *TCF8+/-* mice. It may be that the flux through both are slightly altered but the net effect is increased lipid deposition.

One possible explanation for the increased adipocyte hypertrophy is the increase in perilipin (Chapter IV, Figure 11). Perilipin coats the lipid droplet preventing its breakdown via HSL-mediated lipolysis [208-210]. This could be a consequence of increased adiposity as perilipin does increase with obesity [222]. While the male and Faslodex-treated mice do not have increased weight or fat mass, their adipocytes are larger with presumably more lipid. The number of cells in a fat pad would have provided useful information, as the discrepancy between hypertrophy and fat mass could be attributed to a decrease in the number of adipocytes in both the male and the Faslodex-treated female mice.

Further studies are required to determine a mechanism for the increased adiposity in ZEB1 haploinsufficient mice. To extend these studies a better model system should be employed. One significant problem with the *TCF8+/-* mouse model is that there are residual ZEB1 levels, which are not always significantly different from WT mice when the mice gain weight. Female *TCF8+/-* mice did not maintain increased body weight and fat mass throughout their life but within a small window (Chapter III, Figure 2). We show that ZEB1 increases in WT mice as they gain weight (Chapter III, Figure 1). This is problematic because by 4 months of age the *TCF8+/-* female mice do not have significantly different ZEB1 expression from WT mice (Chapter III, Figure 4). While this may enhance our argument that ZEB1 opposes obesity, the rise in endogenous ZEB1 levels limits our studies. In addition, if ZEB1 is mediating some of the anti-obesity effects of estrogen one might also expect increased adiposity in *TCF8+/-* male mice as they are able to produce estradiol and have ER expression comparable to

females in adipose tissue. However, the C57BL/6 male mice become fat so rapidly that at two months of age there is no significant difference in ZEB1 expression between male TCF8 +/- and WT mice (Chapter III, Figure 4). To really examine the necessity of ZEB1 in adipose tissue and to further investigate a mechanism by which ZEB1 reduces adipose accumulation, an adipose tissue-specific knockout is needed. By eliminating ZEB1, the extent of the adipose phenotype could be determined. This would also provide a better model to examine the sexual dimorphic effects of ZEB1.

Based on the ability of ZEB1 to oppose differentiation of other mesenchymal tissues, we hypothesized that ZEB1 inhibited adipogenesis. To answer this question, endogenous ZEB1 levels were measured throughout adipocyte differentiation *in vitro*. In both a pre-adipocyte model (3T3-L1) and a pluripotent mesenchymal stem cell model (C3H10T1/2), we saw ZEB1 expression increase when cells were confluent, drop following treatment to differentiate, and increase again when lipid accumulation was visible. The dip in ZEB1 expression may be necessary for the cells to differentiate, or it could merely be that ZEB1 increases following contact inhibition. The increase in ZEB1 expression that is observed with lipid accumulation (Chapter V) is in agreement with our mouse studies, where increased body weight and adipose mass result in increased ZEB1 expression (Chapter III, Figure 1; Chapter IV, Figure 4). Consequently this increase in ZEB1 with adiposity is probably a result of lipid accumulation and is not causal. To determine whether ZEB1 was necessary for adipogenesis three different short hairpin microRNA constructs were used to

generate stable ZEB1 knockdown cell lines, two of which (Sh#2, miR200c) efficiently reduced ZEB1 mRNA expression (Chapter 5, Figure 3). Upon differentiation of these cell lines there was a slight reduction in the ability of the cells to round up (Chapter 5, Figure 7) suggesting they were not differentiating as well. But more surprisingly, there was a marked reduction in lipid accumulation by Oil Red O staining and in morphological changes as measured by electrical impedance (Chaper 5, Figures 6 and 7). This may be in part due to a proliferation defect as the Sh#2 cell lines do show reduced proliferation and increased cell death resulting in fewer adipocytes and consequently reduced lipid accumulation (Chapter V, Figures 4 and 5). However, miR200c, which also has reduced ZEB1 levels although admittedly not as much as Sh#2, has no obvious proliferation defect as measured by MTS or increase in capase-3 activity, yet it still has reduced lipid accumulation. The decrease in ZEB1 expression reduced the cells ability to round up and accumulate lipid, suggesting that ZEB1 normally functions to promote adipogenesis and possibly lipid accumulation. ZEB1 may accomplish this through the reduction of resistin expression. Resistin has been shown to prevent the differentiation of 3T3-L1 cells [223], and our data show a dramatic increase in resistin mRNA and circulation when ZEB1 is reduced (Chapter IV, Figure 7). Diminished lipid accumulation may be a result of impaired differentiation. However, the mature adipocytes in Sh#2 and miR200c cell lines have smaller lipid droplets implying they have reduced lipid accumulation in addition to reduced differentiation (Chapter V, Figures 6 and 7). This is in contrast to what we observed in *TCF8+/-* mice (Chapter IV, Figure 8).

An explanation for these discrepancies is that ZEB1 has a bimodal role in adipocyte differentiation and lipid accumulation. ZEB1 may be necessary for adipocyte differentiation by enhancing proliferation of pre-adipocytes to achieve contact inhibition and initiate mitotic clonal expansion. Following adipocyte formation, ZEB1 may operate to maintain lipid accumulation. This bimodal role could be a consequence of negative feedback. I propose that ZEB1 acts as a lipid sensor through as yet unknown mechanisms and that its expression increases in response to lipid accumulation whereby it reduces lipid uptake and/or increases lipid expenditure (Figure 1). This may be accomplished through the downregulation of resistin (Chapter IV, Figure 7). A decrease in resistin would allow for pre-adipocyte differentiation *in vitro* [223], and increase AMPK signaling [224], a master regulator of fatty acid oxidation.

The concept of a bifunctional role for ZEB1 is not unique as both estrogen and IGF-1 are similar with regard to having dual roles in adipocyte biology. Estrogen is pro-proliferative [121], yet it opposes lipid accumulation in cells by increasing HSL-mediated lipolysis and decreasing LPL-mediated lipogenesis and *de novo* lipogenesis [84, 86, 118, 119, 123, 125]. IGF-1 promotes adipogenesis yet eliminating its receptor in the mouse results in increased adipose accumulation in the perigonadal fat pads [225]. Thus, IGF-1 also appears to have to opposing functions in fat tissues. Based on my data with the mice and the somewhat conflicting data with the stable cell lines, I hypothesize that ZEB1 also has multiple functions in adipogenesis and fat accumulation.

To more extensively examine the role of estrogen and ZEB1 in adipogenesis and lipid accumulation, stable cell lines expressing ER could provide a biologically relevant system. It is difficult to determine what is the cause and what is the effect of a long-term consequence such as adipose accumulation *in vivo*. There are several positive and negative feedback regulatory networks striving to maintain homeostasis. By measuring fat accumulation and other parameters at isolated time points in the mice, it was only possible to catch a glimpse at what is occurring at that specific time and all the dynamic changes that occurred to elicit these responses can only be inferred.

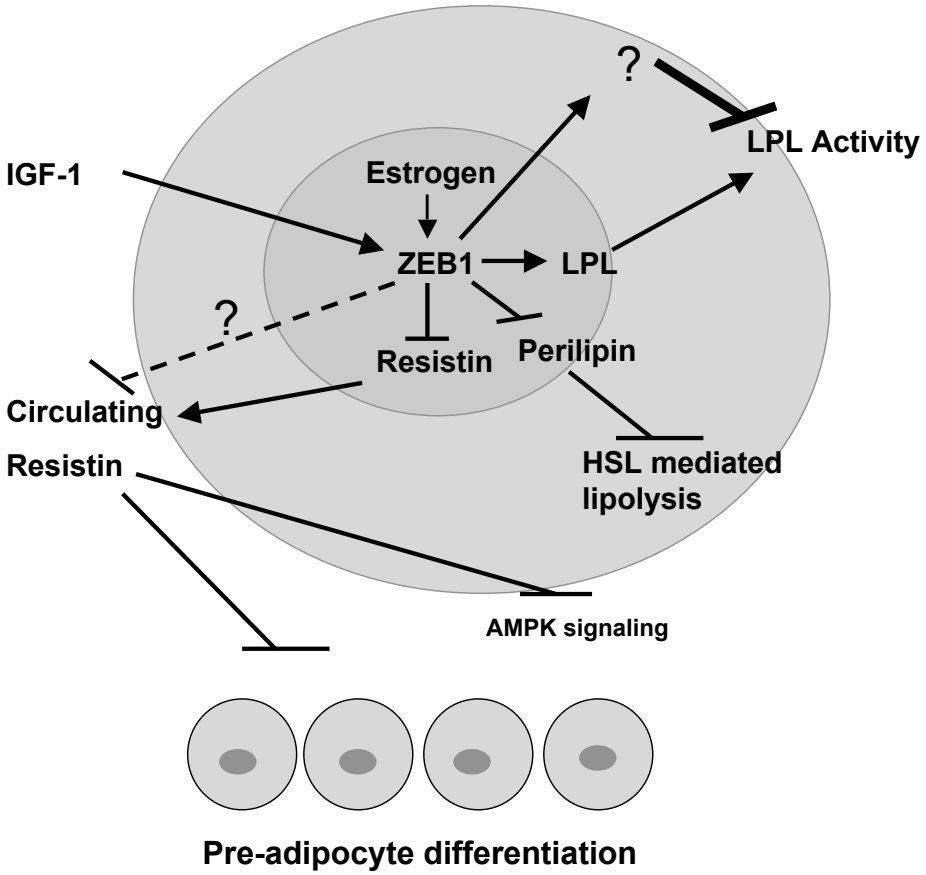
To discern what mechanistic effects ZEB1 has on lipid accumulation, stable cell lines that overexpress or knock down ZEB1 under the control of the aP2 promoter could be made. In this system endogenous ZEB1 would permit the differentiation into adipocytes, and the induction of the aP2 promoter in mature adipocytes would either reduce or increase ZEB1 expression. Subsequent measurements of lipid accumulation and of the expression of LPL, HSL, perilipin, and fatty acid transporters may provide some answers. In addition, using these cells in genome-profiling studies such as a microarray throughout differentiation could identify ZEB1 target genes. A comparable study is currently underway in the laboratory comparing MEFs from *TCF8* null and WT mice. Comparing the measurement in cells with or without ER could provide insight into what effects of estrogen are mediated by ZEB1. One caveat is the health of the cell lines that are stably expressing multiple genes under several drug selections may not permit these experiments. Overexpression of ZEB1 in

the C3H10T1/2 cell lines should further support a role for ZEB1 in differentiation of adipocytes. Based on our results with reduced ZEB1 levels, we would expect an increase in ZEB1 expression to augment adipogenesis. For this, 3T3-L1 cells that are already efficient at differentiation (90-95%) may not be the best choice, and a cell line that is not as effective at adipocyte differentiation such as the C3H10T1/2 cells may be a more suitable model.

In summary, this thesis has identified ZEB1 as a novel modulator of adipose tissue in female mice. Reduction of ZEB1 in mice resulted in increased adiposity due to adipocyte hypertrophy, reduced glucose tolerance, and increased adipokine production. Cell culture data suggest that not only does ZEB1 influence lipid accumulation within cells but that a certain level of ZEB1 is necessary for adipocyte differentiation. Finally, we have shown that this research is relevant to humans as there is linkage between the *TCF8* gene and childhood obesity. Sequencing of the *TCF8* coding region in a subset of obese patients identified two polymorphisms that may contribute to their obese phenotype.

Not only has this thesis identified a novel transcription factor in adipocyte development and lipid accumulation, but it has also enhanced our understanding of sexual dimorphism in metabolism.

Figure 1. A model of how ZEB1 may promote differentiation of pre-adipocytes and inhibit lipid accumulation. ZEB1 is a novel modulator of adipose tissue that may be mediating the effects of estrogen and/or IGF1. An upregulation of ZEB1 by either IGF-1 or estrogen leads to the downregulation of the adipokine resistin. Reduced resistin levels relieves inhibition on pre-adipocyte differentiation and AMPK signaling, a central regulator of lipid metabolism. Thus the transcription factor ZEB1 acts as a lipid sensor and in a hypertrophic cell ZEB1 expression increases in response to lipid accumulation reduces lipid uptake, promotes lipolysis, and the differentiation of new adipocytes.


?
LPL Activity
IGF-1
Estrogen
ZEB1
LPL
?
Resistin
Perilipin
Circulating
Resistin
HSL mediated
lipolysis
AMPK signaling
Pre-adipocyte differentiation

REFERENCES

1. Williams, T.M., et al., *The TCF8 gene encoding a zinc finger protein (Nil-2-a) resides on human chromosome 10p11.2.* Genomics, 1992. **14**(1): p. 194-6.
2. Sekido, R., et al., *Organization of the gene encoding transcriptional repressor deltaEF1 and cross-species conservation of its domains.* Gene, 1996. **173**(2): p. 227-32.
3. Manavella, P.A., et al., *The ZFHX1A gene is differentially autoregulated by its isoforms.* Biochem Biophys Res Commun, 2007. **360**(3): p. 621-6.
4. Chamberlain, E.M. and M.M. Sanders, *Identification of the novel player deltaEF1 in estrogen transcriptional cascades.* Mol Cell Biol, 1999. **19**(5): p. 3600-6.
5. Hurt, E.M., et al., *Expression of the ZEB1 (deltaEF1) transcription factor in human: additional insights.* Mol Cell Biochem, 2008. **318**(1-2): p. 89-99.
6. Spoelstra, N.S., et al., *The transcription factor ZEB1 is aberrantly expressed in aggressive uterine cancers.* Cancer Res, 2006. **66**(7): p. 3893-902.
7. Wang, J., et al., *Opposing LSD1 complexes function in developmental gene activation and repression programmes.* Nature, 2007. **446**(7138): p. 882-7.
8. Carroll, J.S., et al., *Genome-wide analysis of estrogen receptor binding sites.* Nat Genet, 2006. **38**(11): p. 1289-97.
9. Klinge, C.M., *Estrogen receptor interaction with estrogen response elements.* Nucleic Acids Res, 2001. **29**(14): p. 2905-19.
10. Nilsson, S., et al., *Mechanisms of estrogen action.* Physiol Rev, 2001. **81**(4): p. 1535-65.
11. Petz, L.N. and A.M. Nardulli, *Sp1 binding sites and an estrogen response element half-site are involved in regulation of the human progesterone receptor A promoter.* Mol Endocrinol, 2000. **14**(7): p. 972-85.
12. Graham, T.R., et al., *Insulin-like growth factor-I-dependent up-regulation of ZEB1 drives epithelial-to-mesenchymal transition in human prostate cancer cells.* Cancer Res, 2008. **68**(7): p. 2479-88.
13. Shirakihara, T., M. Saitoh, and K. Miyazono, *Differential regulation of epithelial and mesenchymal markers by deltaEF1 proteins in epithelial mesenchymal transition induced by TGF-beta.* Mol Biol Cell, 2007. **18**(9): p. 3533-44.
14. Shi, Y. and J. Massague, *Mechanisms of TGF-beta signaling from cell membrane to the nucleus.* Cell, 2003. **113**(6): p. 685-700.
15. Otto, T.C., R.R. Bowers, and M.D. Lane, *BMP-4 treatment of C3H10T1/2 stem cells blocks expression of MMP-3 and MMP-13.* Biochem Biophys Res Commun, 2007. **353**(4): p. 1097-104.
16. Ponticos, M., et al., *Regulation of collagen type I in vascular smooth muscle cells by competition between Nkx2.5 and deltaEF1/ZEB1.* Mol Cell Biol, 2004. **24**(14): p. 6151-61.
17. Yang, S., et al., *deltaEF1 represses BMP-2-induced differentiation of C2C12 myoblasts into the osteoblast lineage.* J Biomed Sci, 2007. **14**(5): p. 663-79.

18. Hurteau, G.J., S.D. Spivack, and G.J. Brock, *Potential mRNA degradation targets of hsa-miR-200c, identified using informatics and qRT-PCR.* Cell Cycle, 2006. **5**(17): p. 1951-6.
19. Hurteau, G.J., et al., *Overexpression of the microRNA hsa-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin.* Cancer Res, 2007. **67**(17): p. 7972-6.
20. Gregory, P.A., et al., *The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1.* Nat Cell Biol, 2008. **10**(5): p. 593-601.
21. Funahashi, J., et al., *Delta-crystallin enhancer binding protein delta EF1 is a zinc finger-homeodomain protein implicated in postgastrulation embryogenesis.* Development, 1993. **119**(2): p. 433-46.
22. Genetta, T., D. Ruezinsky, and T. Kadesch, *Displacement of an E-box-binding repressor by basic helix-loop-helix proteins: implications for B-cell specificity of the immunoglobulin heavy-chain enhancer.* Mol Cell Biol, 1994. **14**(9): p. 6153-63.
23. Williams, T.M., et al., *Identification of a zinc finger protein that inhibits IL-2 gene expression.* Science, 1991. **254**(5039): p. 1791-4.
24. Vandewalle, C., F. Van Roy, and G. Berx, *The role of the ZEB family of transcription factors in development and disease.* Cell Mol Life Sci, 2009. **66**(5): p. 773-87.
25. Costantino, M.E., et al., *Cell-specific phosphorylation of Zfhep transcription factor.* Biochem Biophys Res Commun, 2002. **296**(2): p. 368-73.
26. Long, J., D. Zuo, and M. Park, *Pc2-mediated sumoylation of Smad-interacting protein 1 attenuates transcriptional repression of E-cadherin.* J Biol Chem, 2005. **280**(42): p. 35477-89.
27. Wacker, I., et al., *zag-1, a Zn-finger homeodomain transcription factor controlling neuronal differentiation and axon outgrowth in C. elegans.* Development, 2003. **130**(16): p. 3795-805.
28. Fortini, M.E., Z.C. Lai, and G.M. Rubin, *The Drosophila zfh-1 and zfh-2 genes encode novel proteins containing both zinc-finger and homeodomain motifs.* Mech Dev, 1991. **34**(2-3): p. 113-22.
29. Remacle, J.E., et al., *New mode of DNA binding of multi-zinc finger transcription factors: deltaEF1 family members bind with two hands to two target sites.* EMBO J, 1999. **18**(18): p. 5073-84.
30. Fontemaggi, G., et al., *The transcriptional repressor ZEB regulates p73 expression at the crossroad between proliferation and differentiation.* Mol Cell Biol, 2001. **21**(24): p. 8461-70.
31. Shi, Y., et al., *Coordinated histone modifications mediated by a CtBP co-repressor complex.* Nature, 2003. **422**(6933): p. 735-8.
32. Hlubek, F., et al., *Tip60 is a cell-type-specific transcriptional regulator.* J Biochem, 2001. **129**(4): p. 635-41.
33. Dillner, N.B. and M.M. Sanders, *The zinc finger/homeodomain protein deltaEF1 mediates estrogen-specific induction of the ovalbumin gene.* Mol Cell Endocrinol, 2002. **192**(1-2): p. 85-91.

34. Lazarova, D.L., M. Bordonaro, and A.C. Sartorelli, *Transcriptional regulation of the vitamin D(3) receptor gene by ZEB.* Cell Growth Differ, 2001. **12**(6): p. 319-26.
35. Postigo, A.A., et al., *Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins.* EMBO J, 2003. **22**(10): p. 2453-62.
36. Takagi, T., et al., *DeltaEF1, a zinc finger and homeodomain transcription factor, is required for skeleton patterning in multiple lineages.* Development, 1998. **125**(1): p. 21-31.
37. Su, A.I., et al., *A gene atlas of the mouse and human protein-encoding transcriptomes.* Proc Natl Acad Sci U S A, 2004. **101**(16): p. 6062-7.
38. Higashi, Y., et al., *Impairment of T cell development in deltaEF1 mutant mice.* J Exp Med, 1997. **185**(8): p. 1467-79.
39. Brabletz, T., et al., *Negative regulation of CD4 expression in T cells by the transcriptional repressor ZEB.* Int Immunol, 1999. **11**(10): p. 1701-8.
40. Inuzuka, T., et al., *Transcription factor 8 activates R-Ras to regulate angiogenesis.* Biochem Biophys Res Commun, 2009. **379**(2): p. 510-3.
41. Inuzuka, T., et al., *Integral role of transcription factor 8 in the negative regulation of tumor angiogenesis.* Cancer Res, 2009. **69**(4): p. 1678-84.
42. Taylor, S.M. and P.A. Jones, *Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine.* Cell, 1979. **17**(4): p. 771-9.
43. Tylzanowski, P., et al., *Smad-interacting protein 1 is a repressor of liver/bone/kidney alkaline phosphatase transcription in bone morphogenetic protein-induced osteogenic differentiation of C2C12 cells.* J Biol Chem, 2001. **276**(43): p. 40001-7.
44. Sooy, K. and M.B. Demay, *Transcriptional repression of the rat osteocalcin gene by deltaEF1.* Endocrinology, 2002. **143**(9): p. 3370-5.
45. Jethanandani, P. and R.H. Kramer, *Alpha7 integrin expression is negatively regulated by deltaEF1 during skeletal myogenesis.* J Biol Chem, 2005. **280**(43): p. 36037-46.
46. Nishimura, G., et al., *DeltaEF1 mediates TGF-beta signaling in vascular smooth muscle cell differentiation.* Dev Cell, 2006. **11**(1): p. 93-104.
47. Murray, D., et al., *The transcription factor deltaEF1 is inversely expressed with type II collagen mRNA and can repress Col2a1 promoter activity in transfected chondrocytes.* J Biol Chem, 2000. **275**(5): p. 3610-8.
48. Terraz, C., et al., *delta Ef1 binds to a far upstream sequence of the mouse pro-alpha 1(I) collagen gene and represses its expression in osteoblasts.* J Biol Chem, 2001. **276**(40): p. 37011-9.
49. Liu, Y., et al., *Zeb1 links epithelial-mesenchymal transition and cellular senescence.* Development, 2008. **135**(3): p. 579-88.
50. Bui, T., et al., *ZEB1 links p63 and p73 in a novel neuronal survival pathway rapidly induced in response to cortical ischemia.* PLoS One, 2009. **4**(2): p. e4373.
51. Postigo, A.A., *Opposing functions of ZEB proteins in the regulation of the TGFbeta/BMP signaling pathway.* EMBO J, 2003. **22**(10): p. 2443-52.

52. Krafchak, C.M., et al., *Mutations in TCF8 cause posterior polymorphous corneal dystrophy and ectopic expression of COL4A3 by corneal endothelial cells.* Am J Hum Genet, 2005. **77**(5): p. 694-708.
53. Liskova, P., et al., *Novel mutations in the ZEB1 gene identified in Czech and British patients with posterior polymorphous corneal dystrophy.* Hum Mutat, 2007. **28**(6): p. 638.
54. Liu, Y., et al., *Zeb1 mutant mice as a model of posterior corneal dystrophy.* Invest Ophthalmol Vis Sci, 2008. **49**(5): p. 1843-9.
55. Grooteclaes, M.L. and S.M. Frisch, *Evidence for a function of CtBP in epithelial gene regulation and anoikis.* Oncogene, 2000. **19**(33): p. 3823-8.
56. Aigner, K., et al., *The transcription factor ZEB1 (deltaEF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity.* Oncogene, 2007. **26**(49): p. 6979-88.
57. Aigner, K., et al., *The transcription factor ZEB1 (deltaEF1) represses Plakophilin 3 during human cancer progression.* FEBS Lett, 2007. **581**(8): p. 1617-24.
58. Adachi, Y., et al., *Zeb1-mediated T-cadherin repression increases the invasive potential of gallbladder cancer.* FEBS Lett, 2009. **583**(2): p. 430-6.
59. Singh, M., et al., *ZEB1 expression in type I vs type II endometrial cancers: a marker of aggressive disease.* Mod Pathol, 2008. **21**(7): p. 912-23.
60. Spaderna, S., et al., *The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer.* Cancer Res, 2008. **68**(2): p. 537-44.
61. Schmalhofer, O., S. Brabletz, and T. Brabletz, *E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer.* Cancer Metastasis Rev, 2009. **28**(1-2): p. 151-66.
62. Park, S.H., et al., *Estrogen regulates Snail and Slug in the down-regulation of E-cadherin and induces metastatic potential of ovarian cancer cells through estrogen receptor alpha.* Mol Endocrinol, 2008. **22**(9): p. 2085-98.
63. Spaderna, S., et al., *A transient, EMT-linked loss of basement membranes indicates metastasis and poor survival in colorectal cancer.* Gastroenterology, 2006. **131**(3): p. 830-40.
64. Rosen, E.D. and O.A. MacDougald, *Adipocyte differentiation from the inside out.* Nat Rev Mol Cell Biol, 2006. **7**(12): p. 885-96.
65. Uldry, M., et al., *Complementary action of the PGC-1 coactivators in mitochondrial biogenesis and brown fat differentiation.* Cell Metab, 2006. **3**(5): p. 333-41.
66. Klaus, S., et al., *The uncoupling protein UCP: a membraneous mitochondrial ion carrier exclusively expressed in brown adipose tissue.* Int J Biochem, 1991. **23**(9): p. 791-801.
67. Nicholls, D.G. and R.M. Locke, *Thermogenic mechanisms in brown fat.* Physiol Rev, 1984. **64**(1): p. 1-64.
68. Otto, T.C. and M.D. Lane, *Adipose development: from stem cell to adipocyte.* Crit Rev Biochem Mol Biol, 2005. **40**(4): p. 229-42.
69. Rosen, E.D., et al., *Transcriptional regulation of adipogenesis.* Genes Dev, 2000. **14**(11): p. 1293-307.

70. Bowers, R.R., et al., *Stable stem cell commitment to the adipocyte lineage by inhibition of DNA methylation: role of the BMP-4 gene.* Proc Natl Acad Sci U S A, 2006. **103**(35): p. 13022-7.
71. Tang, Q.Q., T.C. Otto, and M.D. Lane, *Commitment of C3H10T1/2 pluripotent stem cells to the adipocyte lineage.* Proc Natl Acad Sci U S A, 2004. **101**(26): p. 9607-11.
72. Lin, F.T. and M.D. Lane, *Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes.* Genes Dev, 1992. **6**(4): p. 533-44.
73. Tanaka, T., et al., *Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene.* EMBO J, 1997. **16**(24): p. 7432-43.
74. Tontonoz, P., et al., *PPAR gamma 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene.* Mol Cell Biol, 1995. **15**(1): p. 351-7.
75. Barak, Y., et al., *PPAR gamma is required for placental, cardiac, and adipose tissue development.* Mol Cell, 1999. **4**(4): p. 585-95.
76. Kubota, N., et al., *PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance.* Mol Cell, 1999. **4**(4): p. 597-609.
77. Lefterova, M.I., et al., *PPARgamma and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale.* Genes Dev, 2008. **22**(21): p. 2941-52.
78. Eckel, R.H., *Lipoprotein lipase. A multifunctional enzyme relevant to common metabolic diseases.* N Engl J Med, 1989. **320**(16): p. 1060-8.
79. Kraemer, F.B. and W.J. Shen, *Hormone-sensitive lipase: control of intracellular tri-(di-)acylglycerol and cholesteryl ester hydrolysis.* J Lipid Res, 2002. **43**(10): p. 1585-94.
80. Ackerman, G.E., et al., *Potentiation of epinephrine-induced lipolysis by catechol estrogens and their methoxy derivatives.* Endocrinology, 1981. **109**(6): p. 2084-8.
81. Palin, S.L., et al., *17Beta-estradiol and anti-estrogen ICI:compound 182,780 regulate expression of lipoprotein lipase and hormone-sensitive lipase in isolated subcutaneous abdominal adipocytes.* Metabolism, 2003. **52**(4): p. 383-8.
82. Arner, P., *Regional adipocity in man.* J Endocrinol, 1997. **155**(2): p. 191-2.
83. Mayes, J.S. and G.H. Watson, *Direct effects of sex steroid hormones on adipose tissues and obesity.* Obes Rev, 2004. **5**(4): p. 197-216.
84. Shi, H., R.J. Seeley, and D.J. Clegg, *Sexual differences in the control of energy homeostasis.* Front Neuroendocrinol, 2009.
85. Wajchenberg, B.L., *Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome.* Endocr Rev, 2000. **21**(6): p. 697-738.
86. Power, M.L. and J. Schulkin, *Sex differences in fat storage, fat metabolism, and the health risks from obesity: possible evolutionary origins.* Br J Nutr, 2008. **99**(5): p. 931-40.
87. Simpson, E.R., *Role of aromatase in sex steroid action.* J Mol Endocrinol, 2000. **25**(2): p. 149-56.
88. Simpson, E.R. and S.R. Davis, *Minireview: aromatase and the regulation of estrogen biosynthesis--some new perspectives.* Endocrinology, 2001. **142**(11): p. 4589-94.

89. Hall, J.M., J.F. Couse, and K.S. Korach, *The multifaceted mechanisms of estradiol and estrogen receptor signaling.* J Biol Chem, 2001. **276**(40): p. 36869-72.
90. Carroll, J.S. and M. Brown, *Estrogen receptor target gene: an evolving concept.* Mol Endocrinol, 2006. **20**(8): p. 1707-14.
91. Kamalakaran, S., S.K. Radhakrishnan, and W.T. Beck, *Identification of estrogen-responsive genes using a genome-wide analysis of promoter elements for transcription factor binding sites.* J Biol Chem, 2005. **280**(22): p. 21491-7.
92. Lin, C.Y., et al., *Whole-genome cartography of estrogen receptor alpha binding sites.* PLoS Genet, 2007. **3**(6): p. e87.
93. O'Lone, R., et al., *Genomic targets of nuclear estrogen receptors.* Mol Endocrinol, 2004. **18**(8): p. 1859-75.
94. Nawaz, Z., et al., *Proteasome-dependent degradation of the human estrogen receptor.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 1858-62.
95. Preisler-Mashek, M.T., et al., *Ligand-specific regulation of proteasome-mediated proteolysis of estrogen receptor-alpha.* Am J Physiol Endocrinol Metab, 2002. **282**(4): p. E891-8.
96. Lonard, D.M., et al., *The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation.* Mol Cell, 2000. **5**(6): p. 939-48.
97. Reid, G., et al., *Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling.* Mol Cell, 2003. **11**(3): p. 695-707.
98. Prossnitz, E.R., et al., *Estrogen signaling through the transmembrane G protein-coupled receptor GPR30.* Annu Rev Physiol, 2008. **70**: p. 165-90.
99. Green, S., et al., *Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A.* Nature, 1986. **320**(6058): p. 134-9.
100. Greene, G.L., et al., *Sequence and expression of human estrogen receptor complementary DNA.* Science, 1986. **231**(4742): p. 1150-4.
101. Kuiper, G.G., et al., *Cloning of a novel receptor expressed in rat prostate and ovary.* Proc Natl Acad Sci U S A, 1996. **93**(12): p. 5925-30.
102. Matthews, J. and J.A. Gustafsson, *Estrogen signaling: a subtle balance between ER alpha and ER beta.* Mol Interv, 2003. **3**(5): p. 281-92.
103. Bunone, G., et al., *Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation.* EMBO J, 1996. **15**(9): p. 2174-83.
104. Tremblay, A., et al., *Ligand-independent recruitment of SRC-1 to estrogen receptor beta through phosphorylation of activation function AF-1.* Mol Cell, 1999. **3**(4): p. 513-9.
105. Weigel, N.L. and Y. Zhang, *Ligand-independent activation of steroid hormone receptors.* J Mol Med, 1998. **76**(7): p. 469-79.
106. Weihua, Z., et al., *Update on estrogen signaling.* FEBS Lett, 2003. **546**(1): p. 17-24.
107. Wrenn, C.K. and B.S. Katzenellenbogen, *Structure-function analysis of the hormone binding domain of the human estrogen receptor by region-specific*

mutagenesis and phenotypic screening in yeast. J Biol Chem, 1993. **268**(32): p. 24089-98.
108. Harris, H.A., *Estrogen receptor-beta: recent lessons from in vivo studies.* Mol Endocrinol, 2007. **21**(1): p. 1-13.
109. Naaz, A., et al., *Effect of ovariectomy on adipose tissue of mice in the absence of estrogen receptor alpha (ERalpha): a potential role for estrogen receptor beta (ERbeta).* Horm Metab Res, 2002. **34**(11-12): p. 758-63.
110. Pedersen, S.B., et al., *Demonstration of estrogen receptor subtypes alpha and beta in human adipose tissue: influences of adipose cell differentiation and fat depot localization.* Mol Cell Endocrinol, 2001. **182**(1): p. 27-37.
111. Price, T.M. and S.N. O'Brien, *Determination of estrogen receptor messenger ribonucleic acid (mRNA) and cytochrome P450 aromatase mRNA levels in adipocytes and adipose stromal cells by competitive polymerase chain reaction amplification.* J Clin Endocrinol Metab, 1993. **77**(4): p. 1041-5.
112. Dieudonne, M.N., et al., *Evidence for functional estrogen receptors alpha and beta in human adipose cells: regional specificities and regulation by estrogens.* Am J Physiol Cell Physiol, 2004. **286**(3): p. C655-61.
113. Heine, P.A., et al., *Increased adipose tissue in male and female estrogen receptor-alpha knockout mice.* Proc Natl Acad Sci U S A, 2000. **97**(23): p. 12729-34.
114. Mueller, S.O. and K.S. Korach, *Estrogen receptors and endocrine diseases: lessons from estrogen receptor knockout mice.* Curr Opin Pharmacol, 2001. **1**(6): p. 613-9.
115. Ohlsson, C., et al., *Obesity and disturbed lipoprotein profile in estrogen receptor-alpha-deficient male mice.* Biochem Biophys Res Commun, 2000. **278**(3): p. 640-5.
116. Hall, J.M. and D.P. McDonnell, *The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens.* Endocrinology, 1999. **140**(12): p. 5566-78.
117. Mizutani, T., et al., *Identification of estrogen receptor in human adipose tissue and adipocytes.* J Clin Endocrinol Metab, 1994. **78**(4): p. 950-4.
118. Cooke, P.S. and A. Naaz, *Role of estrogens in adipocyte development and function.* Exp Biol Med (Maywood), 2004. **229**(11): p. 1127-35.
119. Pallottini, V., et al., *Estrogen regulation of adipose tissue functions: involvement of estrogen receptor isoforms.* Infect Disord Drug Targets, 2008. **8**(1): p. 52-60.
120. Lea-Currie, Y.R., D. Monroe, and M.K. McIntosh, *Dehydroepiandrosterone and related steroids alter 3T3-L1 preadipocyte proliferation and differentiation.* Comp Biochem Physiol C Pharmacol Toxicol Endocrinol, 1999. **123**(1): p. 17-25.
121. Anderson, L.A., et al., *The effects of androgens and estrogens on preadipocyte proliferation in human adipose tissue: influence of gender and site.* J Clin Endocrinol Metab, 2001. **86**(10): p. 5045-51.
122. Homma, H., et al., *Estrogen suppresses transcription of lipoprotein lipase gene. Existence of a unique estrogen response element on the lipoprotein lipase promoter.* J Biol Chem, 2000. **275**(15): p. 11404-11.

123. D'Eon, T.M., et al., *Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways.* J Biol Chem, 2005. **280**(43): p. 35983-91.
124. Wade, G.N., J.M. Gray, and T.J. Bartness, *Gonadal influences on adiposity.* Int J Obes, 1985. **9 Suppl 1**: p. 83-92.
125. Pedersen, S.B., et al., *Estrogen controls lipolysis by up-regulating alpha2A-adrenergic receptors directly in human adipose tissue through the estrogen receptor alpha. Implications for the female fat distribution.* J Clin Endocrinol Metab, 2004. **89**(4): p. 1869-78.
126. Musatov, S., et al., *Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome.* Proc Natl Acad Sci U S A, 2007. **104**(7): p. 2501-6.
127. Woods, S.C., K. Gotoh, and D.J. Clegg, *Gender differences in the control of energy homeostasis.* Exp Biol Med (Maywood), 2003. **228**(10): p. 1175-80.
128. Louet, J.F., C. LeMay, and F. Mauvais-Jarvis, *Antidiabetic actions of estrogen: insight from human and genetic mouse models.* Curr Atheroscler Rep, 2004. **6**(3): p. 180-5.
129. Bolinder, J., et al., *Site differences in insulin receptor binding and insulin action in subcutaneous fat of obese females.* J Clin Endocrinol Metab, 1983. **57**(3): p. 455-61.
130. Marin, P., et al., *The morphology and metabolism of intraabdominal adipose tissue in men.* Metabolism, 1992. **41**(11): p. 1242-8.
131. Ray, P., et al., *Repression of interleukin-6 gene expression by 17 beta-estradiol: inhibition of the DNA-binding activity of the transcription factors NF-IL6 and NF-kappa B by the estrogen receptor.* FEBS Lett, 1997. **409**(1): p. 79-85.
132. Stein, B. and M.X. Yang, *Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappa B and C/EBP beta.* Mol Cell Biol, 1995. **15**(9): p. 4971-9.
133. Bjorntorp, P., *Body fat distribution, insulin resistance, and metabolic diseases.* Nutrition, 1997. **13**(9): p. 795-803.
134. Barros, R.P., et al., *Muscle GLUT4 regulation by estrogen receptors ERbeta and ERalpha.* Proc Natl Acad Sci U S A, 2006. **103**(5): p. 1605-8.
135. Le May, C., et al., *Estrogens protect pancreatic beta-cells from apoptosis and prevent insulin-deficient diabetes mellitus in mice.* Proc Natl Acad Sci U S A, 2006. **103**(24): p. 9232-7.
136. Kahn, S.E., R.L. Hull, and K.M. Utzschneider, *Mechanisms linking obesity to insulin resistance and type 2 diabetes.* Nature, 2006. **444**(7121): p. 840-6.
137. Gustafson, B., et al., *Inflamed adipose tissue: a culprit underlying the metabolic syndrome and atherosclerosis.* Arterioscler Thromb Vasc Biol, 2007. **27**(11): p. 2276-83.
138. Fantuzzi, G., *Adipose tissue, adipokines, and inflammation.* J Allergy Clin Immunol, 2005. **115**(5): p. 911-9; quiz 920.
139. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* J Clin Invest, 2003. **112**(12): p. 1796-808.
140. Kuo-cherng Lin, C.-H.W., Fan Chang, Moi-Kim Hong, Hui-Wen chang, Jyuer-Ger Yanh, Gia-Ni Hsieh, *Exogenous estrogen stimulates the increase of*

circulating leptin in normal cyclic women. changhua Journal of Medicine, 2003. **8**(4): p. 6.
141. Ahima, R.S., et al., *Leptin regulation of neuroendocrine systems.* Front Neuroendocrinol, 2000. **21**(3): p. 263-307.
142. Kristensen, K., S.B. Pedersen, and B. Richelsen, *Regulation of leptin by steroid hormones in rat adipose tissue.* Biochem Biophys Res Commun, 1999. **259**(3): p. 624-30.
143. Clegg, D.J., et al., *Gonadal hormones determine sensitivity to central leptin and insulin.* Diabetes, 2006. **55**(4): p. 978-87.
144. Enriori, P.J., et al., *Leptin resistance and obesity.* Obesity (Silver Spring), 2006. **14 Suppl 5**: p. 254S-258S.
145. Munzberg, H. and M.G. Myers, Jr., *Molecular and anatomical determinants of central leptin resistance.* Nat Neurosci, 2005. **8**(5): p. 566-70.
146. Wang, J., et al., *Overfeeding rapidly induces leptin and insulin resistance.* Diabetes, 2001. **50**(12): p. 2786-91.
147. Hager, J., et al., *A genome-wide scan for human obesity genes reveals a major susceptibility locus on chromosome 10.* Nat Genet, 1998. **20**(3): p. 304-8.
148. Hinney, A., et al., *Independent confirmation of a major locus for obesity on chromosome 10.* J Clin Endocrinol Metab, 2000. **85**(8): p. 2962-5.
149. Price, R.A., et al., *A locus affecting obesity in human chromosome region 10p12.* Diabetologia, 2001. **44**(3): p. 363-6.
150. Emilsson, V., et al., *Genetics of gene expression and its effect on disease.* Nature, 2008. **452**(7186): p. 423-8.
151. Barsh, G.S., I.S. Farooqi, and S. O'Rahilly, *Genetics of body-weight regulation.* Nature, 2000. **404**(6778): p. 644-51.
152. Farooqi, S. and S. O'Rahilly, *Genetics of obesity in humans.* Endocr Rev, 2006. **27**(7): p. 710-18.
153. Rankinen, T., et al., *The human obesity gene map: the 2005 update.* Obesity (Silver Spring), 2006. **14**(4): p. 529-644.
154. Dong, C., et al., *Possible genomic imprinting of three human obesity-related genetic loci.* Am J Hum Genet, 2005. **76**(3): p. 427-37.
155. Gu, C. and D.C. Rao, *A linkage strategy for detection of human quantitative-trait loci. II. Optimization of study designs based on extreme sib pairs and generalized relative risk ratios.* Am J Hum Genet, 1997. **61**(1): p. 211-22.
156. Gu, C., A.A. Todorov, and D.C. Rao, *Genome screening using extremely discordant and extremely concordant sib pairs.* Genet Epidemiol, 1997. **14**(6): p. 791-6.
157. Wicks, J., *Exploiting excess sharing: a more powerful test of linkage for affected sib pairs than the transmission/disequilibrium test.* Am J Hum Genet, 2000. **66**(6): p. 2005-8.
158. de Bakker, P.I., et al., *Efficiency and power in genetic association studies.* Nat Genet, 2005. **37**(11): p. 1217-23.
159. Ding, C. and C.R. Cantor, *A high-throughput gene expression analysis technique using competitive PCR and matrix-assisted laser desorption ionization time-of-flight MS.* Proc Natl Acad Sci U S A, 2003. **100**(6): p. 3059-64.

160. Fei, Z., T. Ono, and L.M. Smith, *MALDI-TOF mass spectrometric typing of single nucleotide polymorphisms with mass-tagged ddNTPs.* Nucleic Acids Res, 1998. **26**(11): p. 2827-8.
161. Sabin, M.A. and J.P. Shield, *Childhood obesity.* Front Horm Res, 2008. **36**: p. 85-96.
162. Sekiya, I., et al., *Adipogenic differentiation of human adult stem cells from bone marrow stroma (MSCs).* J Bone Miner Res, 2004. **19**(2): p. 256-64.
163. Gesta, S., Y.H. Tseng, and C.R. Kahn, *Developmental origin of fat: tracking obesity to its source.* Cell, 2007. **131**(2): p. 242-56.
164. Tontonoz, P. and B.M. Spiegelman, *Fat and beyond: the diverse biology of PPARgamma.* Annu Rev Biochem, 2008. **77**: p. 289-312.
165. Xu, Z., et al., *The orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor II is a critical regulator of adipogenesis.* Proc Natl Acad Sci U S A, 2008. **105**(7): p. 2421-6.
166. Comuzzie, A.G. and D.B. Allison, *The search for human obesity genes.* Science, 1998. **280**(5368): p. 1374-7.
167. Bell, C.G., A.J. Walley, and P. Froguel, *The genetics of human obesity.* Nat Rev Genet, 2005. **6**(3): p. 221-34.
168. Clement, K., P. Boutin, and P. Froguel, *Genetics of obesity.* Am J Pharmacogenomics, 2002. **2**(3): p. 177-87.
169. Herbert, A., et al., *A common genetic variant is associated with adult and childhood obesity.* Science, 2006. **312**(5771): p. 279-83.
170. Postigo, A.A. and D.C. Dean, *ZEB, a vertebrate homolog of Drosophila Zfh-1, is a negative regulator of muscle differentiation.* EMBO J, 1997. **16**(13): p. 3935-43.
171. Fontemaggi, G., et al., *deltaEF1 repressor controls selectively p53 family members during differentiation.* Oncogene, 2005. **24**(49): p. 7273-80.
172. Dolinkova, M., et al., *The endocrine profile of subcutaneous and visceral adipose tissue of obese patients.* Mol Cell Endocrinol, 2008. **291**(1-2): p. 63-70.
173. Clark, S.G. and C. Chiu, *C. elegans ZAG-1, a Zn-finger-homeodomain protein, regulates axonal development and neuronal differentiation.* Development, 2003. **130**(16): p. 3781-94.
174. Eger, A., et al., *DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells.* Oncogene, 2005. **24**(14): p. 2375-85.
175. Cleary, M.P., et al., *Prevention of mammary tumorigenesis by intermittent caloric restriction: does caloric intake during refeeding modulate the response?* Exp Biol Med (Maywood), 2007. **232**(1): p. 70-80.
176. Bonorden, M.J., et al., *Cross-sectional analysis of intermittent versus chronic caloric restriction in the TRAMP mouse.* Prostate, 2009. **69**(3): p. 317-26.
177. Landisch, R.M., et al., *Adaptive and nonadaptive responses to voluntary wheel running by mdx mice.* Muscle Nerve, 2008. **38**(4): p. 1290-303.
178. Tchkonia, T., et al., *Identification of depot-specific human fat cell progenitors through distinct expression profiles and developmental gene patterns.* Am J Physiol Endocrinol Metab, 2007. **292**(1): p. E298-307.
179. Layden, M.J., et al., *Zfh1, a somatic motor neuron transcription factor, regulates axon exit from the CNS.* Dev Biol, 2006. **291**(2): p. 253-63.

180. Lee, H.K. and M.J. Lundell, *Differentiation of the Drosophila serotonergic lineage depends on the regulation of Zfh-1 by Notch and Eagle.* Mol Cell Neurosci, 2007. **36**(1): p. 47-58.
181. Ahima, R.S. and M.A. Lazar, *Adipokines and the peripheral and neural control of energy balance.* Mol Endocrinol, 2008. **22**(5): p. 1023-31.
182. Arita, Y., et al., *Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity.* Biochem Biophys Res Commun, 1999. **257**(1): p. 79-83.
183. Trujillo, M.E. and P.E. Scherer, *Adipose tissue-derived factors: impact on health and disease.* Endocr Rev, 2006. **27**(7): p. 762-78.
184. Diez, J.J. and P. Iglesias, *The role of the novel adipocyte-derived hormone adiponectin in human disease.* Eur J Endocrinol, 2003. **148**(3): p. 293-300.
185. Pajvani, U.B., et al., *Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity.* J Biol Chem, 2004. **279**(13): p. 12152-62.
186. Nkhata, K.J., et al., *Mammary tumor development from T47-D human breast cancer cells in obese ovariectomized mice with and without estradiol supplements.* Breast Cancer Res Treat, 2009. **114**(1): p. 71-83.
187. Costacou, T. and T.J. Orchard, *Adiponectin: good, bad, or just plain ugly?* Kidney Int, 2008. **74**(5): p. 549-51.
188. Blakemore, S.J., *The social brain in adolescence.* Nat Rev Neurosci, 2008. **9**(4): p. 267-77.
189. Misso, M.L., et al., *Cellular and molecular characterization of the adipose phenotype of the aromatase-deficient mouse.* Endocrinology, 2003. **144**(4): p. 1474-80.
190. Jones, M.E., et al., *Aromatase-deficient (ArKO) mice accumulate excess adipose tissue.* J Steroid Biochem Mol Biol, 2001. **79**(1-5): p. 3-9.
191. Robertson, J.F. and M. Harrison, *Fulvestrant: pharmacokinetics and pharmacology.* Br J Cancer, 2004. **90 Suppl 1**: p. S7-10.
192. Wakeling, A.E. and J. Bowler, *ICI 182,780, a new antioestrogen with clinical potential.* J Steroid Biochem Mol Biol, 1992. **43**(1-3): p. 173-7.
193. Wakeling, A.E., M. Dukes, and J. Bowler, *A potent specific pure antiestrogen with clinical potential.* Cancer Res, 1991. **51**(15): p. 3867-73.
194. Laborda, J., *36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal phosphoprotein PO.* Nucleic Acids Res, 1991. **19**(14): p. 3998.
195. Fasshauer, M. and R. Paschke, *Regulation of adipocytokines and insulin resistance.* Diabetologia, 2003. **46**(12): p. 1594-603.
196. Srivastava, S., et al., *Estrogen decreases TNF gene expression by blocking JNK activity and the resulting production of c-Jun and JunD.* J Clin Invest, 1999. **104**(4): p. 503-13.
197. Combs, T.P., et al., *Induction of adipocyte complement-related protein of 30 kilodaltons by PPARgamma agonists: a potential mechanism of insulin sensitization.* Endocrinology, 2002. **143**(3): p. 998-1007.
198. Zhang, Y., et al., *Positional cloning of the mouse obese gene and its human homologue.* Nature, 1994. **372**(6505): p. 425-32.

199. Steppan, C.M., et al., *The hormone resistin links obesity to diabetes.* Nature, 2001. **409**(6818): p. 307-12.
200. Chen, Y.H., et al., *17 beta-estradiol stimulates resistin gene expression in 3T3-L1 adipocytes via the estrogen receptor, extracellularly regulated kinase, and CCAAT/enhancer binding protein-alpha pathways.* Endocrinology, 2006. **147**(9): p. 4496-504.
201. Huang, S.W., et al., *Resistin mRNA levels are downregulated by estrogen in vivo and in vitro.* FEBS Lett, 2005. **579**(2): p. 449-54.
202. Zhou, L., et al., *Transcriptional regulation of the resistin gene.* Domest Anim Endocrinol, 2006. **30**(2): p. 98-107.
203. Ahima, R.S. and J.S. Flier, *Leptin.* Annu Rev Physiol, 2000. **62**: p. 413-37.
204. Machinal, F., et al., *In vivo and in vitro ob gene expression and leptin secretion in rat adipocytes: evidence for a regional specific regulation by sex steroid hormones.* Endocrinology, 1999. **140**(4): p. 1567-74.
205. Ainslie, D.A., et al., *Estrogen deficiency causes central leptin insensitivity and increased hypothalamic neuropeptide Y.* Int J Obes Relat Metab Disord, 2001. **25**(11): p. 1680-8.
206. Clegg, D.J., et al., *Differential sensitivity to central leptin and insulin in male and female rats.* Diabetes, 2003. **52**(3): p. 682-7.
207. Pedersen, S.B., et al., *Regulation of UCP1, UCP2, and UCP3 mRNA expression in brown adipose tissue, white adipose tissue, and skeletal muscle in rats by estrogen.* Biochem Biophys Res Commun, 2001. **288**(1): p. 191-7.
208. Clifford, G.M., et al., *Dephosphorylation of perilipin by protein phosphatases present in rat adipocytes.* FEBS Lett, 1998. **435**(1): p. 125-9.
209. Blanchette-Mackie, E.J., et al., *Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes.* J Lipid Res, 1995. **36**(6): p. 1211-26.
210. Londos, C., et al., *Perilipin: unique proteins associated with intracellular neutral lipid droplets in adipocytes and steroidogenic cells.* Biochem Soc Trans, 1995. **23**(3): p. 611-5.
211. Smith, P.J., et al., *Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes.* J Biol Chem, 1988. **263**(19): p. 9402-8.
212. Boney, C.M., R.M. Smith, and P.A. Gruppuso, *Modulation of insulin-like growth factor I mitogenic signaling in 3T3-L1 preadipocyte differentiation.* Endocrinology, 1998. **139**(4): p. 1638-44.
213. Jin, S., et al., *c-Crk, a substrate of the insulin-like growth factor-1 receptor tyrosine kinase, functions as an early signal mediator in the adipocyte differentiation process.* J Biol Chem, 2000. **275**(44): p. 34344-52.
214. Yeh, W.C., B.E. Bierer, and S.L. McKnight, *Rapamycin inhibits clonal expansion and adipogenic differentiation of 3T3-L1 cells.* Proc Natl Acad Sci U S A, 1995. **92**(24): p. 11086-90.
215. Reichert, M. and D. Eick, *Analysis of cell cycle arrest in adipocyte differentiation.* Oncogene, 1999. **18**(2): p. 459-66.
216. Tang, Q.Q., T.C. Otto, and M.D. Lane, *Mitotic clonal expansion: a synchronous process required for adipogenesis.* Proc Natl Acad Sci U S A, 2003. **100**(1): p. 44-9.

217. Farmer, S.R., *Transcriptional control of adipocyte formation.* Cell Metab, 2006. **4**(4): p. 263-73.
218. Smas, C.M. and H.S. Sul, *Control of adipocyte differentiation.* Biochem J, 1995. **309 (Pt 3)**: p. 697-710.
219. Lowell, B.B., *PPARgamma: an essential regulator of adipogenesis and modulator of fat cell function.* Cell, 1999. **99**(3): p. 239-42.
220. Porter, A.G., *Flipping the safety catch of procaspase-3.* Nat Chem Biol, 2006. **2**(10): p. 509-10.
221. Ohnishi, S.T., N. Koshiba, S., Harada T., Watanabe, S. Kigawa, T., *Solution structure of the homeobox domain from human NIL-2-A zinc finger protein, transcription factor 8*. To be Published.
222. Kern, P.A., et al., *Perilipin expression in human adipose tissue is elevated with obesity.* J Clin Endocrinol Metab, 2004. **89**(3): p. 1352-8.
223. Kim, K.H., et al., *A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation.* J Biol Chem, 2001. **276**(14): p. 11252-6.
224. Banerjee, R.R., et al., *Regulation of fasted blood glucose by resistin.* Science, 2004. **303**(5661): p. 1195-8.
225. Kloting, N., et al., *Autocrine IGF-1 action in adipocytes controls systemic IGF-1 concentrations and growth.* Diabetes, 2008. **57**(8): p. 2074-82.