ROLE OF VITAMIN A METABOLISM IN VISCERAL OBESITY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

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2012

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ABSTRACT

Obesity has become a global epidemic in recent years. In the United States, obesity rates are increasing at an alarming rate along with a dramatic rise in morbidity and mortality. A number of studies attribute such mortalities to central/visceral obesity, characterized by increased fat mass in the abdomen. Visceral obesity is closely associated with the prevalence of insulin resistance, type 2 diabetes, cardiovascular diseases, and even some cancers. However, mechanisms underlying visceral obesity are unclear.

This thesis is designed to investigate how vitamin A metabolites retinaldehyde (Rald) and retinoic acid (RA) and their metabolizing enzymes regulate adipogenesis. My work showed that induction of \( \text{PPAR}_\gamma \) in adipogenesis depends on RA generation by \( \text{ALDH}1 \) enzymes that give rise to fat formation in depot-and sex-specific manner.

Chapter 1 is a literature review of white fat depots, \( \text{PPAR}_\gamma \) involvement during adipogenesis and vitamin A metabolism. Chapter 2 investigates the molecular mechanisms by which \( \text{Aldh1a1} \) or its product RA affects \( \text{PPAR}_\gamma \) expression in white fat depots. We demonstrated that adipogenesis \textit{in vitro} is accompanied by RA production, 70% of which was generated by \( \text{Aldh1a1} \). In \( \text{Aldh1a1} \)-deficient adipocytes, adipogenesis was impaired compared with WT adipocytes due to markedly reduced expression of \( \text{PPAR}_\gamma \) regulated through ZFP423. These effects were recovered to some extent either by
RA stimulation or overexpression of any of the ALDH1 enzymes in Aldh1a1−/− cells. We used Aldh1a1 deficient mice (Aldh1a1−/−tm1Gdu), a mouse model of obesity developed by Duester and colleagues, to demonstrate causal role of Aldh1a1 in obesity \textit{in vivo}. We found a fat depot-specific expression of Aldh1 enzymes that suggests distinct RA generation in different adipose depots regulating visceral obesity by vitamin A-metabolizing enzymes or by RA.

Chapter 3 investigates sex- and depot-specific fat formation by Rald and RA on a high-fat (HF) diet. Aldh1a1−/−tm1Gdu mice displayed a lean phenotype compared to wild type (WT) on a HF diet associated with markedly higher ATGL protein levels in visceral fat of Aldh1a1−/−tm1Gdu females vs. males. The sex differences in ATGL protein expression were dependent on disrupted catabolism of Rald in Aldh1−/−tm1Gdu females whereas male mice maintained RA production by Aldha3. The depot and sex-specific RARE activation indicating endogenous RA production was demonstrated in RARELacZ reporter mice.

Chapter 4 examines regulation of ATGL protein translation by mammalian target of rapamycin complex 1 (mTORC1).

In summary, our findings uncover a novel role of vitamin A metabolites in transcriptional regulation of PPARγ through ZFP423 and identifies ATGL as a Rald-sensitive gene that protects from visceral fat accumulation, even on a HF diet. Vitamin A metabolizing enzymes may help to develop new therapeutics preventing and treating visceral obesity.
Dedicated to my parents
ACKNOWLEDGMENTS

First, I would like to express my sincere gratitude to my advisor Dr. Ouliana Ziouzenkova. From my first day in the lab till now, you have been a constant source of support through every hurdle. I feel very fortunate to have a mentor like you during my graduate school years. You are a great scientist and teacher, and I can proudly say that I have learned from one of the best in the field. I could never imagine myself where I am now, if it weren’t for your encouragement, patience and support. My role as a mother often hindered experiment execution in a timely manner, but you always understood and let me be a mother first, and then a scientist. My family and I thank you for that. I would also like to extend my thanks to Drs. Martha Belury, Helen Everts, and Kichoon Lee for taking time out of their busy schedules to serve on my committee. It truly is an honor to be surrounded by such exceptional researchers. Dr. Lee, a special thank you for letting me learn new techniques from your lab.

Among the past lab members in Ziouzenkova lab, Barbie and Fangping, my old pals, I have enjoyed having you in the lab and all the help you offered. Still now, I miss our laughs we have had together. The lab would never be the same without you. To all the other members in the lab, both past and present: Joe, Mike, David, Qiwen, Alex,
Luba and Jaisa, I am thankful to have known you and feel privileged to have worked with such a talented team.

I would also like to thank my parents, for their unconditional love and support. I couldn’t have come this far without their help in every step of the way. Mom, you will always be my true friend and dad - I feel proud to continue your legacy and I know you feel the same way too. My husband, thank you for your encouragement and support throughout the way. Without your support, my dream would have never realized. I thank you all for making this journey so worthwhile.
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LIST OF ABBREVIATIONS

Adh.......................................................... Alcohol dehydrogenase
Aldh.......................................................... Aldehyde dehydrogenase
atRA.......................................................... All-trans retinoic acid
ARAT........................................................ Acyl CoA:retinol acyl transferase
ATGL........................................................ Adipose triglyceride lipase [annotated as patatin-like phospholipase domain-containing protein A2]
AKR.......................................................... Aldo-keto reductase
BAT........................................................... Brown adipose tissue
BCMO1........................................................ β-Carotene 15,15′-carotene monooxygenase 1
βLG.............................................................. beta-Lactoglobulin
C/EBP........................................................ CCAAT enhancer-binding proteins
CRBPI........................................................ Cellular retinol binding protein I
CRABP-I........................................................ Cellular retinoic acid-binding protein I
CRABPII....................................................... Cellular retinoic acid-binding protein type II
CYP.............................................................. Cytochrome p450
DGAT1........................................................ Diacylglycerol acyltransferase
DQRS9........................................................ Dehydrogenase reductase S9
RARE......................................................... Retinoic acid response element
EBF1................................................................. Early B-cell factor 1
4-EBP1..........................................................Elongation binding protein 1
FABP............................................................... Fatty acid binding protein
FAS................................................................. Fatty acid synthase
FBS............................................................... Fetal bovine serum
GR................................................................. Glucocorticoid receptor
HDL................................................................. High-density lipoprotein
HF................................................................. High fat
HOX................................................................. Homeobox proteins
HSL................................................................. Hormone-sensitive lipase
IL-6................................................................. Interleukin 6
JAK2................................................................. Janus kinase 2
KLF................................................................. Krüppel-like transcription factor
L-PGDS........................................................... Lipocalin-type prostaglandin (PG) D synthase
LPL................................................................. Lipoprotein lipase
LRP................................................................. Low density lipoprotein (LDL) receptor-related protein
LRAT............................................................... Lecithin retinol-acyltransferase
MGL................................................................. Monoglyceride lipase
mTORC1.................................Mammalian target of rapamycin complex 1
NAD........................................Nicotinamide adenine dinucleotide
NEFA........................................Non-esterified fatty acid
Pref1........................................Preadipocyte factor -1
15-d-PGJ2.................................15-deoxy-delta (12,14)-Prostaglandin J2
PPARγ .................................Peroxisome proliferator-activated receptor γ
PPRE.........................Peroxisome proliferator-activated receptor response element (DR1)
RAR............................................Retinoic acid receptor
RARE........................................Retinoic acid response element (DR5)
RA............................................Retinoic acid
Rald.............................................Retinaldehyde/retinal
Raldehyde................................Retinaldehyde dehydrogenase
RBP.............................................Retinol-binding protein
RDH..........................................Retinol dehydrogenases (microsomal)
RXR..............................................Retinoid X receptor
SDR...........................................Short-chain dehydrogenase/reductase
SR-BI..........................................Scavenger receptor class B type 1
STAT5......................................Signal transducer and activator of transcription 5
STRA6.....................................Stimulated by retinoic acid 6
SOCS3………………………………………………….. Suppressor of cytokine signaling
S6k1………………………………………………………p70 ribosomal S 6 kinase 1
SREBP1c……………………………………………. Sterol regulatory element-binding protein-1c
TG………………………………………………………… Triglycerides/triacylglycerol
UCP1……………………………………………………….. Uncoupling protein 1
Visceral Fat………………………………………………… Visceral fat
WAT………………………………………………………….. White adipose tissue
ZFP423…………………………………………………. Zinc finger protein 423,transcription factor
CHAPTER 1

Literature Review

1.1 Introduction

Recent studies show that obesity rates in the United States have reached epidemic proportions along with a dramatic rise in morbidity and mortality [1, 2]. Such mortalities are attributed to central/visceral obesity, characterized by increased fat mass in the abdomen [3, 4]. Increased visceral fat accumulation has been implicated as a major contributor to adverse metabolic alterations (i.e. elevated serum cholesterol, triglycerides, fasting glucose levels) in humans [5] that were independent of overall obesity. Harmful metabolic effects exerted by visceral fat contribute to the development of insulin resistance, type 2 diabetes, cardiovascular diseases and even some cancers [3, 5, 6]. In contrast, subcutaneous fat is associated with beneficial metabolic properties such as improved insulin sensitivity and reduced chronic inflammation. White adipose depots, apart from being energy storage depots, also function as major endocrine tissue secreting many cytokines and adipokines, which potentially affect the function of other tissues [7]. Mechanisms underlying the formation of visceral fat are emerging. HOX, PPARγ, and other key genes are differentially expressed in white fat depots [45] (Figure.1.1). Paradoxically, both subcutaneous and visceral adipose tissue are regulated by similar
basic transcription factors and signaling cascades. Therapies directed towards depot specific obesity will provide substantial therapeutic benefits. However, the formulation of these therapies requires understanding the congenital and adaptive transcriptional networks and signaling pathways in adipocytes from different depots. In this dissertation, I searched for auto/paracrine mechanisms that govern transcriptional networks involved in the formation of visceral fat versus subcutaneous fat.

**Figure. 1.1. Fat-depot-specific expression of selected genes.**

Red box, small font- repressed genes; Green box, large font- induced genes. Group 1. Developmental genes. Among genes involved in organogenesis, homeobox genes *HoxA5, HoxA9*, homeobox transcription factor *Engrailed 1 (En1)*, and nuclear receptor *COUP-TF1/NR2F1* are expressed at higher levels in the subcut fat depot, whereas in visceral fat, these genes are downregulated. Vice-versa, *HoxC9* and *En1* have nominal expression in subcut fat, while visceral fat shows their higher expression. Group 2. Fundamental adipogenic transcription factors. Among genes regulating adipogenesis, subcut fat expresses higher levels of *PPARγ, C/EBPα*, and *SREBP1* compared to visceral fat. Group 3. Proteins involved in vitamin A metabolism. Retinol binding protein RBP4 is preferentially expressed in the visceral fat depot. The expression of the Aldh1 family of enzymes regulating endogenouse RA production is also dissimilar in the two fat depots but it differs between humans and mice (indicated by H and M respectively) [165].
Vitamin A derivatives (retinoids) have a broad range of pharmacological action in adipose tissue [8]. RA, the active metabolite of vitamin A, plays a crucial role in adipocytes differentiation, embryogenesis and apoptosis [8,11,13]. It is also a signaling molecule that controls gene expression at the transcriptional level by acting as a ligand for two types of nuclear receptors, the RA receptors (RARα, β and γ), and the retinoid X receptors (RXRα, β γ) [14,15,16,17]. Heterodimers of RAR with RXR, bind to a retinoic acid response element (RARE), and regulate transcription of target genes [16].
Rald is generated from retinol by alcohol dehydrogenases (ADH1, ADH3, and ADH4) and retinol dehydrogenases (members of the short-chain dehydrogenase/reductase (Sdr) family). RA is produced solely from Rald by the cytosolic aldehyde dehydrogenase-1 family of enzymes (ALDH1A1, ALDH1A2, and ALDH1A3). This family is also known as the retinaldehyde dehydrogenase family, and alias names of these enzymes are Raldh1, Raldh2, and Raldh3. Excess RA is oxidized primarily by Cyp26B1 and by Cyp26A1 [9,10,11,12].

Vitamin A in the form of retinol is oxidized to Rald by cytosolic alcohol dehydrogenases (ADHs) of the medium-chain dehydrogenase/reductase (MDR) superfamily and microsomal retinol dehydrogenases (RDHs) of the short-chain dehydrogenase/reductase (SDR) superfamily in the liver. In adipose tissue the expression of these enzymes and their role was not examined. Rald is further oxidized to retinoic acid (RA) by aldehyde dehydrogenase1 (ALDH1) family of enzymes [9,10,11,12] (Figure 1.2). ALDH1 family comprise of three cytosolic enzymes. Regulation of depot-specific fat formation by Aldh1 is yet to be explored. Limited understanding of the role vitamin A-metabolizing enzymes in adipogenesis is a barrier for the understanding of formation of different fat depots. During adipogenesis, RA stimulation represses transcription factors C/EBPα and β by a retinoic acid receptor (RAR) dependent mechanism that prevents PPARγ expression and inhibits adipogenesis [18]. PPARγ, the master regulator of adipogenesis, is known to be regulated to a large extent by some other

Figure 1.2. Vitamin A Metabolism [13]
pathways [19, 20, 21, 22], including the transcription factor zinc finger protein-423 (ZFP423) [23].

The mammalian target of rapamycin (mTORC1) is a known key regulator of protein translation in adipocytes [24,25,26] and can also influence expression and activation state of PPARγ [27, 28,29]. mTORC1, acts as a nutrient sensor for branched chain amino acids, and, possibly, interacts with RAR receptor in the neurons stimulated by RA [31,32]. Despite the evidence of participation of these pathways in fat formation, the molecular mechanisms by which Aldh1a1 or metabolites RA and Rald affect obesity and especially visceral fat formation is still to be determined.

My research has been directed towards understanding the role of vitamin A metabolizing enzymes, mainly, Aldh1a1, in regulation of depot and sex-specific fat formation.

1.2 White fat depots & estrogen influence on fat distribution

Abdominal/visceral obesity is characterized by the increase in fat mass in the abdomen [1,2]. Excessive fat mass results from the increase in the size of adipocytes as well as in the number of these cells during the expansion of adipose tissue in obesity [3, 4, 5]. Omentectomy, i.e. removal of visceral fat, improves glucose metabolism in humans [33] and rodents [34,35]. However, liposuction, a common procedure of removing subcutaneous fat (peripheral obesity) in obese patients, could not improve metabolic effects [36]. Studies in humans and rodents characterize visceral fat as a unique, pathogenic fat depot, associated with an adverse metabolic profile, while subcutaneous fat seems to improve insulin sensitivity and lower the risk of developing metabolic
syndrome [33,34,35]. Such differences, however, prove not to be due to their different anatomical location. Transplantation of subcutaneous fat into visceral or subcutaneous regions of mice exhibited improved insulin sensitivity, decreased body weight and total fat mass. Visceral fat transplantation into subcutaneous fat had no effects, further emphasizing specific properties of fat from different depots [37].

Unlike subcutaneous fat depots, visceral fat formation is associated with elevated production of pro-inflammatory cytokines, i.e. TNF-α, IL-6, resistin, and leptin [38-40], which provokes a local and systemic state of chronic low-grade inflammation [41] and induce insulin resistance [42]. Adiponectin, an adipokine specifically secreted at higher levels in subcutaneous than in visceral adipose tissue, improves insulin sensitivity [43]. Ob/ob mice overexpressing adiponectin showed a massive increase in subcutaneous fat; however, this subcutaneous obesity was associated with improved insulin sensitivity, increased lipid clearance and improved β-cell function [44]. Despite the notoriety of visceral fat, little is known about regulation of visceral fat formation. Hormonal changes encountered during menopause are known to alter fat distribution in women [161], resulting in excess deposition of visceral fat. On a regular diet, men tend to gain more visceral fat than women [162]. However, this pattern of fat accumulation changes as women reach menopause and consume more high fat foods [163,164]. The mechanism for this sudden switch in fat distribution still remains unclear.
1.3 Transcriptional network defining fat depots

1.3.1 Developmental Genes

Recent efforts to identify differences in human and rodent adipocytes isolated from different white fat depots using gene array profiling yielded explicit evidence of participation of developmental genes in the formation of subcutaneous and visceral tissues [45-47]. These genes include numerous transcription factors participating in organogenesis, including members of homeobox (HOX) and nuclear receptor (e.g. COUP-TF1/ NR2F1) families of transcription factors, such as HoxA4, A5, C8, C9, En1 [45]. These findings suggest that adipocytes from different white fat depots originate from different precursors and maintain their distinct developmental transcriptional profile [46] (figure1.1). Consistent with the hypothesis that different fat depots have inherited characteristics, multiple propagations, immortalization of human preadipocytes isolated from omental and subcutaneous fat depots of different donors did not alter their depot-specific expression [46]. These developmental genes comprised approximately 18% of the adipocyte genes [46], which reveal the heterogeneity of visceral depots in humans, consisting of omental and mesenteric fat. Mesenteric fat shares characteristics with subcutaneous fat that is distinct from omental fat [47,48]. WAT, both subcutaneous and visceral, also harbor multilocular adipocytes resembling thermogenic brown adipocytes [200,201]. Multilocular adipocytes also contain numerous mitochondria involved in thermogenesis, and their lipids are organized as multilocular droplets accessible for lipolysis [200,202]. Despite their similar morphological features, multilocular adipocytes from white fat and brown adipocytes appear to derive from different lineages, whereas
myocytes and brown adipocytes share a common precursor [203]. Uni- and multilocular adipocytes, derived from mesenchymal progenitor cells, are able to change their morphology depending upon their environment [204]. Cold exposure and β-adrenergic stimulation can increase the number of multilocular adipocytes in visceral fat, whereas HF feeding enhances the proportion of unilocular adipocytes [204]. In white fat depots, not only the proportion of uni- and multilocular adipocytes differs widely among species; even factors such as sex, age, nutritional and hormonal changes can influence their numbers [200]. An increase in the number of multilocular adipocytes can improve metabolic parameters, leading to enhanced insulin sensitivity and reduced inflammation [200,203,205,206].

The role of HOX genes in adipocyte biology and the mechanisms of their regulation in response to nutrients deserve further study. Even though many genes are expressed in both visceral and subcutaneous adipose tissue, their mRNA levels are depot-specific [165].

1.3.2 Peroxisome proliferator-activated receptor-γ (PPARγ)

In all depots, adipose tissue formation during development depends on peroxisome proliferator-activated receptor-γ (PPARγ), a master regulator of transcription during adipogenesis [49, 50]. PPARγ is expressed as two isoforms, PPARγ1 and PPARγ2, produced by alternative promoter usage (functions and regulation reviewed in [51], appendix 1). Many of the in vitro studies related to adipocyte differentiation have been
performed using NIH-3T3 cell lines. These NIH-3T3 fibroblasts lack the ability to undergo adipocyte differentiation due to the absence of PPARγ2 [52]. In the course of development, both human and rodent adipocytes express more PPARγ in subcutaneous than in visceral fat depots [53] and maintain this expression pattern even in cultured adipocytes isolated from these fat depots for several generations, which highlight their origin from different lineages [54,55]. Correspondently, PPARγ activity is higher in subcutaneous than in visceral fat tissues [53]. Consequently, high expression of PPARγ target genes, such as, adiponectin, defines properties of subcutaneous fat. PPARγ-mediated activation of adiponectin has already been recognized as a major mechanism for insulin sensitivity associated with subcutaneous fat [55,56]. Moreover, increased adiponectin production by PPARγ is the basis of the insulin sensitizing effects of PPARγ ligands, thiazolidinediones [56]. Differences in PPARγ expression can contribute causally to the formation of specific fat depots. Impaired transcriptional PPARγ2 performance leads to weight gain, prevailing loss of subcutaneous fat, insulin resistance, and other metabolic dysfunctions in children and lean adults [57-62]. In contrast, treatment of rodents and humans with PPARγ agonists increase formation of subcutaneous depots [62], further underscoring the regulatory role of this transcription factor in regional fat formation.

It is currently unknown whether congenital and acquired depot-specific differences in PPARγ expression are regulated by the same transcriptional mechanisms. It is clear, however, that regional obesity in abdominal regions induced by overfeeding in humans is also associated with higher PPARγ expression [63]. Hormone-dependent fat distribution
also proceeds through PPAR\(\gamma\) regulation, although PPAR\(\gamma\) involvement could be somewhat paradoxical in the context of autocrine mechanisms for glucocorticoid production [64]. Adipose-specific transgenic expression of 11\(\beta\)-hydroxysteroid dehydrogenase 2, inactivating the hormone corticosterone into cortisol, is associated with resistance to diet-induced obesity, but accompanied by increased expression of PPAR\(\gamma\) in mouse fat [64]. The factors defining depot-specific PPAR\(\gamma\) expression remain poorly understood, whereas numerous potential transcriptional inducers of PPAR\(\gamma\) expression have been reported and reviewed in chapters 1.3.3 & 1.3.4 (Figure 1.3).

**Figure 1.3. Transcriptional Network Regulating PPAR\(\gamma\) Expression**

IBMX activates cAMP signaling cascade resulting in induction of C/EBP\(\beta\) expression in vitro. C/EBP\(\delta\) expression is induced by dexamethasone in vitro and mediated through glucocorticoid receptor (GR). C/EBP\(\beta\) forms homo/heterodimer with C/EBP\(\delta\) and binds to the C/EBP regulatory region of C/EBP\(\alpha\) and PPAR\(\gamma\), activating both transcription factors. C/EBP\(\alpha\), in a positive feedback loop can further induce PPAR\(\gamma\). Furthermore, C/EBP\(\beta\) activates KLF5 promoter, which in turn activates PPAR\(\gamma\) promoter, in conjunction with C/EBP \(\delta\). SREBP1c, a transcription factor acting downstream of C/EBP\(\beta\), upon insulin stimulation, activates PPAR\(\gamma\), possibly through endogenous PPAR\(\gamma\) ligand production. Some evidence suggested that fetal bovine serum (FBS) stimulates STAT5/GR pathway, which induces PPAR\(\gamma\) expression, although its role in vivo is yet to be explored. Early B cell factor (EBF1) regulates C/EBP\(\beta\) expression. Recently, ZFP423 was identified as a potent inducer of PPAR\(\gamma\)2 and adipogenesis. Pathways that are dispensable in vivo are represented by dashed lines [165].
1.3.3 Transcriptional Inducers of \( \text{PPAR}\gamma \) Expression

CCAAT/enhancer binding proteins (C/EBP) were the first identified family of transcription factors contributing to \( \text{PPAR}\gamma \) expression and activation [65]. The C/EBP family belongs to the large family of basic leucine zipper transcription factors. They can both homodimerize and heterodimerize with each other and bind to the same C/EBP consensus sequences (reviewed in [51]). The expression of \( C/EBP\beta \) in cultured preadipocytes results from activation of the cAMP signaling cascade by \( \beta \)-adrenergic signaling \textit{in vivo} and 3-isobutyl-1-methylxanthine (IBMX) \textit{in vitro}. \( C/EBP\delta \) expression
in these cells is mediated by the glucocorticoid pathway, which is stimulated through
dexamethasone in vitro. C/EBPβ homodimers or C/EBPβ-C/EBPδ heterodimers bind to
the C/EBP regulatory elements in PPARγ and C/EBPα promoters, activating these
transcription factors [66-68].

In a positive feedback regulatory loop, C/EBPα can further induce PPARγ
expression [67, 68, 69]. Global gene expression studies revealed that both PPARγ and
C/EBPα occupy similar regulatory sites of proteins required for terminal adipocyte
differentiation [62]. PPARγ, however, plays the chief role and can rescue adipogenesis in
C/EBPα−/− adipocytes [68]. Similar to PPARγ, C/EBPα is also preferentially expressed in
subcutaneous compared to visceral fat [48], where it is likely to contribute to the increase
in PPARγ activation in this tissue. The complex relationship between C/EBPβ and
C/EBPδ on PPARγ induction was shown in mouse models lacking C/EBPβ and C/EBPδ
expression (Figure 1.3). Whereas isolated mouse embryonic fibroblasts (MEF) obtained
from these mice were not capable of PPARγ and C/EBPα expression, significantly
reduced epididymal WAT mass in the C/EBPβ−/−,δ−/− mice, expressed both PPARγ and
C/EBPα to a similar extent compared to WT [69]. This observation argues for the
presence of an alternative pathway(s) that induces PPARγ in different white adipose
depots.
1.3.4 Alternative inducers of $PPAR_\gamma$ expression

STAT5 and STAT5A/glucocorticoid receptor (GR) complexes have been proposed to induce $PPAR_\gamma$ expression and adipogenesis in NIH-3T3 cells [70], but the role of this pathway in fat formation in vivo has not been elucidated yet. Emerging evidence suggests that many growth factors and hormones in fetal bovine serum act through STAT-mediated pathways in adipogenesis [71,72]. Recently, the RBP-retinol/STRA6/JAK2/STAT5 signaling cascade was identified as an inducer of STAT target genes, including suppressor of cytokine signaling 3 ($SOCS3$), which inhibits insulin signaling and $PPAR_\gamma$, enhancing lipid accumulation [73].

The breakthrough in the understanding of $PPAR_\gamma$ expression mechanisms came from recent studies by Spiegelman and associates [23], who convincingly demonstrated that ectopic expression of transcription factor $ZFP423$ ($ZNF423$ is a human analog) is sufficient to rescue $PPAR_\gamma2$ expression and adipogenesis in NIH-3T3 cells which lack $PPAR_\gamma$ (Figure 1.3). This pathway appears to bypass $C/EBPs$, $KLFs$, and other known $PPAR_\gamma$ inducers, in its activation of $PPAR_\gamma2$. Surprisingly, $PPAR_\gamma1$ expression is not influenced in the presence of $ZFP423$. Although a detailed mechanism for $PPAR_\gamma2$ regulation by $ZFP423$ remains to be elucidated, it is clear that bone morphogenetic protein (BMP) activates $PPAR_\gamma2$ in adipogenesis through $ZFP423$/SMAD interaction [23]. The estimation of $ZFP423$’s contribution to $PPAR_\gamma$ regulation in vivo is problematic given the embryonic lethality of $ZFP423^{-/-}$ mice, but the initial observations showed a fewer number of adipocytes in the $ZFP423^{-/-}$ embryo [23]. Thus far, $ZFP423$ appears to
be a powerful alternative pathway for PPARγ regulation. The potent role of ZFP423 in the regulation of PPARγ raises questions about mechanisms controlling the expression of this transcription factor and the contribution of this pathway to PPARγ induction in fat depots.

1.4 mTOR Regulation: obesity vs. thermogenesis

Apart from important regulatory transcriptional events during adipogenesis, multiple signaling pathways are involved in the development of adipose tissue. mTORC1 is a critical signaling pathway that regulates translation of 15% of cellular proteins, and decreases longevity in mammals [24,74,75]. mTOR, a serine/threonine kinase, exists in two complexes: mTOR Complex1 (mTORC1), and mTOR Complex2 (mTORC2). mTORC1 contains raptor, MTOR associated protein LST8 homolog (mLST8), and prolin-rich Akt substrate of 40kilodaltons (PRAS40). mTORC1 is inhibited by the immunosuppressant and anticancer drug rapamycin [74]. mTORC1 regulates protein synthesis through the hyperphosphorylation of eukaryotic translation initiation factor 4E binding proteins (4E-BPs), a repressor of mRNA translation, and through the phosphorylation and activation of p70 ribosomal S6 kinase S6K1 [76,77] (Figure 1.4).
mTORC1, positively regulates lipid synthesis, cell cycle progression, and negatively regulates mitochondrial biogenesis, thermogenesis, autophagy, apoptosis and mRNA degradation. mTORC1 phosphorylates downstream targets S6K1 and 4EBP1, thereby regulating translation and cell growth [76,77]. This regulation is not limited to adipocytes.

4-EBPs repress translation by binding to eIF4E. Hyperphosphorylation of 4-EBPs weakens the interaction between 4-EBPs and eIF4E, resulting in dissociation of the 4-EBPs from eIF4E and initiating translation [77]. S6K1-deficient mice are glucose tolerant and are protected against obesity, which further suggests participation of mTOR in insulin signaling and fat formation [75,76]. mTORC2 consists of rictor, mammalian stress-activated protein kinase-interacting protein 1 (mSIN1), mLST8. mTORC2, is insensitive to rapamycin. It phosphorylates protein kinase B (PKB, also known as Akt).
Both mTOR complexes are stimulated by mitogens, but only mTORC1 is under the control of nutrient and energy inputs and its upregulation leads to obesity and insulin resistance [28,29]. Rapamycin inhibits phosphorylation of the two downstream effectors of mTORC1 (S6K1, 4EBP1) \textit{in vivo}. Thus, S6K1 or 4E-BP1 phosphorylation is representative for mTOR activation. Rapamycin, reduces adipocyte clonal expansion and differentiation in human and rodent cells [29, 74]. Rapamycin also decreases expression (mRNA) of 2 late-phase adipocyte differentiation transcription factors, PPARγ and C/EBPα [28]. Adipose tissue specific deletion of raptor, a regulatory protein in mTORC1 complex, is sufficient to prevent high-fat diet induced obesity in mice through thermogenic remodeling of white fat [30,207]. Much evidence indicates that mTORC1, the rapamycin-sensitive complex, controls adipogenesis by activating PPARγ [28,79], however, identification of metabolites regulating mTORC1 and PPARγ in fat depots is yet to be explored.

1.5 \textbf{Enzymes involved in vitamin A metabolism}

Retinol is a major substrate for NAD+ or NADP+-dependent ADH and RDH enzymes oxidizing this metabolite to Rald, which is subsequently converted to RA by ALDH enzymes [11,12,13]. ADH1 (isozymes A, B1, B2, C1), ADH2, ADH3, ADH4/7, and approximately 17 different SDR/RDH enzymes are capable of oxidizing retinol [80, 81,87, 88, 89, 91, 92, 93, 209-217].
Alcohol dehydrogenase (ADH) family of enzymes:

These enzymes (ADH1, ADH3, ADH7) are members of the medium chain dehydrogenase family. They are involved in the oxidation of retinol to Rald in the presence of nicotinamide adenine dinucleotide (NAD) [9,10]. These cytosolic enzymes are also capable of oxidizing ethanol to acetaldehyde and can utilize other substrates.

ADH1 is highly expressed in the liver, kidney, intestine [141] and also adipose tissue (our unpublished data). ADH1 is known to be highly efficient in oxidizing free retinol (high retinol activity) and works in tandem with ALDH1A1 enzyme to produce RA [9]. Through studies in Adh1<sup>−/−</sup>tm1Gdu mice, it is known that ADH1 can clear excess retinol and thus protect from vitamin A toxicity. Adh1<sup>−/−</sup>tm1Gdu mice had increased retinyl ester stores (compared to WT mice), suggesting the existence of some free retinol which is continuously being oxidized by ADH1 for retinol turnover [9]. ADH3 is ubiquitously expressed. ADH3 has less retinol oxidizing activity compared to ADH1 and ADH4. Adh3<sup>−/−</sup>tm1Gdu mice have a growth deficiency which can be rescued with retinol supplementation [10]. ADH4 is expressed at higher levels in the stomach, and skin, but is not expressed in the liver. Adh4<sup>−/−</sup>tm1Gdu mice do not exhibit any growth defects when maintained on a normal chow diet but experience 100% postnatal lethality on a vitamin A deficient diet. ADH4 appears to promote survival, whereas ADH3 probably participates in the prevention of retinol toxicity and vitamin A deficiency [84].
Retinol dehydrogenases:

Retinol dehydrogenases are members of the short chain retinol dehydrogenases/reductase (SDR/RDH) family which can also oxidize retinol into Rald. Retinol to Rald step is reversible, which means Rald can be converted back to retinol by some RDHs such as RDH12 or some aldo-keto reductases such as AKR1B10 [85,86]. To date, 17 different short chain dehydrogenases have been identified, among which Rdh1, Rdh10 and DHRS9 have been known to be significant ones involved in retinoic acid biosynthesis [87,88,89]. SDR enzymes are membrane bound and not inhibited by ADH inhibitors, such as ethanol, 4-methylpyrazole, and citral [82].

Rdh1<sup>-/-tm1.1jln</sup> mouse model gives us information about its role in retinoid homeostasis [90]. RDH1 is known to be expressed in mouse embryo as early as e7.5. It is highly expressed in hepatic stellate cells. The knock out mouse develop normal, however, Rdh1<sup>-/-tm1.1jln</sup> mice gain weight on a vitamin A deficient diet (due to increase in fat mass) with increased retinyl ester levels in the kidney and liver. Cyp26A1 was significantly downregulated, suggesting an autocompensatory mechanism in the absence of RDH1 (since RDH1 oxidizes retinol to Rald) [90].

Rdh10 was first cloned and evaluated in retinal epithelial cells [88]. Like RDH1, Rdh10<sup>tm1ics</sup> mutant mice experience embryonic lethality, yet still produces some RA, which suggests that RDH10 is essential for embryogenesis, however in its absence, other enzymes can produce RA [91]. Dehydrogenase reductase SDR family member 9 (DHRS9), like Rdh1, can utilize both free and CRBP1 bound retinol and oxidize to Rald. Its function in RA generation was identified in Zebra fish where it is required for normal
gut development and differentiation [218]. DHRS9 is highly expressed in the epidermis, hair follicle and sebaceous gland, where it is the key enzyme for RA synthesis [92, 93]. It has not been knocked out in mice to study its effect in RA biogenesis in vivo.

Multiple RDH/SDR enzymes are known to regulate the visual cycle, especially RDH5, RDH10 and RDH11 convert 11 cis-retinol to 11 cis-retinal in the retinal pigment epithelium, whereas RDH8, RDH12, and retSDR1 reduce all-trans retinal [94, 95].

Despite known functions of these enzymes in various tissues, roles of Rald-generating ADH and SDR/RDH enzymes in WAT are largely unexplored.

**Retinol Saturase:**

Retinol saturase was identified and characterized by Palczewski lab as an enzyme converting retinol to (R)-all-trans-13,14-dihydroretinol (13,14-dihydroretinol) 13,14-dihydroretinol [96]. However, its role in adipose tissue is not clear due to opposite in vitro and in vivo findings [97, 98].

**Aldehyde dehydrogenase family of enzymes:**

RA is produced predominantly by a family of cytosolic, NADPH-dependent aldehyde dehydrogenases, also known as retinaldehyde dehydrogenases, that comprises of three members ALDH1A1, ALDH1A2, and ALDH1A3 [11]. All three enzymes are known to participate in embryonic development. Compared with other ALDH1 enzymes,
ALDH1A1 has many unique characteristics. It is expressed in many tissues where it generates RA working downstream of ADH1. *In vitro*, ALDH1A1 can also use other substrates [99, 222]. It is known as a stem cell marker and is the only aldehyde dehydrogenase expressed in the pituitary gland of adult rat where its expression is inhibited by estrogen [100,101,102]. ALDH1A1 is also known as a protein phosphorylated on tyrosine in nasopharyngeal tissues, indicating that ALDH1A1, in addition to transcriptional regulation, may undergo post-translational regulation by all-trans RA [103]. The role of ALDH1A1 in fat formation was suggested based on phenotype in *Aldh1a1*^−/−^tm1.Gdu mice fed a high-fat diet. These mice were resistant to high-fat diet induced obesity and have significantly decreased fat mass [104,105].

ALDH1A2 and A3 are much more specific in using Rald as a substrate [106]. They have distinct expression patterns and function in specific tissues. For example, ALDH1A2 is critical for RA production in the embryonic mesoderm [107]. ALDH1A3 is critical for RA generation in the embryonic eye as well as regulation of insulin and glucagon from pancreatic islets in diabetic mice [106,108]. *Aldh1a2*^−/−^tm1.Gdu mice die at e9.5 due to defects in heart development [12]. The embryonic lethality caused by ALDH1A2 deletion shows its importance in embryonic development. *Aldh1a2* promoter has an estrogen receptor response element and, therefore, expect to respond to estrogen stimulation [174].

*Aldh1a3*^−/−^tm1.Gdu mice suffer from congenital abnormalities, which can be rescued with retinoic acid supplementation during pregnancy [109]. Such findings emphasize the importance of ALDH1A3 in RA generation. *Aldh1a3* expression is induced by RA in
keratinocytes [110]. Thus, all the three equally important Rald-generating enzymes, in concert, participate in RA generation and also exert distinct functions in specific tissues.

RA can also be produced by NADP(H)-dependent cytosolic aldo-keto reductases (AKR), such as AKR1B3 (aldose reductase), AKR1B7, AKR1B8 (fibroblast-growth factor 1-regulated protein), and AKR1B9 (Chinese hamster ovary reductase) from all-trans Rald in vitro [111]. Among the AKR1B subfamily, AKRB3, in particular, is expressed in adipocytes and suppresses adipogenesis, however, this effect was mediated by prostaglandin F2α also synthesized by this enzyme [112]. The role of this enzyme in RA synthesis in WAT has not been reported.

1.6 Fat-depot-specific aspects of vitamin A metabolism

Although liver is the primary storage and utilization tissue of vitamin A in mammals, several studies have shown that adipose tissue plays an active role in the metabolism and homeostasis of vitamin A. Retinol bound to retinol-binding protein (holo-RBP4) in the circulation is taken up by membrane receptor STRA6 [113]. Comparison of RBP4 expression in 66 lean and 130 obese patients revealed higher expression of RBP4 in visceral than in subcutaneous fat [114]. Although some subsequent studies did not find such associations in other patient populations [115], these studies draw attention to possible depot-specific processing of vitamin A.

In the postprandial period in addition to holo RBP4, retinyl ester is transported to adipose tissue in remnant chylomicrons. Lipoprotein lipase present on endothelial cells
facilitates the uptake of the postprandial retinoids into adipose tissue [116] (Figure 1.5). Beta-carotene, another source of vitamin A, is transported in lipoproteins and taken up by SR-B1 receptor [119]. Beta-carotene 15,15'-monooxygenase 1 (BCMO1) in adipose tissue cleaves β-carotene to two molecules of Rald [117, 118].

![Retinoid Transport & Metabolism](image)

**Figure 1.5. Retinoid Transport & Metabolism [RY]**

Retinol bound to retinol-binding protein (RBP4) enters cells by the receptor STRA6, and LRAT converts retinol to retinyl esters for storage. Retinyl esters in chylomicron remnants are hydrolyzed by lipoprotein lipase (LPL) to retinol. In adipose tissue, hormone sensitive lipase (HSL) is the major retinyl ester hydrolase. Retinol is reversibly oxidized to Rald by either alcohol dehydrogenase (ADH) or retinol dehydrogenase (RDH). Rald can be transported intracellularly by CRBPs to the nucleus where it suppresses PPARγ target gene transcription. Rald is irreversibly oxidized to RA by aldehyde dehydrogenase1 (ALDH1) family of enzymes. The produced RA can act in paracrine (embryonic development) or autocrine (beta pancreatic cells) manner. Cytochrome P450 (CYP26) initiate degradative oxidation of RA. Cellular-RA binding protein-2 (CRABP2) t facilitates RA transport to the nucleus where RA binds to RA receptor (RAR). RA could be delivered into cells bound to lipocalins and albumin. β-carotene, transported in LDL, HDL, is taken up by SR-B1.
Retinol in the blood is taken up by RBP4 and transported into adipose tissue through membrane bound receptor STRA6 [219]. Lecithin-retinol acyltransferase LRAT is a key enzyme responsible for retinol esterification (~75%) in adipose tissue [120,121,122,124]. On a vitamin A deficient diet, adipose stores are depleted of retinol in \( LRAT^{-/-} \) mice indicating that retinol uptake through RBP4/STRA6 pathway depends primarily on LRAT in adipose tissue [122, 123]. Currently, it is not known what enzyme is responsible for 25% of retinyl esters in adipose tissue; however, on the chow diet, WAT showed an increase in retinyl esters in \( LRAT^{-/-} \) mice [124], which suggests the presence of other esterases in adipose tissue. Literature suggests the existence of acyl CoA: retinol acyltransferase (ARAT) enzymes in tissues, which use fatty acyl-CoA and unbound retinol as substrates through \textit{in vitro} studies [125], however the molecular characterization of ARAT has been unsuccessful. In 2005, Yen et al. demonstrated that mouse DGAT1 possesses ARAT activity \textit{in vitro} [126] and it is expressed in the small intestine, adipose tissue and mammary glands [127]. However, DGAT1 does not catalyze retinyl ester synthesis in adipose tissue \textit{in vivo} [128]. Recently, DGAT1 is found to be an important ARAT in murine skin [129]. Hormone sensitive lipase HSL null mice (\( HSL^{-/-} \)) revealed retinol hydrolase activity of this lipase and has increased retinyl ester levels and decreased levels of retinol, Rald and RA. Reduced white fat mass observed in HSL null mice on a high fat diet could partially be restored by RA administration, which demonstrates its importance in WAT development [130].

\( STRA6 \) expression increases in the late phase (\( \geq 5 \) days) of adipogenesis [131]. The binding of RBP-retinol to STRA6 triggers phosphorylation of STRA6 by JAK2, which
then recruits and activates STAT5. This activation of STAT5 upregulates expression of SOCS3 and PPARγ in mature adipocytes [131]. In the future, adipose specific RBP4 and/or STRA6 knockout mouse models will help characterize functions of these proteins in WAT \textit{in vivo}.

In adipose tissue, retinol is oxidized to Rald by alcohol dehydrogenase family of enzymes. Rald can be potentially transported intracellularly by CRBP1, because it has similar binding affinity to this protein as retinol [104]. White adipose tissue expresses CRBPI, II and III [124]. Rald is further oxidized to RA by aldehyde dehydrogenase family of enzymes. RA could be delivered into cells bound to beta-lactoglobulin and lipocalin-type prostaglandin D synthase [148].

Further studies are needed to determine concentrations of vitamin A (retinol and retinyl esters) and its metabolites, Rald and RA, in human and rodent visceral and subcutaneous adipose tissues. In an early study in rats, it was shown that visceral and subcutaneous adipose depots have comparable amounts of retinol, i.e. 6.4 and 6.9 µg retinol/g tissue [132]. There are substantial differences between hepatic and adipose tissue stores. In the liver, retinol is re-esterified to retinyl ester by LRAT and stored in hepatic stellate cells, the major storage site for vitamin A in the body. Hepatic vitamin A accumulation is in the form of retinyl esters. Adipose tissues mostly store the retinol form (75%), while the esterified form accounts for only 25% of total retinol stores in the lipid droplets [132]. The estimated vitamin A content (both retinol and retinyl esters) in adipose tissues is 15-20% of the total body retinoid stores in rodents [132]. Even less is known about concentrations of retinol metabolites, retinol and RA, in different depots,
although these compounds have been detected in adipose tissue [133] and their concentrations were dependent on retinyl hydrolase activity of hormone sensitive lipase [130, 134] and Aldh1a1 [104].

Both adipocytes and fat tissue express Rald-generating Adh1 and RA-generating Aldh1a1 enzymes [104]. Rald, the intermediate metabolite in the vitamin A metabolic pathway, is a transcriptional repressor of PPARγ [104]. RA is produced solely from Rald by the cytosolic Aldh1 family of enzymes. Thereby, Aldh1 enzymes control concentrations of both Rald and RA [13]. All three Aldh1 enzymes – Aldh1A1, A2, A3 have redundant function with respect to Rald oxidation to RA. Specific pattern of expression of Aldh1 enzymes defines amount of Rald and RA in adipose tissue and could potentially influence fat formation in specific depots.

1.7 Summary/Significance

My research will aid in understanding the role of vitamin A metabolizing enzymes and their metabolites, RA and Rald, in the regulation of fundamental transcriptional and translational pathways that diverge differentiation of white adipocytes into subcutaneous and visceral tissue in males and females.

Significance: Understanding of these vitamin A metabolizing pathways and the therapeutic modulation of these pathways in the future will help to prevent and treat visceral obesity.
CHAPTER 2

Concerted Action of Aldehyde Dehydrogenases Influences Depot-Specific Fat Formation

Published in Molecular Endocrinology, 2011.

2.1 Introduction

Vitamin A and its metabolites regulate embryogenesis and differentiation of various cells in adults, including adipocytes [13, 18]. These functions are mediated by the three major vitamin A metabolites: retinol, Rald, and retinoic acid (RA) [135,136]. These metabolites are ligands for nuclear RA receptors (RAR), and exert specific transcriptional effects [13,18, 136, 137]. Among retinoids, RA has the highest affinity for binding to RAR [138]. Binding of RA activates RAR heterodimerization with retinoid X receptor (RXR), releases transcriptional co-repressors, and recruits transcriptional co-activators [135]. Heterodimers of RAR with RXR bind to a RA response element (RARE) and regulate transcription of target genes [135]. During adipogenesis, RA stimulation represses peroxisome proliferator-activated receptor (PPARγ) expression and inhibits adipogenesis by an RAR-dependent down-regulation of the transcription factors C/EBP [18, 139]. In contrast, in preadipocytes expressing PPARγ, RA stimulation enhances adipogenesis [18, 139]. Rald can act as a PPARγ inhibitor and suppress adipogenesis
throughout differentiation [104]. Although the role of retinoids in transcriptional control of adipogenesis is widely recognized, the mechanisms of Rald and RA production during adipogenesis are far less clear.

The expression of vitamin A-metabolizing enzymes is under spatiotemporal control by endo-, para-, and autocrine hormones as well as signaling and transcription factors and varies among different tissues [13]. Both adipocytes and fat tissue express Rald-generating ADH1 and RA-generating ALDH1A1 (Raldh1) enzymes [104]. In the absence of Aldh1a1, both adipogenesis in vitro and diet-induced fat formation in vivo are markedly impaired [104]. These findings in Aldh1a1−/− adipocytes and mice were unforeseen, given the redundant function of the other members of the ALDH1 family (ALDH1A2 and A3) with respect to Rald oxidation and RA production. Compared with other enzymes in the Aldh1 family, Aldh1a1 has many unique characteristics. Aldh1a1 is expressed in abundance in many tissues, where it has been shown to generate RA in vivo [140,141]; however, this enzyme can potentially use other aldehyde substrates [99]. A combination of specific and redundant functions among ALDH1 enzymes could potentially influence adipogenesis and fat formation. Here, we provide evidence that RA generation for adipogenesis is supported predominantly by the ALDH1A1 enzyme, whereas in vivo, different fat formation in visceral and subcutaneous depots in mice and humans result in part from different expression and concerted action of all ALDH1 enzymes.
2.2 Materials & Methods

Reagents: We purchased reagents from Sigma-Aldrich (St. Louis, MO) and cell culture media from Invitrogen (Grand Island, NY) unless otherwise indicated; Cignal Lenti inducible RARE reporter vectors were purchased from SA Biosciences/Qiagen (Valencia, CA).

Human subjects: mRNA was isolated from fat tissue, which was obtained from four healthy women who underwent surgery as kidney donors and had given informed consent. The protocol was approved by the Mayo Clinic Institutional Review Board for Human Research. Subjects were 41 ± 2 yrs of age. The subjects’ mean body mass index (BMI) was 30.3± 0.4 kg/m².

Animal studies and MRI: Generation and characterization of Aldh1a1−/−tm1Gdu mice, including metabolic responses in high-fat fed mice, have been previously described [13,104]. All experimental protocols were approved by the Institutional Animal Care and Use Committee. Age- (13 month old) and sex-matched Aldh1a1−/− (9 females) and C57BL/6J (Wild type, 10 females) mice were fed regular chow (Research Diets Inc., New Brunswick, NJ). Water and food was ad libitum. Weight was measured monthly. The absence of glucose intolerance in both genotypes fed regular chow was confirmed by glucose tolerance testing (data not shown). Glucose tolerance tests were performed in overnight fasted mice by intraperitoneal injection of a single 25% dextrose injection (0.004 ml/g body weight) using a glucometer for measurements (Accu-Chek Advantage, Roche or OneTouch Ultra, LifeScan). Body fat distribution was measured by magnetic
resonance imaging (MRI) using 9.4 T Bruker BioSpin wide-bore scanner. Multi Slice Multi Echo (MSME) sequence (repetition time 1400 ms; echo time 12 ms; flip angle 180°; matrix 128x128; 4 averages) was used to acquire a total of 80 1-mm-thick images per mouse scanning from the tail to the head. ParaVision 4.0 software was used for MR acquisition and reconstruction. All image processing and analysis was performed in OsiriX software (The Osirix Foundation, Geneva, Switzerland). Representative coronal whole-body images were obtained using maximum intensity projection technique (MIP). Visceral (perigonadal) and subcutaneous fat pads were dissected and analyzed for mRNA expression.

**Cell culture:** Murine 3T3-L1 preadipocytes were cultured, maintained, and differentiated using standard procedures (7, 16). Preadipocyte cell lines were derived from embryonic fibroblasts of WT (F<sub>WT</sub>) and Aldh1a1<sup>−/−</sup> (F<sub>Aldh1a1KO</sub>) as previously described (99, 96). In all preadipocyte cultures, differentiation was induced (day 0) with a standard differentiation mixture of 3-isobutyl-1-methylxanthine (0.5mM), dexamethasone (1μM), and insulin (1.7μM) in DMEM containing 10% fetal bovine serum (FBS). For differentiation into adipocytes, cells were maintained for 7 days in DMEM medium containing 10% FBS and insulin (1.7μM), which was replaced every 48h.

**Analysis of mRNA:** mRNA was isolated from adipose tissue and adipocyte cultures according to the manufacturer’s instructions (Qiagen, Valencia, CA). For semi-quantitative analysis of expression, cDNA was prepared from purified mRNA and analyzed using 7900HT Fast Real-Time PCR System and TaqMan fluorogenic detection system (Applied Biosystems, Carlsbad, CA). Validated primers were also purchased from
Applied Biosystems. Comparative real-time PCR was performed in triplicate, including no-template controls. The mRNA expression of the genes of interest was compared to 18S expression levels. Occasionally, we used TATA-box binding protein as a control in genes expressing at low levels. Relative expression was calculated using the comparative delta Ct method. All data shown in the Figures is based on delta Ct method normalized to 18S.

Transfection studies:

1) Stably transfected RARE-reporter clones.

We derived stably transfected 3T3-L1, WT (A\textsubscript{WT}), and Aldh1a1\textsuperscript{+/−} (A\textsubscript{Aldh1a1KO}) cell lines according to the manufacturer’s instructions (SA Bioscience, Valencia, CA). Briefly, after reaching 70% confluence, cells were transfected with Cignal Lenti RARE-LUC reporter suspension (25 MOI/10\textsuperscript{4} cells) in the presence of Polybrene (Millipore, Billerica, MA) transduction reagent in serum-free MEM-medium. After 3hrs, cells were supplemented with 10% heat inactivated calf serum. Stable clones were selected and derived from the single cells selected with puromycin (0.75-1.5mg/mL, Invitrogen, Grand Islan,NY). We measured luciferase activity using a dual-luciferase reporter assay (Promega, Madison, WI).

2) Clones stably transfected with shRNA.

3T3-L1 preadipocytes were transfected with shRNA sequences corresponding to nonspecific (scrambled) siRNA, Aldh1a2 siRNA, or Aldh1a3 siRNA gene silencer sequences (Santa Cruz, Santa Cruz, CA). shRNA lentiviral particles contained three to
five expression constructs each, encoding target-specific 19-25 nt (plus hairpin) shRNA to knockdown gene expression after transduction. Transfection and selection of specific clones were performed according to the manufacturer’s instructions. Briefly, preadipocytes were plated at 85% confluence in 6 well plates. Three hours later, plating medium was replaced with OptiMEM, and cells were transfected with a transduction mixture (200uL/well). Transduction mixture was comprised of 10% viral particles (4x10^8 infectious units of virus) and 3% Polybrene (Millipore, Billerica, CA) solutions in Opti-MEM, which were incubated for 15 min prior to the addition to cells. 3T3-L1 preadipocytes were incubated overnight, and selected with puromycin (1.0 mg/mL, Invitrogen, Grand Island, NY). Clones were derived from a single transfected cell tested for presence of all Aldh1 genes before and after differentiation using a TaqMan assay.

3) *Transiently transfected Aldh1a1−/− (Aldh1a1KO) preadipocytes.*

RARE_{AldhA1KO} preadipocytes were transfected with human full-length Aldh1a1 and Aldh1a3 vectors (Origene, Rockville, MD). Cells were transfected with FugeneHD (Roche, South Sanfrancisco, CA) in OptiMEM medium according to manufacturer instructions. After 12 h, transfection medium was replaced with growth medium. Next day, the cells were differentiated by a standard procedure for 6 days (143). Differentiated cells were then harvested and assayed for luciferase activity according to Promega manufacturers’ instructions. Another set of similarly treated cells was used for mRNA expression analysis.
**Oil-Red-O Staining:** This assay was performed in all in vitro adipocyte differentiation experiments according to Thermo Scientific protocol.

**Statistical analysis:** Data were shown as mean±SD or mean±SE of experiments which were performed at least in triplicate. Group comparisons were performed using Mann Whitney U test unless otherwise indicated, and correlations were examined by Pearson test.

### 2.3 Results

*Adh1a1 is the major enzyme expressed during adipocyte differentiation in vitro.*

Vitamin A metabolism is dependent on a number of vitamin A-metabolizing enzymes (Fig. 1.1). We analyzed expression of RA-generating enzymes in non-differentiated and differentiated 3T3-L1 fibroblasts, a classic model for adipogenesis (Fig.2.1). In adipose tissue, short chain dehydrogenases are negligibly expressed (unpublished data from our lab). Our lab differentiated 3T3-L1 fibroblasts and found high expression of Adh1 (306%), Adh3 (143%) in whole cell lysates, while Adh4 and Rdh1 and Rdh10 (59%) were expressed at very low levels (differentiated vs. non-differentiated). Such finding led us to omit SDRs from our research focus. Adipose tissue is the second largest storage organ of vitamin A after the liver and also involved in RA and Rald production [208]. However, SDRs, enzyme family appears to play only minor role in retinol oxidation in adipose tissue, in contrast to their major role in these processes in the liver.
Among efficient RA-generating enzymes, *Aldh1al* was the only enzyme significantly induced during 3T3-L1 differentiation (201%, *P*<0.035); moreover, *Aldh1al* had the highest expression among Aldh1 enzymes (Fig. 2.1A). *Aldh1al* expression increased proportionally to adipocyte differentiation as indicated by a significant correlation between *Fabp4* and *Aldh1al* expression (*P* < 0.001, Pearson correlation; Fig. 2.1A, inset). Stimulation of mature adipocytes (differentiated for 6 d) with RA markedly inhibited *Aldh1al* expression in a concentration-dependent manner, whereas expression of *Aldh1a2* and *a3* was not altered (Fig. 2.1B). This experiment suggests a potential feedback mechanism, primarily for *Aldh1al* in response to administration of high RA concentrations in adipocytes. Since expression profiles suggested a possible *Aldh1al* role in adipogenesis, our next studies dissected the role of this enzyme in RA production and adipogenesis and analyzed possible redundancies in functions of other members of the *Aldh1* gene family *in vitro*.

**Aldh1al** deficiency impairs RA production and adipogenesis *in vitro*.

To elucidate time-dependent endogenous production of nuclear receptor ligands in adipogenesis, previous studies employed sensitive and specific reporter assays in permanently transfected 3T3-L1 fibroblasts [141].

Using a similar approach, we transfected the RARE luciferase reporter in wild-type (WT) and *Aldh1al*−/− fibroblasts to create RARE*WT* and RARE*Aldh1alKO* cell lines, respectively. The RARE activation allows us to examine whether *Aldh1al* is functional (capable of producing endogenous RA). Adipogenesis in these RARE*WT* and
RARE\textsubscript{Aldh1a1KO} cell lines was induced by a standard protocol [104,143]. Kinetics of RARE activation during adipogenesis are shown in Fig. 2.2A. Differentiation in RARE\textsubscript{WT} fibroblasts was accompanied by RARE activation that reached a maximum (384\%) at day 7 of differentiation compared with non-differentiated RARE\textsubscript{WT} fibroblasts. The RARE activation suggested that endogenous RA was generated during adipogenesis. In RARE\textsubscript{Aldh1a1KO} fibroblasts, differentiation was accompanied by a minor (165\%) increase in RARE activation compared with non-differentiated RARE\textsubscript{Aldh1a1KO} fibroblasts. At all time points, RARE activation by endogenous RA generation was significantly lower in RARE\textsubscript{Aldh1a1KO} than in RARE\textsubscript{WT} fibroblasts. Importantly, stimulation of RARE\textsubscript{Aldh1a1KO} and RARE\textsubscript{WT} fibroblasts with RA or TTNPB (4-((E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)benzoic acid), a synthetic RAR ligand, yielded similar activation in these cell lines (Fig. 2.2A, inset). Therefore, decreased RARE activation in RARE\textsubscript{Aldh1a1KO} fibroblasts might result from impaired endogenous RA production, consistent with the loss of Aldh1a1, the predominantly expressed enzyme of the ALDH1 family. RARE\textsubscript{Aldh1a1KO} fibroblasts had impaired adipogenesis, resulting in the markedly lower expression of \textit{PPAR\textgamma} than in RARE\textsubscript{WT} fibroblasts (Fig. 2.2B).

To elucidate whether the changes in PPAR\textgamma were attributed to RA generation, we performed rescue experiments by overexpression of human \textit{Aldh1a1} and \textit{Aldh1a3} enzymes or their combination. We took advantage of the similar catalytic function of human \textit{Aldh1} enzymes in RA generation combined with their distinct sequence to distinguish them from the endogenous mouse Aldh1 enzymes. We did not overexpress \textit{Aldh1a2} because this gene was expressed at negligible levels in both non-differentiated
and differentiated 3T3-L1 fibroblasts. Overexpression of Aldh1a1, Aldh1a3, or both led to a significant increase in RARE-luciferase activity (from 240 to 260% compared with control) in Aldh1a1−/− fibroblasts (Fig. 2.2C).

Overexpression of Aldh1a1, Aldh1a3, or both resulted in significantly increased PPARγ expression that was correlated with the expression levels of all Aldh1 enzymes and was not related to the expression of a specific member of this family (Fig. 2.2D).

To elucidate whether Aldh1a1 effects were dependent on RA production, we stimulated Aldh1a1−/− fibroblasts with low concentrations of RA (10–100 nM) during differentiation. In agreement with the role of Aldh1a1 in RA production, RA stimulation helped to rescue, in part, PPARγ expression (Fig. 2.2E) and differentiation (Fig. 2.2, F and G) in Aldh1a1−/− fibroblasts after 8 d of differentiation. The increase in differentiation was characterized by an increased ratio of adipogenesis/preadipogenesis markers Fabp4/Pref-1 (Fig. 2.2F) and formation of adipocytes with large lipid droplets (Fig. 2.2G) seen in Aldh1a1−/− fibroblasts stimulated with RA. Both administered RA or RA produced by overexpression of Aldh1 enzymes can partially regain PPARγ expression; thus, the impact of Aldh1a1 on adipogenesis is linked, at least in part, to its dominant role in RA production during adipogenesis.
Decreased expression of *Aldh1a3* influences *Pref1* and visfatin expression.

Consistent with the expression profile, the impact of ALDH1A3 on adipogenesis in 3T3-L1 fibroblasts was minor (Fig. 2.3).

We studied the effects of the *Aldh1a3* expression profile in two representative cell lines stably transfected with scrambled (3T3-L1_shCo) or *Aldh1a3* short hairpin RNA (shRNA) containing control or specific shRNA. A scrambled shRNA control cell line principally expressed *Aldh1* enzymes in a similar fashion as non-transfected 3T3-L1 fibroblasts (data not shown). As expected, stable transfection of 3T3-L1 fibroblasts with *Aldh1a3* shRNA (3T3-L1_sh3a3) decreased *Aldh1a3* expression that was significantly different in differentiated 3T3-L1_sh3a3 vs. 3T3-L1_shCo fibroblasts, but *Aldh1a1* remained the major ALDH1 enzyme expressed in all differentiated 3T3-L1 clones. *Aldh1a2* expression was decreased in non-differentiated 3T3-L1_sh3a3 and remained the same in differentiated 3T3-L1_sh3a3 compared with control cells (data not shown).

The small decrease in *Aldh1a3* expression in 3T3-L1_sh3a3 vs. 3T3-L1_shCo did not influence expression of *PPARγ* or other adipogenesis markers, including *Fabp4*, *C/EBPa*, *and C/EBPβ*, and adiponectin (Fig. 2.3, A–D, and data not shown). In contrast, the expression of preadipocyte marker *Pref1* and visceral adipocyte marker visfatin was significantly increased in differentiated 3T3-L1_sh3a3 vs. 3T3-L1_shCo fibroblasts (Fig. 2.3, E and F), suggesting an ALDH1A3 role in the regulation of preadipocyte responses and some depot-specific adipokine production. Importantly, the effects on *PPARγ* expression and overall adipogenesis were dependent on RA production and could be recovered to a significant extent by expression of any member of Aldh1 family.
Aldh1 enzymes and RA regulate PPARγ expression by mechanisms involving zinc-finger protein 423 (ZFP423) expression.

Two major transcriptional pathways, C/EBP and ZFP423, have been shown to induce PPARγ expression during adipogenesis [18, 23], however, direct regulation of PPARγ by retinoids is unknown [137]. In our studies on rescuing PPARγ expression and adipogenesis in Aldh1a1−/− cells with Aldh1a1 and/or Aldh1a3 expression (Fig. 2.2D), we found a significant correlation between Aldh1 and ZFP423 expression levels, whereas expression of transcription factors C/EBPβ (Fig. 2.4A) and C/EBPa (data not shown, coefficient of determination of a linear regression, R^2 = 0.05, not significant) was not influenced by the expression of Aldh1 enzymes.

To strengthen the link between expression of Aldh1 enzymes and ZFP423, we analyzed expression in differentiated fibroblasts (adipocytes) that were stably transfected with shRNA. The WT adipocytes expressed WT levels of Aldh1 enzymes (high Aldh1a1/low Aldh1a2 and a3), the Aa1− adipocytes were deficient in Aldh1a1 and other Aldh1 enzymes. Aa1−a2+a3+ adipocytes were deficient in Aldh1a1 but expressed stable increased levels of Aldh1a2 and a3 enzymes (data not shown). ZFP423 expression was markedly decreased (99.9%) in Aa1− adipocytes compared with WT adipocytes (WT adipocytes, 100%) and recovered, partially, in Aa1−a2+a3+ adipocytes expressing Aldh1a2 and a3 enzymes (Fig. 2.4B). In these adipocytes, the expression of all Aldh1 also correlated with ZFP423 (R^2 = 0.75; P < 0.000, ANOVA). In contrast, C/EBPβ expression was increased in Aa1− adipocytes (158%) but not altered in Aa1−a2+a3+ (104%) compared with WT adipocytes. Similarly, C/EBPa expression was also marginally
influenced by Aldh1 expression (data not shown), in contrast to the expression of lipogenic genes Fabp4 and adiponectin (data not shown).

Finally, administration of RA during adipogenesis rescued both PPARγ (Fig. 2.2E) and ZFP423 expression in a dose-dependent manner in Aldh1a1−/− fibroblasts (Fig. 2.4C). C/EBPβ expression at these nanomolar RA concentrations was not changed. Together these experiments suggest that intrinsic RA production in adipocytes promotes adipogenesis through the expression of PPARγ that is mediated primarily by ZFP423.

**Fat depot-specific Aldh1 expression underlies the distinct expression of transcription factors.**

The difference in PPARγ expression is a distinguishing characteristic between sc and visceral adipose tissues [144], but mechanisms establishing this specific expression pattern are unknown.

We compared Aldh1 expression in sc and visceral tissue of seven C57BL/6J WT and six Aldh1a1−/−tm1Gdu females fed a regular chow diet (Fig. 2.5, A and B). Aldh1a1 was predominantly expressed in both sc (Fig. 2.5A) and visceral (Fig. 2.5B) fat compared with other Aldh1 enzymes, although the expression of Aldh1a2 and a3 was lower in visceral than in sc fat. In Aldh1a1−/−tm1Gdu mice, the Aldh1a3 enzyme was expressed in sc fat (Fig. 2.5A), whereas visceral fat did not express any Aldh1 enzymes (Fig. 2.5B). The decrease in RA-generating enzymes in sc fat of Aldh1a1−/−tm1Gdu mice did not influence ZFP423 expression levels (Fig. 2.5C); however, in visceral fat, a marked 70% decrease in
ZFP423 was observed in Aldh1a1<sup>−/−</sup>tm1Gdu compared with WT mice (Fig. 2.5D). In these fat tissues, ZFP423 expression correlated with the PPARγ expression (Fig. 2.5E). Accordingly, whereas PPARγ and Fabp4 underwent moderate or no change in expression in sc fat of Aldh1a1<sup>−/−</sup>tm1Gdu mice (Fig. 2.5F), expression of both PPARγ and Fabp4 was markedly (70%) reduced in visceral fat of these mice as compared with WT mice (Fig. 2.5G).

Noting the altered expression of PPARγ, the key transcriptional regulator of adipogenesis, in response to depot-specific RA production in Aldh1a1<sup>−/−</sup>tm1Gdu mice, we could also expect to change fat distribution. Aldh1a1<sup>−/−</sup>tm1Gdu mice weighed less than WT mice (Fig. 2.6A).

We employed magnetic resonance imaging (MRI) to analyze fat distribution in WT and Aldh1a1<sup>−/−</sup>tm1Gdu mice. Mice were scanned throughout their length, and the resulting 69 cross-sectional images were analyzed for fat content (Fig. 2.6B). We observed significantly higher fat accumulation in visceral regions between sections 18–31 in WT compared with Aldh1a1<sup>−/−</sup>tm1Gdu mice (Fig. 2.6, B–D). In the other regions, fat accumulation varied among different animals and was not significantly different between genotypes. Representative perigonadal and pericardial sections in WT and Aldh1a1<sup>−/−</sup>tm1Gdu mice are shown in Fig. 2.6, C and D. The fat reduction in these areas was in agreement with some loss in PPARγ expression seen in sc fat.
Fat depot-specific expression of Aldh1 enzymes in women.

To gain insight into the relevance of Aldh1 enzymes in human fat biology, we analyzed expression of Aldh1 enzymes in sc and visceral (omentumal) fat isolated from four healthy women (Fig. 2.7). In these women, Aldh1a1 mRNA expression was the most abundant among the Aldh1 enzymes in both sc and omental fat. However, Aldh1a2 and a3 expression were significantly higher in omental fat than in sc fat. Therefore, in both humans and rodents, Aldh1 enzyme expression was fat depot-specific, but Aldh1a2 and a3 expression was higher in human visceral fat than in sc fat.

2.4 Discussion

Although the etiology of visceral obesity is not fully understood, it is known that hormone and cytokine production in adipose tissue contributes to depot-specific fat formation [145-147]. Here we show that the Aldh1 family of enzymes generating RA is an essential autocrine pathway in adipogenesis that regulates depot-specific fat formation by transcriptional mechanisms involving PPARγ activation through ZFP423.

The importance of vitamin A metabolism in adipogenesis and fat formation was established based on evidence that retinoids suppress adipogenesis and fat formation [18, 137] as well as recent studies in animals deficient in single genes responsible for the generation of vitamin A metabolites [104, 90,148]. These studies reported somewhat paradoxical observations that RA supplementation [137,149] and deficient RA generation in Aldh1a1−/− mice both render resistance to diet-induced obesity [104]. Our studies here take into account that endogenous retinoid production is a result of the concerted action
of multiple enzyme families [13]. Families of enzymes can produce similar retinoids, e.g. the Aldh1 enzyme family members, Aldh1a1, a2, and a3, can all use Rald for RA generation [13]. Here we show that adipogenesis is accompanied by RA generation predominantly by Aldh1a1 (200% expression). Importantly, stimulation with RA specifically inhibits Aldh1a1 expression suggesting a probable negative feedback mechanism regulating Aldh1a1 expression and RA generation. Inhibition of Aldh1a1 expression by RA also provides a possible explanation of the paradoxical suppression of fat formation seen in previous studies with Aldh1a1\textsuperscript{-/-}tm1Gdu mice and obese rodents treated with RA [104,137,149]. The Aldh1a1-dependent inhibition of adipogenesis is mediated by physiological RA (10–100 nM) concentrations. At higher RA concentrations (10 µM) used in previous seminal studies on adipogenesis inhibition [18, 139], RA induced an array of effects [165].

We show that Aldh1 expression is necessary for endogenous RA production. We measured RA during adipogenesis in sensitive and specific RARE-reporter 3T3-L1, WT, and Aldh1a1\textsuperscript{-/-} cell lines. Earlier, an analogous approach was used to demonstrate endogenous PPAR\textgreek{y} ligand generation in the course of adipogenesis (16). It is generally accepted that RARE activation is due to increased RA generation, because RA has high specificity and affinity for RAR [13, 104,142], and RARE reporters are frequently used to assay spatiotemporal RA production in embryogenesis [13]. In our experiments, both WT and 3T3-L1 RARE preadipocytes exhibited increased RA generation during the course of adipogenesis, with maximum RA production at day 5 in WT and day 4 in 3T3-L1 adipocytes. The Aldh1a1 enzyme was responsible for approximately 70% of
generated RA, based on comparison of RARE\textsubscript{WT} and RARE\textsubscript{Aldh1a1KO} differentiated fibroblasts. The major Aldh1a1 contribution to RA production was consistent with predominant expression of this enzyme during differentiation. Nevertheless, ALDH1A1 and ALDH1A3 appear to be redundant with respect to RA generation; hence, overexpression of either of these enzymes can partially recover RA generation. This redundant function of ALDH1A1 and ALDH1A3 is also manifested \textit{in vivo} in sc fat of \textit{Aldh1a1}\textsuperscript{-/-}\textsuperscript{tm1Gdu} mice. In the absence of \textit{Aldh1a1} expression, the remaining Aldh1a3 partially supported sc fat formation, whereas the loss of all \textit{Aldh1} enzymes in visceral fat markedly impaired formation of this fat depot in \textit{Aldh1a1}\textsuperscript{-/-}\textsuperscript{tm1Gdu} mice. Specific expression of different \textit{Aldh1} enzymes in sc and visceral depots offers a therapeutic opportunity to regulate RA production in a tissue-specific fashion.

Our data provide evidence that production of RA by Aldh1a1 and other Aldh1 enzymes was causatively linked to regulation of \textit{PPAR}\gamma expression. \textit{Aldh1a1}\textsuperscript{-/-} fibroblasts expressed 29-fold reduced \textit{PPAR}\gamma levels compared with WT adipocytes and failed to differentiate. Overexpression of one or a combination of \textit{Aldh1} enzymes significantly increased \textit{PPAR}\gamma in differentiated \textit{Aldh1a1}\textsuperscript{-/-} fibroblasts. RA was similarly effective in rescuing \textit{PPAR}\gamma expression and adipogenesis in \textit{Aldh1a1}\textsuperscript{-/-} fibroblasts, arguing that RA is a central mediator of Aldh1-dependent responses in adipocytes. In the absence of Aldh1 enzymes, Rald spared from conversion to RA [104,141], can potentially contribute to the suppression of \textit{PPAR}\gamma activation as an inhibitor of \textit{PPAR}\gamma and its heterodimeric partner RXR [104]. Given the master role of PPAR\gamma in the transcriptional regulation of adipogenesis, Aldh1 enzymes could be considered as a key autocrine pathway in adipogenesis acting as a \textit{PPAR}\gamma switch.
The mechanisms regulating PPARγ expression in adipogenesis are not completely understood, although recent studies demonstrate the essentiality of transcription factor ZFP423 in this process [23]. It has been shown that ZFP423 overexpression is sufficient to rescue adipogenesis through PPARγ in NIH3T3 fibroblast lines that lack PPARγ and resist differentiation [23]. The means by which ZFP423 is regulated during adipogenesis remained unclear. Here we show that RA produced by Aldh1 enzymes is a critical mediator of ZFP423 expression. ZFP423 expression levels were reduced by 99% in Aldh1a1−/− compared with WT fibroblasts; however, ZFP423 expression could be rescued by either expression of any Aldh1 enzymes or RA stimulation. In consonance with these loss- and gain-of-function studies, ZFP423 and PPARγ expression correlated in vivo in visceral and sc fat of WT and Aldh1a1−/−tm1Gdu mice. Our results suggested that Aldh1 enzymes regulate PPARγ expression predominantly through ZFP423-dependent mechanisms. Given the importance of ZFP423 regulation in adipogenesis, more studies are needed in the future to elucidate mechanistic aspects of ZFP423 dependence on RA.

The ALDH1A1-dependent induction of adipogenesis is mediated by physiological RA (10nM) concentration. Previous studies have demonstrated that RA at higher concentrations (10 μM) blocks PPARγ expression and activity via RAR interaction with C/EBPa and/or C/EBPβ transcription factors [13,23]. It is plausible that at these concentrations, RA activates different pathways that inhibit differentiation independent of the induction of ZFP423 and PPARγ or before expression of these transcription factors. The potential mechanisms can involve transcriptional RA-dependent pathways, including RARγ autoregulation [150] and interaction with C/EBP as well as direct protein
modifications through retinoylation [151]. In our studies, expression of the Aldh1a1 enzyme was associated with a modest induction C/EBPα and reduction of C/EBPβ expression levels; however, these effects could not be rescued by other Aldh1 enzymes. These unique responses to Aldh1a1 may indicate specific roles of the ALDH1A1 enzyme in both adipogenesis and fat-depot differences. In addition to the common function of all ALDH1 enzymes of using Rald for RA generation, each member of the ALDH1 family may have a specific function. ALDH1A1 can use different aldehyde substrates, including lipid aldehydes and acetaldehydes [141]. Other ALDH1 enzymes also exerted distinct effects. For example, decreased Aldh1a3 expression, but not Aldh1a1 (data not shown), in vitro was associated with increased expression of visfatin. This adipokine mediates insulin responses mainly in visceral fat [220]. Therefore, ALDH1A3 can potentially regulate endocrine responses in visceral fat. More future studies would provide insight into the distinct roles of ALDH1A1, A2, or A3 in the regulation of cytokines specific for visceral or sc fat and the mechanisms of their actions.

These transcriptional effects in response to RA production by ALDH1 enzymes could have important implications for the regulation of fat depots in vivo. Many differences between visceral and sc depots stem from the different PPARγ expression in these tissues. Our studies in Aldh1a1⁻/⁻tm1Gdu mice shed light on the mechanisms that establish depot-specific transcription patterns. In visceral adipose tissue, ZFP423, PPARγ, and Fabp4 expression was decreased by 70% in Aldh1a1⁻/⁻tm1Gdu compared with WT mice. In sc fat, PPARγ was reduced by only 40%, and the expression of both inducing factor ZFP423 and gene Fabp4 was similar in Aldh1a1⁻/⁻tm1Gdu and WT mice.
These differences in $PPAR_\gamma$ expression in $Aldh1a1^{-/-}\text{tm1Gdu}$ mice could be attributed to $Aldh1a3$ expression in sc but not visceral fat. ALDH1A3 helped to maintain some RA production and was capable of inducing both $ZFP423$ and $PPAR_\gamma$ in vitro and could be expected to exert similar effects in sc fat. These data suggest that concerted $Aldh1$ function in vivo may include regulation of transcription in a fat depot-specific manner.

Our findings raise questions as to whether $Aldh1$ enzymes act in a species-specific manner. We found substantial differences between $Aldh1$ expression patterns in visceral and omental fat isolated from female mice and women, respectively. The visceral fat in mice and omental fat in humans may originate from different progenitors that may be predisposed to express dissimilar amounts and types of $Aldh1$. Another possibility could be differences in cholesterol/fat content in food consumed by humans and rodents. Cholesterol can directly regulate expression of $Aldh1a1$ and $a2$ enzymes through transcriptional activation of sterol regulatory element-binding protein 1c transcription factor [152]. In our studies, mice received a regular chow diet, but high-fat diets increase expression of $Aldh1a1$ and $a2$, at least in the liver [152]. Intriguingly, fat accumulation in specific depots, insulin resistance, lipolysis, lipogenesis, response to high-fat feeding, and other adipose tissue characteristics vary across different mouse strains and species [153]. It remains to be investigated whether different expression patterns of $Aldh1$ enzymes contribute to distinct characteristics of fat tissue depots.

Importantly, $Aldh1a1$, which regulates fat formation, was abundantly expressed in both human and mouse fat depots. ALDH1A1 was the major enzyme for RA production and regulation of adipogenesis by tandem expression of $ZFP423$ and $PPAR_\gamma$. Even so,
only concerted action of Aldh1 enzymes had diverged fat accumulation and transcription responses in visceral and sc fat depots. Visceral obesity in humans increases the risk of premature death related to the development of type 2 diabetes, certain cancers, and cardiovascular disease, whereas sc fat produces a variety of cytokines with anti-inflammatory and insulin-sensitizing properties [154,155]. Our data outline ALDH1-dependent mechanisms that preferentially reduce visceral fat and can potentially lead to the development of preventive measures and treatment of visceral obesity.
Figure 2.1. *Aldh1a1* expression is increased following adipogenesis in 3T3-L1 preadipocytes and inhibited by stimulation with exogenous RA [168].

(A) The expression of RA-generating enzymes in non-differentiated (Non-D) and differentiated 3T3-L1 adipocytes (6 days of differentiation). In all studies, mRNA expression was measured by TaqMan. Data were normalized using 18S as an endogenous control. Relative expression was calculated based on the comparative Ct method. *, *P*<0.05 (Mann Whitney U test, n=3, mean±SD). Insert shows a correlation between mRNA expression of *Fabp4* and *Aldh1a1* in differentiation experiments (n=9, Pearson correlation test, *P*<0.001).

(B) 3T3-L1 fibroblasts were differentiated for 6 days. These adipocytes were stimulated with the indicated RA concentrations for 12 hours, and mRNA was then isolated and analyzed for *Aldh1* expression by TaqMan as in (C). *, *P*<0.007 (Mann Whitney U test, n=3, mean±SD).
Figure 2.2. *Aldh1a1* and *Aldh1a3* generate RA and regulate *PPARγ* mRNA expression

(A) RARE<sub>WT</sub> (open circles) and RARE<sub>Aldh1a1-/-</sub> (RARE<sub>Aldh1a1KO</sub> black circles) fibroblast cell lines were derived from fibroblasts stably transfected with lentiviral RARE reporter vector. Differentiation was induced using a standard differentiation procedure. Cells were harvested every day and assayed for luciferase activity, while the other cell set was used to measure mRNA (A). Data are shown as a ratio of luciferase activity on the indicated day to that seen on day 0 in the same cell line. All data are shown as mean±SD (n=4). *, statistically different compared to day 0; #, statistically different between RARE<sub>WT</sub> and RARE<sub>Aldh1a1-/-</sub> on the same day of differentiation (Both: P<0.05, Mann Whitney U test).

Insert shows RARE<sub>WT</sub> (open bar) and RARE<sub>Aldh1a1-/-</sub> (RARE<sub>KO</sub> black bar) fibroblasts stimulated with all-trans-retinoic acid (0.1µM) for 3 hours. Luciferase activity was not statistically different in these fibroblasts.

(B) mRNA expression of *PPARγ* and other adipogenic markers (data not shown) was measured at day 6 of differentiation in RARE<sub>WT</sub> and RARE<sub>Aldh1a1-/-</sub> fibroblasts. (Mann Whitney U test, n=3, P<0.001).

(C) RARE<sub>Aldh1a1-/-</sub> fibroblasts were transiently transfected with human full-length control vector (Co), Aldh1a1 (a1+), Aldh1a3 (a3+), or both of these vectors (a1+a3+) as described in Methods. After transfection, differentiation was induced by standard procedure. Luciferase activity was assayed on day 6 of differentiation. Data are shown as fold-induction compared to activity in RARE<sub>Aldh1a1-/-</sub> fibroblasts transfected with a control vector (black bar). *, significant difference compared to RARE<sub>Aldh1a1KO</sub> fibroblasts transfected with control vector (Mann Whitney U test, n=3, P<0.01).

(D) mRNA samples from these cells were used to measure expression of mouse and human Aldh1a1, a2, and a3 as well as *PPARγ*. The summarized expression levels of all Aldh1 enzymes correlate with *PPARγ* expression (ANOVA test, n=12, P<0.016).

(E) RARE<sub>Aldh1a1-/-</sub> fibroblasts were differentiated in the presence of 10nM all-trans retinoic acid (RA) for 8 days. RA (10nM) was added in the differentiation mix and in the medium every 48 h. *PPARγ* mRNA expression was significantly increased in RARE<sub>Aldh1a1-/-</sub> fibroblasts stimulated with RA (Mann Whitney U test, mean±SD, 3 independent experiments, P<0.025).

(F) Significantly increased ratio of *Fabp4* to *Pref1* expression (Mann Whitney U test, mean _ SD, three independent experiments, P > 0.05) in RARE*Aldh1a1-/-* fibroblasts stimulated with RA compared with those treated with vehicle (Veh, ethanol/dimethyl sulfoxide, 50%).

(G) Representative oil red O staining of neutral lipids in these differentiated RARE*Aldh1a1-/-* fibroblasts stimulated with vehicle and RA.
Figure 2.2. *Aldh1a1* and *Aldh1a3* generate RA and regulate *PPARγ* mRNA expression [168].
Figure 2.3. Decreased expression of Aldh1a3 influences Pref1 and visfatin expression [168].

3T3-L1 fibroblast cell lines were stably transfected with scrambled shRNA or Aldh1a3 shRNA and differentiated for 6 days. mRNA expression of the adipogenic markers: PPARγ (A), Fabp4 (B), C/EBPβ (B), adiponectin (Adipoq, D), as well as markers of preadipocytes: preadipocyte factor-1 (Pref1, E), and visceral fat: visfatin (F) in differentiated Aldh1a3shRNA 3T3-L1 adipocytes (black bars) compared to differentiated scrambled shRNA 3T3-L1 adipocytes (white bars). P value indicates differences in differentiated Aldh1a3s hRNA vs. scrambled 3T3-L1 adipocytes (Mann Whitney U test, n=3, mean±SD).
Figure 2.4. Aldh1 enzymes and RA stimulation induce ZFP423 expression.

(A) RARE<sub>Aldh1a1<sub>−−</sub></sub> fibroblasts were transiently transfected with human full-length control vector (Co), Aldh1a1 (a1+), Aldh1a3 (a3+), or both of these vectors (a1+a3+) and differentiated for 6 days as described in Fig. 2C and D. Along with Aldh1 and PPARγ expression, mRNA samples from these cells were used to measure expression of ZFP423, C/EBPα, and C/EBPβ expression. The summarized expression levels of all Aldh1 enzymes correlate with ZFP423, but not C/EBPβ expression (ANOVA test, n=12, n.s.-not significant). C/EBPα did not correlate with Aldh1 (ANOVA test, n=12, $R^2$ =0.05, n.s.).

(B) WT and Aldh1a1<sup>−−</sup> fibroblast cell lines were stably transfected with scrambled and Aldh1a2 and a3 shRNA to obtain clones deficient in all Aldh1 enzymes (Aa-) as well as those expressing no Aldh1a1, but high Aldh1a2 and a3 levels (Aa-a2+a3+) upon differentiation. mRNA expression of ZFP423 and C/EBPβ was measured in cells differentiated for 6 days. Asterisks indicate significant differences as compared to Awt, # indicates significant differences between Aa1- and Aa1-a2+a3+ (Mann Whitney U test, n=3, mean±SD). PPARγ and ZFP423 had similar expression patterns in these cells (data not shown). C/EBPα was modestly reduced (40%) in both Aa1- and Aa1-a2+a3+ clones compared to Awt (data not shown).

(C) ZFP423 and C/EBPβ expression in RARE Aldh1a1<sup>−−</sup> fibroblasts that were differentiated in the absence and in the presence of different RA concentrations (indicated in the Figure) for 8 days. P values indicate significant increase in ZFP423 expression in cells stimulated with RA compared to vehicle (ethanol, Veh) (Mann Whitney U test, n=3, mean±SD).
Figure 2.4. Aldh1 enzymes and RA stimulation induce ZFP423 expression [168].
Figure 2.5. Fat depot-specific expression of Aldh1a3 and Aldh1a1 enzymes manifests as decreased ZFP423, PPARγ, and Fabp4 expression in visceral, but not in subcutaneous fat of Aldh1a1<sup>−/−</sup>tm1Gdu mice.

mRNA was isolated from subcutaneous and perigonadal visceral fat of WT (n=7) and Aldh1a1<sup>−/−</sup> (n=6) female mice fed regular chow. All data are shown as mean±SD unless otherwise indicated, statistical significance was examined by Mann Whitney U test.

(A) Expression of Aldh1 enzymes in subcutaneous fat. *, significant decrease in Aldh1a1 and a3 expression in Aldh1a1<sup>−/−</sup> compared to WT mice.

(B) Expression of Aldh1 enzymes in visceral fat. *, significant decrease in Aldh1a1 and a3 expression in Aldh1a1<sup>−/−</sup> compared to WT mice. #, significant differences compared to Aldh1 expression in subcutaneous fat (A).

(C, D) ZFP423 expression in subcutaneous (C) and visceral (D) fat. Here and throughout this figure: *, significant differences in gene expression in Aldh1a1<sup>−/−</sup> compared to WT fat (same fat depot), P<0.002 (Mann-Whitney U test); #, significant differences in gene expression in visceral compared to subcutaneous fat within the same genotype. (paired t-test, P<0.001).

(E) Correlation between expression levels of ZFP423 and PPARγ (ANOVA test, n=25, P<0.002)

(F, G) PPARγ and Fabp4 expression in subcutaneous (F) and visceral (G) fat.
Figure 2.5. Fat depot-specific expression of Aldh1a3 and Aldh1a1 enzymes manifests as decreased ZFP423, PPARγ, and Fabp4 expression in visceral, but not in subcutaneous fat of Aldh1a1<sup>−/-</sup>tm1Gdu mice [168].
Figure 2.6. Aldh1a1 deficiency influences fat formation in specific visceral regions [168].

(A) Whole body weights are shown in WT (n=10) and Aldh1a1⁻⁻ (n=9) female mice on regular chow (same as in Fig.4). (B) Analysis of fat area of 69 MRI cross-sectional images performed in frozen WT and Aldh1a1−/− female mice from this study (n=3 per group). *, significant differences in fat areas in WT and Aldh1a1−/− mice. Dashed lines show approximate position of significantly affected fat areas.

(C, D) Cross-sectional (two right panels of pericardial and peribladder regions) and coronal (left image) MRI images from representative WT (C) and Aldh1a1−/− (D) mice. H, heart; Fat, v, visceral fat; Fat, s, subcutaneous fat; B, bladder.
Figure 2.7. Predominant expression of *Aldh1a1* in subcutaneous compared to omental fat in women [168].

Relative mRNA expression of *Aldh1* enzymes in subcutaneous (A) and omental (B) adipose tissue isolated from four women. *Aldh1* expression is shown as mean±SD. #, P<0.03, differences between *Aldh1* expression in subcutaneous and omental fat in each donor (paired t-test).
CHAPTER 3

Autocrine Function of Aldehyde Dehydrogenase 1 as a Determinant of Diet – and Sex-specific Differences in Visceral Adiposity

Published in Diabetes, 2012

3.1 Introduction

The higher prevalence rates of obesity in women (61.3% vs. 42% prevalence in men) correlate with a higher risk for type 2 diabetes, cardiovascular disease, cancer, and premature death [156-158]. The onset of adiposity occurs on a Western diet in premenopausal women [159,160] or after menopause [161]. On a regular diet, preferential distribution of fat to visceral depots is atypical for females but occurs in males [162]. Obesity is a polygenic and multifactorial disorder with various predisposing factors, including sex hormones and obesogenic diets [163]. The effector mechanisms modulating visceral fat (VF) accumulation in females and, in particular, its relationship to HF diets are poorly characterized [163,164].

We showed that low autocrine RA generation by the cytosolic aldehyde dehydrogenase-1 (ALDH1A1, A2, and A3) enzyme family stimulates adipogenesis via mechanisms dependent on transcription factors ZFP423 and PPARγ [168].

RA is produced primarily by Aldh1a1 in VF and by Aldh1a1 and Aldh1a3 in subcutaneous fat [137]. In consonance with their tissue-specific distribution,
Aldh1a1−/−tm1Gdu mice develop less VF than sc when compared to WT mice on a chow diet [137,168]. HF diet in the liver and estrogen in the uterus induced Aldh1 expression in mice, possibly directly through ER sites in the promoter of Aldh1a2 [173,174] and SREBP sites in the promoter of Aldh1a1 and Aldh1a2 [152,175]. On a HF diet Rald repressed adipogenesis by inhibiting RXR and PPARγ [104], whereas Kiefer et al. [172] showed that both RA and Rald regulated Ucp1-expression through RAR in vitro with Rald being a weaker RAR ligand than RA [138,171]. This interpretation is in disagreement with observations in vivo, where only Aldh1a1−/−tm1Gdu female mice developed thermogenic adipocytes in white fat in the absence of all RA-generating enzymes and C57BL/6J WT mice producing RA by ALDH1A1, A2, and A3 remained obese. In humans, therapeutic RA doses can also cause RA syndrome demonstrating increased adiposity [169] by unknown mechanisms. We hypothesized that adipose tissue responds to HF feeding or sex hormones by intrinsic RA production stimulating obesity. Here we provide evidence of HF-diet induced RA generation in sex- and depot-specific manner contributing to fat depot formation in mouse and showed an evidence of the relevance of this pathway in human adipose.

3.2 Material & Methods

Reagents: We purchased reagents from Sigma-Aldrich (St. Louis, MO) and cell culture media from Invitrogen (Carlsbad, CA) unless otherwise indicated. ATGL and GAPDH antibodies were from Cell Signaling Technology (Danvers, MA), UCP1, β-ACTIN and TUBULIN were from Abcam (Cambridge, MA); secondary antibodies were from LI-
COR Biosciences (Lincoln, NE). 17-beta-Estradiol was obtained from Cayman (Ann Arbor, MI) and ELISA kits for E2, insulin were from Abnova (Walnut, CA) and Millipore (Billerica, MA). All-trans retinoid isomers were stored under argon, protected from light.

**Human studies:** VF was obtained from the greater omentum during endoscopic repair of hernias from overnight fasted lean subjects (BMI<30) and bariatric surgeries (laparoscopic banding and gastric bypass) in obese patients (BMI≥40). IRB-approved informed consent was obtained for the patients' medical records. Stromal vascular fraction (SVF) was isolated from VF using Ficoll-Hypaque (GE Healthcare) as described [178].

**Animal studies** were approved by the IACUC. Study 1: *Aldh1a1*<sup>-/-</sup>tm1Gdu mice were constructed by Gregg Duester [13] and initially characterized by Reichert et al. and Ziouzenkova et al.[104,137]. Age- (8 weeks old) and sex-matched C57BL/6J (WT) and *Aldh1a1*<sup>-/-</sup>tm1Gdu mice were fed either a regular chow (RC) obtained from Harlan Laboratories, Indianapolis, IL (irradiated 7912) or HF diet (45% kcal from fat with standard 4IU vitamin A/g, DIO series diet, D12451, Research Diets Inc., New Brunswick, NJ) for 180d and 300d. The number of mice used: WT, both males and females, n=18, and *Aldh1a1*<sup>-/-</sup>tm1Gdu males, n=18, females, n=23 on RC diet and WT males, n=8, females, n=9 and *Aldh1a1*<sup>-/-</sup>tm1Gdu males, n=9, females, n=11 on HF diet for 180d. 7 WT males, 9 females and 9 *Aldh1a1*<sup>-/-</sup>tm1Gdu male and female mice were used for 300d HF feeding study. Food intake and metabolic rate were measured after mouse
acclimation to a powdered HF diet (4d) in metabolic cages (Ancare, Charles River Laboratories).

Study 2: Seven-month-old C57BL/6J (WT) and Aldh1a1<sup>−/−</sup>tm1Gdu females on regular chow diet were ovarioctomized or sham-operated. For WT : sham,n=10, OVX,n=8 and Aldh1a1<sup>−/−</sup>tm1Gdu : sham, n=9,OVX,n=5 mice were used. Mice continued on RC diet for three months after surgery.

Study 3: Three-month-old Tg(RARE-Hspa1b/lacZ)12Jrt/J (denoted as RARE-lacZ) mice were purchased from The Jackson laboratory (Bar Harbor, ME). These mice were developed by Dr. Rossant using a transgenic construct containing 3 copies of the 32bp RARE placed upstream of the mouse heat shock protein 1B promoter and β-galactosidase gene (lacZ) [176]. Two RARE-lacZ mouse groups received RC or a HF diet (same as in study 1) for 150d. In all animal studies, weight and food consumption were measured weekly. Five mice per group was used for this study.

**Glucose tolerance test** (GTT) was performed in overnight fasted mice by intraperitoneal injection of 0.004 mL 25% glucose/g body weight.

**Cell culture:** Murine NIH3T3-L1 (3T3-L1) preadipocytes were cultured and differentiated using standard procedures (20). Preadipocytes differentiated for 7-12d were denoted as 'mature’ and stimulated in 1% UV-treated FBS depleted of retinoids. Human SGBS preadipocytes were cultured and differentiated in DMEM/Ham's F12 containing 10% FBS as described [177]. NEFA were measured in media at 45min and 3h using NEFA-HR kit (Wako Diagnostics, Richmond, VA).
SVF: SVF was isolated from VF of 16m- and 11m-old WT and Aldh1al<sup>−/−</sup>tm1Gdu female mice on RC diet as described [179].

**Explant Cultures:** VF was isolated from three Aldh1al<sup>−/−</sup>tm1Gdu or RARE-lacZ males fed RC. Each fat pad was excised into 4 equal by weight sections for stimulation with retinoids (~87mg/fat section, 5mL 1% UV treated FBS DMEM/g fat). Explants were stimulated with isoproterenol (10QM) in DMEM containing 2% fatty acid-free BSA (Sigma-Aldrich, St. Louise, MO) (5ml medium/g fat). Medium was collected every 30min for 2h for NEFA detection. Other explants were homogenized in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Roche, Indianapolis, IN) or used for mRNA isolation.

**Western blot:** Cell/tissue lysates normalized by protein content were separated on 10% acrylamide gel under reducing conditions, transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Billerica,CA). Proteins were analyzed using an Odyssey Infrared Imaging System (LI-COR).

**Proteomic 2-D Fluorescence Difference Gel Electrophoresis (DIGE):**

This experiment was carried out by the proteomic facility at OSU.

Four RIPA homogenates each from VF of males and females of WT and Aldh1al<sup>−/−</sup>tm1Gdu genotypes were precipitated with 10% trichloroacetic acid and used for DIGE (GE Healthcare, Pataskala, OH). Samples were labeled with DIGE fluor minimal-label dyes and focused on 18cm pH 4-7 Immobiline strips using an IPGphor IIIEF system.
(GE Healthcare). After SDS-PAGE, individual gels were spot mapped using an internal standard. Independent $t$-test ($P<0.05$) between WT and $Aldh1a1^{-/-}$ groups in males and females was performed to identify spots with more than 1.5-fold differences in abundance with spots appearing in at least 3 of the 4 gels. Significantly changed spots were cored from preparative gels (Ettan workstation) and identified using a LTQ mass spectrometer (MS) detector.

**Immunohistochemistry**: VF embedded in paraffin was stained with ATGL or UCP1 polyclonal rabbit antibodies (1:1000 dilution). [see appendix 2].

**Semi-quantitative mRNA analysis**: cDNA was prepared from purified mRNA (Qiagen, Valencia, CA), and analyzed using a 7900HT Fast Real-Time PCR System, TaqMan detection system, and Validated primers (Applied Biosystems, Foster City, CA) in triplicate, including no-template controls. The mRNA expression was calculated based on 18S expression using the delta Ct method. The β-galactosidase assay was designed based on GenBank: U46489.1 standard LacZ (22bp, underlined) surrounded by 50bp (Applied Biosystems probe design):

GCCGATACTGTCTCGTCCCCCTCAAAPTGGCATGCACCGTTACGATGCGC
CCATCTACACCAACGTAACCTATCCCATTACCGTGCAATCCGGCCGTTTTGTTCCC
ACGGAGAATCCCGACGGG.

**Statistical analysis**: Data are shown as mean±SD. *In vitro* experiments were performed at least in triplicate. Group comparisons were performed using 2-way ANOVA test unless otherwise indicated.
3.3 Results

*Aldh1a1* deficiency suppresses HF diet-induced fat formation in a sex- and fat depot-specific manner.

The RA role in HF diet-induced obesity was studied in male and female mice deficient in *Aldh1a1*, a predominant RA-generating enzyme in white adipose [168]. WT and *Aldh1a1*−/−tm1Gdu mice received a RC or a HF diet for 180 and 300 days, to explore long-term effects of HF consumption (Figure 3.1). WT and *Aldh1a1*−/−tm1Gdu females weighed less than male mice on a RC diet (Figure 3.1A, Day 0). On a HF diet (Figure 3.1A, Days: 180, 300), WT females and males gained weight, and sex-specific differences in weight were only modestly different after 180d and 300d on a HF diet, respectively. *Aldh1a1*−/−tm1Gdu females weighed less than *Aldh1a1*−/−tm1Gdu males throughout this study. However, after 300d on a HF diet, both male and female *Aldh1a1*−/−tm1Gdu mice had significantly reduced weight compared to WT groups.

Fat accumulation on a HF diet occurred in a depot- and sex-specific manner. Subcutaneous fat accumulated to a similar extent in WT males and females (Figure 3.1B) and was reduced to a similar extent in *Aldh1a1*−/−tm1Gdu males and females. Strikingly, VF accumulation was markedly increased in WT females compared to males on a HF diet (Figure 3.1C). In contrast, VF mass in *Aldh1a1*−/−tm1Gdu females was suppressed (4.2-fold lower than in WT females), whereas VF in *Aldh1a1*−/−tm1Gdu males was identical to that seen in WT males after 180d on a HF diet (Figure 3.1C). Longer durations of HF feeding (300d) decreased VF mass in *Aldh1a1*−/−tm1Gdu females (12.3-fold lower than WT females). TG content in VF of *Aldh1a1*−/−tm1Gdu males underwent significant reduction.
(2.3-fold) compared to WT males after 300d on a HF diet in VF (Figure 3.1C). Whereas metabolic rate and RQ were similar in WT males and females (Figure 3.1D&E), Aldh1a1<sup>−/−</sup> females had a higher metabolic rate and lower RQ (therefore, oxidizing fat) compared to Aldh1a1<sup>−/−</sup> males, which reached significance at some time points. Brown fat mass was similar in WT and Aldh1a1<sup>−/−</sup> females, and lower in WT than in Aldh1a1<sup>−/−</sup> males (data not shown). Both Aldh1a1<sup>−/−</sup> males and females had improved glucose tolerance compared to respective WT groups (Figure 3.1F); insulin levels in fasted mice were not significantly different among groups (Figure 3.1G). Food intake monitored for 24h in metabolic cages were comparable among all groups (Figure 3.1H). Plasma TG levels were also similar among groups (data not shown). In contrast, NEFA plasma levels were higher in WT vs. Aldh1a1<sup>−/−</sup> females and showed sexual dimorphism in Aldh1a1<sup>−/−</sup> mice. Therefore, Aldh1a1 may regulate sexual divergence in metabolic responses, e.g. VF in females.

To identify retinoid-sensitive effector proteins responsible for VF reduction in Aldh1a1<sup>−/−</sup> females, we performed a comparative proteomic analysis of VF in: 1) WT versus Aldh1a1<sup>−/−</sup> males (Figure 3.2A, left panel), and 2) WT versus Aldh1a1<sup>−/−</sup> females (Figure 3.2A, right panel).

**Atgl is a Rald-sensitive protein contributing to visceral obesity resistance in Aldh1a1<sup>−/−</sup> females.**

Proteomic analysis of WT versus Aldh1a1<sup>−/−</sup> VF revealed that 176 proteins in group 1 (male) and 167 proteins in group 2 (female) differed. Among them, 13 proteins varied significantly in abundance in VF after false discovery rate correction (Figure 3.2A,
Table 1). Based on statistical significance, proteins were subdivided into groups different in: 1) females, 2) males, and 3) both males and females. Among proteins differentially expressed in females was a key adipose triglyceride lipase (ATGL), a PPARγ-regulated gene [180,181], which is increased in mouse VF following HF feeding [182]. This lipase is also known as palatin-like phospholipase domain-containing protein A2 (Pnpla2). We analyzed mRNA and protein expression of ATGL in VF from each group (Figure 3.2B, C). Atgl mRNA expression was similar in all mice. SVF contributed to a minor extent to Atgl mRNA and protein expression (Figure 3.2B, insert), in consonance with previous reports [186]. In contrast, ATGL protein levels and NEFA release were increased in VF of Aldh1a1−/−tm1Gdu females compared to males (Figure 3.2C, D). Correspondently, adipocyte size was also smaller in VF of Aldh1a1−/−tm1Gdu females compared to other groups (Figure 3.2E). Immunohistological analysis (Figure 3.2F) revealed multiple ATGL-positive multilocular clusters only in VF of Aldh1a1−/−tm1Gdu females, in agreement with the expected lipolytic ATGL function. Recently [183,184,185], ATGL’s function was coupled to thermogenesis in brown multilocular fat. We and others found higher expression of thermogenic genes in the Aldh1a1−/− adipocytes and VF [172,187]. The immunohistological analysis of the same VF sections, revealed Ucp1 expression in multilocular ATGL-positive adipocytes only in Aldh1a1−/−tm1Gdu females, whereas VF in other mice had rare interspersed UCP1-positive adipocytes. The Ucp1 mRNA expression was 9.6 fold higher in Aldh1a1−/−tm1Gdu females than WT females, whereas the values in male groups were not different between genotypes (suppl. table 1 in appendix 2). Thus, VF underwent more profound remodeling in Aldh1a1−/−tm1Gdu females than males.
To examine whether the sex-specific ATGL expression were dependent on Aldh1 products, we investigated the effects of Rald and RA in VF of Aldh1a1<sup>−/−</sup>tm1Gdu male mice ex-vivo (Figure 3.3A). Short-term stimulation (2h) of male VF explants by Rald, but not RA, resulted in significantly increased ATGL protein levels. The estrogen effect on ATGL was examined in VF of ovariectomized WT females (Figure 3.3B, C) with reduced estrogen levels (Figure 3.3B, insert). Estrogen deficiency in ovariectomized WT females moderately increased ATGL protein levels compared to sham-operated animals, but this did not reach statistical significance (Figure 3.3B). Atgl mRNA expression was identical in both groups (Figure 3.3C). Given the minor effects of estrogen on ATGL protein levels, we examined the effect of retinoids on the regulation of this protein. Short-term stimulation with Rald increased ATGL protein levels in mature 3T3-L1 adipocytes (Figure 3.3D). Stimulations with retinol or RA did not influence ATGL protein levels. The potential dependence of Rald’s effects on ATGL translation was examined in the presence of cycloheximide, an inhibitor of protein biosynthesis (Figure 3.3E). Long-term (30h) pretreatment with cycloheximide prevented Rald-mediated induction of ATGL protein, seen at short (6h) pre-treatment of 3T3-L1 adipocytes suggesting mechanisms dependent on protein biosynthesis. Next, we examined sexual dimorphisms in Aldh1 enzymes responsible for Rald catabolism.

Expression of Aldh1 enzymes is sex-specific in VF.

Aldh1 enzymes were expressed in a sex-specific fashion that was particularly apparent in Aldh1a1<sup>−/−</sup>tm1Gdu mice (Figure 3.4A). Aldh1a1, the major RA-producing enzyme in adipocytes [104], was expressed at markedly higher levels than Aldh1a2 and
Aldh1a3 in VF of WT male and female mice. In SVF isolated from VF, Aldh1a1 expression was low and comparable with Aldh1a2 and a3 expression levels (Figure 3.4A, insert). In the genetic absence of Aldh1a1, expression of Aldh1a2 and Aldh1a3 was lower in Aldh1a1<sup>−/−</sup>tm1<sup>Gdu</sup> females than males. Expression of Cyp26A1, a RA-sensitive gene [188] was also significantly lower in Aldh1a1<sup>−/−</sup>tm1<sup>Gdu</sup> female, but not male mice compared to their respective WT groups (Figure 3.4B). To investigate whether inhibition of Aldh1a2 and/or Aldh1a3 was mediated by estrogen, we measured the expression of these enzymes in ovariectomized mice (Figure 3.4C). Only Aldh1a3 expression was significantly increased (180%) in ovariectomized compared to sham-operated WT females. Interestingly, Aldh1a3 expression was 188% higher in male compared to female WT mice on a regular diet without surgical intervention (Figure 3.4C insert). In consonance with the role of ALDH1A3 in RA generation, Cyp26A1 expression also was higher in the ovariectomized female group (Figure 3.4D). Ovariectomized WT mice gained more weight and VF compared to sham-operated WT mice (120% and 217%, respectively) (Figure 3.4E, F). VF in ovariectomized Aldh1a1<sup>−/−</sup>tm1<sup>Gdu</sup> females underwent a moderate (167%), but not significant increase in VF compared to the sham-operated Aldh1a1<sup>−/−</sup>tm1<sup>Gdu</sup> group and reached levels seen in WT sham-operated mice (Figure 3.4F). Ovariectomy in Aldh1a1<sup>−/−</sup>tm1<sup>Gdu</sup> females could not significantly increase Aldh1a2 and a3 expression (Figure 3.4F insert). Correspondent to VF changes, ovariectomized groups had impaired glucose tolerance; however, Aldh1a1<sup>−/−</sup>tm1<sup>Gdu</sup> females were more glucose tolerant than WT in both sham and ovariectomized groups (Figure 3.4G). Post-prandial plasma insulin levels were similar in all groups. Thus, sex-specificity in Rald catabolism
may depend on estrogen, which reduced Aldh1a3 expression in female mice, decreased VF formation, and improved glucose metabolism.

Although the Aldh1a1<sup>−/−</sup>/tm1Gdu model established causal relationships between Aldh1 enzymes and obesity, it does not provide insight into intrinsic RARE activation in relation to RA-generating ALDH1 enzymes in vivo, which is heavily influenced by sex hormone production and diet. For example, ultradian estrogen production and feeding-starvation cycles may alter RA production and RAR activation in a spatiotemporal fashion in adipocytes, which cannot be well characterized in the previously used animal models. In order to better examine RAR regulation in subcutaneous and visceral adipose overtime, a RARE-lacZ reporter mouse model was utilized.

**Sex- and depot-specific RA formation accompanies HF diet-induced obesity in RARE-LacZ mice.**

The RARE-lacZ reporter model was developed and is widely used to monitor progressive spatiotemporal changes in auto- and paracrine RAR activation during embryogenesis in Aldh1-deficient mouse models, reviewed by Duester in [13]. In this model, activated RARs, e.g. due to increased intracellular RA concentrations, bind to native and transgenic RARE coupled to β-galactosidase expression. Thus, β-galactosidase expression in these mice provides cumulative information about RAR regulation. We verified that adult RARE-lacZ mice responded to nanomolar RA concentrations in circulation (Fig.B.2, see appendix 2). Intraperitoneal RA administration increased β-galactosidase protein expression in liver and adipose tissue in RARE-lacZ, but not in WT mice. Accordingly, only RARE-lacZ mice expressed β-galactosidase mRNA in the liver.
Stimulation of VF explants from RARE-lacZ males with RA increased β-galactosidase expression (Figure 3.5A), whereas Rald did not influence β-galactosidase expression, in agreement with the low binding affinity of this metabolite to RAR [138]. The natural RARE-containing target genes Cyp26A1 and Cyp26B1 were also regulated by RA, but not by Rald (Figure 3.5B,C). RARE-lacZ male and female mice gained weight on a HF compared to a chow diet (data not shown) due to increased subcutaneous and VF mass (Figure 3.5D,E). Formation of subcutaneous fat was increased at comparable levels in male (261%) and female (221%) RARE-lacZ mice on a HF compared to a chow diet (Figure 3.5D). HF diet consumption resulted in a significant increase in VF formation in both groups (Figure 3.5E) and an increased ratio of visceral to subcutaneous fat in females (1.6 in males vs. 2.6 in females) (insert).

β-galactosidase expression, a surrogate measure of RARE activation, was similar in subcutaneous fat of males and females on regular chow, whereas in VF, males expressed significantly more β-galactosidase than females (Figure 3.5F). On the HF compared to chow diet, subcutaneous fat formation was accompanied by increased β-galactosidase expression in both male (363%) and female (495%) RARE-lacZ mice (Figure 3.5G). Strikingly, VF formation in RARE-lacZ males was not associated with β-galactosidase expression, whereas in RARE-lacZ females, VF formation was accompanied by an 860% increase in β-galactosidase expression in HF vs. chow diet groups. These experiments suggest that HF diet induces RARE in VF, and retinoid production may underlie sex-specific effects.

In a pilot study, we compared fat depot weights in C57BL/6J (WT) and Aldh1al<sup>tm1Gdu</sup><sup>−/−</sup> mice on two kinds of diet: high fat/4IU vitamin A diet versus high fat/20IU...
vitamin A diet to study vitamin A effects on weight gain in these mice. We did not find any difference in weight gain and fat accumulation in mice on the two different diets, and such finding validates our study design and results described above [see appendix 2 for more details].

Obese men and women express higher levels of Aldh1 enzymes in SVF than lean subjects.
In a pilot investigation, we examined omental adipose from lean and obese patients. Aldh1 expression in the whole VF was not different in obese and lean patients. We then examined Aldh1 expression in the SVF. Aldh1a1 was the most abundantly expressed isoforms in the SVF, and was expressed at significantly higher levels in obese than in lean women (333%) (Figure 3.6A). Aldh1a3 expression was higher in obese than in lean (422%) men. Thus, increases in the major Aldh1 isoform may preferentially drive RA production and its obesity responses in females more than in males. Next we examined whether retinoids or E2 also influences NEFA release in differentiated human simpson-golabi-behmel syndrome (SGBS) adipocytes (Figure 3.6B). SGBS cells are used to study human adipocyte biology. Stimulation of SGBS with Rald, but not RA, increased NEFA release, in agreement with the response seen in mouse VF ex-plants (Figure 3.2D) supporting a possible role of retinoids regulated by Aldh1 in adipocytes.

3.3 Discussion
HF diet and hormonal changes associated with menopause are well known to alter adipose distribution in women [9] and C57BL/6J female mice [42] from a predominantly subcutaneous pattern to excess visceral deposition. The mechanisms for this
redistribution are hitherto undescribed. Here we show that HF feeding induces autocrine RA formation that, in turn, governs fat formation in a depot- and sex-specific fashion. Our data suggest that a HF diet and/or lack of estrogen mediates VF formation through a sex-specific autocrine Aldh1 switch, in which lipolysis, mediated by an induction of ATGL through Rald, is replaced by RA-mediated lipid accumulation. These results in mice were paralleled by increased expression of Aldh1a1 in obese women.

Our previous studies demonstrated that ALDH1A1 is the major RA-generating enzyme in mice [168]. Here we showed that disruption of RA production by Aldh1a1 impaired VF formation in females more than in males. Aldh1a1 deficiency in females prevented development of HF diet-induced visceral obesity in C57BL/6J mice. In males, HF diet modestly increased VF formation that was significantly reduced in Aldh1a1<sup>−/−</sup> tm1Gdu males only after being on a HF diet for a prolonged (>180d) period of time. Notably, WT male and female mice had equal increases in the formation of subcutaneous fat on a HF diet that were reduced to comparable levels in Aldh1a1<sup>−/−</sup> tm1Gdu males and females.

Comparison of the proteomic analysis of VF in WT and Aldh1a1<sup>−/−</sup> tm1Gdu male and female groups enabled the identification of candidate proteins that could explain divergent lipid metabolism and VF formation in Aldh1a1<sup>−/−</sup> tm1Gdu males and females. We scrutinized the regulation of ATGL (amongst 13 others), considering its central role in triglyceride hydrolysis and adipose homeostasis [181]. Increased triglyceride lipolysis is required to support thermogenic function through a PPARα-mediated mechanism [184,185]. Thermogenesis in both white and brown fat was elevated in Aldh1a1<sup>−/−</sup> tm1Gdu mice as shown previously [104, 172,187]. Consistent with a potential role, ATGL protein
levels, and NEFA release were markedly higher in Aldh1a1<sup>−/−</sup>tm1Gdu females than in males. In vivo, adipocytes were smaller, multilocular and expressed ATGL and UCP1 protein in VF in Aldh1a1<sup>−/−</sup>tm1Gdu females, but not in males. These increased ATGL levels in numerous thermogenic visceral adipocytes [184,185] along with increased metabolic rate may provide a potential explanation for the ability of Aldh1a1<sup>−/−</sup>tm1Gdu females to resist pronounced visceral obesity seen in WT females in response to a HF diet. In consonance with this interpretation, the implantation of thermogenic Aldh1a1<sup>−/−</sup> preadipocytes into VF of WT mice limited VF development on a HF diet compared to mice treated with WT preadipocytes [187]. To further delineate the role of Rald and estrogen in sex-specific responses, we studied the effects of these two mediators on ATGL. In the absence of estrogen, ovariectomized WT females had increased expression of Aldh1a3 and RAR target genes (Cyp26A), suggestive of RA generation. These experiments highlight an indirect participatory role of estrogen in ATGL regulation, perhaps via its inhibition of Aldh1 expression. Estrogen regulation of ALDH1 enzymes has been previously reported in other tissues [173]. In both obese mice and obese women, Aldh1a1 overrides expression of Aldh1a2 and a3 enzymes (Fig. 3.4 & 3.6). Consequently, Rald catabolism was markedly impaired in Aldh1a1<sup>−/−</sup>tm1Gdu females, whereas Aldh1a1<sup>−/−</sup>tm1Gdu male mice utilized Rald for RA production by the alternative ALDH1A2 and ALDH1A3 enzymes. The experiments with Rald in cultured tissue and adipocytes suggest that this metabolite can induce ATGL protein but not mRNA levels in VF within hours of stimulation. A non-genomic/non-transcriptional action of Rald was confirmed using cycloheximide. Thus, Rald may exert both non-genomic and genomic
effects (Figure 3.6C), as has been described for a number of hormones, including estrogen and RA [190,191,192].

A unique aspect of this investigation was the assessment of cumulative RARE activation, possibly by RA production in adipose tissue in response to a HF diet over extended periods of time, when RA generation may be under the control of protean influences, including circadian/ultradian (rapid recurrence in cycles of less than 24hrs) hormones and food intake. We took advantage of the RARE-lacZ reporter mice, which express β-galactosidase upon RAR activation by endogenous or added RA. By using this model in embryogenesis studies, researchers eventually mapped ALDH1 enzyme expression, and identified RAR-dependent transcriptional responses in tissues and single cells [176, 189], reviewed in [13]. RARE was activated in subcutaneous fat in comparable amounts in males and females, while VF formation was sex-specific. On a chow diet, VF in females generated substantially less RARE activity than in males. This was changed on a HF diet. Although VF was formed in both groups, only visceral obesity in females was associated with the induction of RARE. Our hypothesis is that autocrine RA production leads to fat formation. Genetic disruptions of several enzymatic pathways responsible for vitamin A metabolism led to markedly altered fat formation [104,117,130,168]. The Aldh1a1 and hormone-sensitive lipase-deficient models are characterized by impaired RA production and resistance to HF diet-induced obesity [130,168]. Moreover, the administration of RA in these mice partially compensated for gene loss and improved adipogenesis in vitro and in vivo, which was consistent with RA’s role in the induction of PPARγ expression and adipogenesis [168]. Physiologic RARE activation, seen in RARE-lacZ mice in response to HF diets, supports RA’s auto-
or paracrine participation in female visceral obesity. In additional studies of morbid obesity, we demonstrate a shift towards higher RA production in the SVF of human visceral (omental) adipose. These changes were related to higher Aldh1 expression in obese compared to lean patients (Figure 3.6A). Obese men demonstrated an increase in the expression of the minor isoform, Aldh1a3. In obese women, RA generation was supported by elevated Aldh1a1 expression, in line with possible Aldh1a3 suppression by estrogen. In our previous studies we showed that ectopic expression of either Aldh1a1 or Aldh1a3 increases intrinsic RA formation and promotes adipogenesis [168]. Notably, although Aldh1a1 induction accompanies adipogenesis, its levels remained similar in mature adipocytes [168], underscoring the role of ALDH1A1 in preadipocytes. The increased Aldh1 expression in SVF may indicate accelerated preadipocyte differentiation in obese vs. lean patients. The changes in Rald catabolism can potentially influence NEFA hydrolysis in differentiated human adipocytes, seen in a model SGBS cell line. Aldh1 effects on lipogenesis, thermogenesis, glucose utilization, immune and other responses in human VF remain to be investigated.

We described a mechanism by which ALDH1A1 integrates dietary and hormonal responses that could account for the regulation of susceptibility to visceral obesity in women. The identified sexual dimorphism in RA generation offers an opportunity for further investigation of gender differences in pathways determining visceral adiposity and in devising sex-specific treatment of obesity.
Figure 3.1. Deficiency in Aldh1a1 suppresses fat accumulation in response to HF-feeding in a depot-and sex-specific manner.

(A) Whole body weights are shown in WT (males, open triangles, n=18 and females, open circles, n=17) and in Aldh1a1−/− (closed triangles males, n=18, and closed circles, females, n=23) on regular chow; in WT (males, n=8 and females, n=9) and in Aldh1a1−/− (males, n=9 and females, n=11) mice on a high-fat diet (HF) for 180d; in WT (males, n=7 and females, n=9) Aldh1a1−/− (males, n=9 and females, n=9) mice for 300d. *, - P<0.001 differences between genotypes; SWT (black) or SKO (blue), - differences between sexes within one genotype. Throughout Figure Legends data are shown as mean±SD and statistical differences were examined by 2 way ANOVA unless otherwise indicated. X-Ray images showed randomly-selected mice from each group on a regular chow and HF diet for 180d.

(B&C) Subcutaneous (B) and visceral (perigonadal) (C) fat pads were dissected as shown in the insert. WT (males, n=9 and females, n=8) and in Aldh1a1−/− (KO, males, n=9 and females, n=8) on regular chow; in WT (males, n=5 and females, n=5) and in Aldh1a1−/− (males, n=4 and females, n=4) mice on a high-fat diet (HF) for 180d; in WT (males, n=7 and females, n=9) Aldh1a1−/− (males, n=9 and females, n=9) mice for 300d. *, P<0.001 differences between genotypes in the same sex group; PS, differences between sexes within one genotype. Red arrow indicates that VF formation seen in WT females on a HF diet did not occur in KO females. Images show one VF pad dissected from randomly-selected WT and KO male and female mice that consumed a HF diet for 180d.

(D&E) Metabolic rate (D) and respiratory quotient (RQ, E) in WT (dashed line, males (blue triangles, n=4) and females (red circles, n=5)) and Aldh1a1−/− (solid line, males (n=3) and females (n=5)). Data were calculated (Weir equation (36)) based on the food and oxygen consumption as well as CO2 release in metabolic cages. *, - P<0.05 differences between genotypes; S, - P<0.05 differences between sexes within one genotype (black font, WT, blue font, KO). Mann-Whitney U test (for D, E, and F panels)

(F) Glucose tolerance test (GTT) in WT (dashed line, males (blue triangles, n=6) and females (red circles, n=7)) and Aldh1a1−/− (solid line, males (n=9) and females (n=8)).

(G) Food consumption was measured in WT (white bars) and Aldh1a1−/− (black bars) mice in metabolic cages (n=5 in all groups). The values among groups were not statistically different.
Figure 3.1. Deficiency in Aldh1a1 suppresses fat accumulation in response to HF-feeding in a depot-and sex-specific manner [105].
Figure 3.2. Sex-specific proteome and ATGL protein levels in VF of WT and Aldh1a1<sup>−/−</sup> mice.

(A) Representative images of the 2-D Fluorescence Difference Gel Electrophoresis (DIGE) proteomic gels comparing cy2, cy3 and cy5 stained proteins from VF isolated from WT and Aldh1a1<sup>−/−</sup> males (180d HF groups). Four gels from four pairs of different WT and Aldh1a1<sup>−/−</sup> VF pads were run in each experiment. Spots, 176 in male and 167 in female groups, were different between WT and Aldh1a1<sup>−/−</sup> mice without false discovery rate correction (FDRC) and 13 proteins after FDRC analysis. The identified 13 spots are shown on the gel image as a square around a spot that was significantly changed by at least 1.5-fold between groups with \( P \leq 0.05 \) after FDRC using an independent \( t \)-test.

(B&C) Atgl mRNA expression levels were measured by TaqMan (B) and Atgl protein levels (C) were analyzed by Western blot in VF from WT and Aldh1a1<sup>−/−</sup> male and female mice (all mean±SD, n=4) on a HF diet. Insert shows Atgl protein levels in two animals from each group. \( P_S \), significantly different between male and female Aldh1a1<sup>−/−</sup> mice.

(D) Release of NEFA from VF explants stimulated with isopreterenol for 1.5 h in a DMEM containing 2% of delipidated BSA. VF was isolated from 3 mice per group. Data were normalized to the levels seen in WT male mice (100%). \( P_S \), significantly different between male and female Aldh1a1<sup>−/−</sup> mice.

(E) Representative Atgl staining of paraffin embedded VF from WT and Aldh1a1<sup>−/−</sup> male and female mice (all n=4, mean±SD) on a HF diet (180d). In Aldh1a1<sup>−/−</sup> female mice Atgl protein was found in multilocular adipocytes. 10x, 40x, 100x indicates magnification. Arrows in 100x magnification group show cytosolic and peri-lipid droplet Atgl staining in a multilocular adipocyte.

(F) Area of unilocular adipocytes was quantified from the images (10x) shown in Fig. 2E. 300 adipocytes were quantified per group. \( P_S \), significantly different between male and female Aldh1a1<sup>−/−</sup> mice.
Figure 3.2. Sex-specific proteome and ATGL protein levels in VF of WT and
*Aldh1a1<sup>−/−</sup>* mice [105].
Table 3.1. Proteins identified in proteomic analysis [105].
Proteins identified in proteomic analysis for which protein levels were altered in VF from 1) WT and Aldh1a12/2 male groups; 2) WT and Aldh1a12/2 female groups; or 3) both differences in protein levels of proteomic markers measured in the two following groups: 1) male WT and Aldh1a12/2 mice and 2) female WT and Aldh1a12/2 mice, using proteomic DIGE. Average ratio represents fold change (+, increase; 2, decrease) of this protein compared with standardized control containing all samples; t test (n = 4). TaqMan (quantitative PCR (QPCR)) or Western blot (W) analyses that were performed to verify these findings are denoted in the last column. Bckdh, 2-oxoisovalerate dehydrogenase subunit b; Bpnt1, 39(29),59-bisphosphate nucleotidase 1; Cmbp-5, charged multivesicular body protein 5; Glod4, glyoxalase domain-containing protein 4.

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Figure 3.3. Rald regulates ATGL protein levels in Aldh1a1−/− male visceral adipose
tissue ex-vivo and in differentiated 3T3-L1 adipocytes in vitro.

(A) ATGL protein levels in VF explants isolated from Aldh1a1−/− males (n=3) were
measured by Western blot. Three VF pads dissected from each animal (equal by weight)
were immediately stimulated with vehicle, RA, and Rald (all 10nM) for 2h. Atgl levels
were normalized to housekeeping proteins, β-actin or Gapdh. Data for each set (Insert)
were normalized to vehicle control (100%) for VF from each animal. *, difference
between control (Veh) and Rald-explants (P<0.05) (all mean±SE, n=3).

(B) ATGL protein levels were analyzed by Western blot in VF from C57BL/6J (WT)
ovariectomized and sham-operated female mice (all mean±SE, n=4). Insert shows
estrogen (E2) levels in plasma using ELISA. (C) Atgl mRNA expression levels were
measured by TaqMan in the same VF. Mann-Whitney U test analysis showed no
significance between groups.

(D) ATGL levels in differentiated (8d) 3T3-L1 fibroblasts measured by Western blot.
Cells were stimulated for 45min with retinol (Rol), RA, and Rald (all retinoids 100nM) in
100nM insulin/20%FBS medium. Atgl levels were normalized to tubulin. Data are shown
as a percent of vehicle control. Insert on the top shows a representative example of a
Western blot. *, difference between control (Veh) and Rald-stimulated cells; #, difference
between Rald- and RA-stimulated cells, both P<0.05 (mean±SE, n=3).

(E) ATGL levels in differentiated (8d) 3T3-L1 fibroblasts stimulated with cycloheximide
(20µg/mL) for 6 or 30h. The cycloheximide-treated adipocytes were stimulated with
vehicle (Veh) and Rald (10nM) for an additional 45 min. Atgl and β-actin levels were
measured by Western blot. Prolonged (30h) cycloheximide treatment inhibited protein
translation and abolished the Rald-dependent increase in Atgl protein levels.
Figure 3.3. Rald regulates ATGL protein levels in Aldh1a1−/− male visceral adipose tissue ex-vivo and in differentiated 3T3-L1 adipocytes in vitro [105].
Figure 3.4. Estrogen contributes to sex-specific expression of Aldh1 enzymes in VF.

(A&B) Relative expression of RA-generating Aldh1 enzymes (A) and RARE containing RA target genes Cyp26A1 and Cyp26B1 (B) in VF isolated from WT male (n=5), female (n=4), and Aldh1a1−/− male and female mice (both, n=4) on a HF diet (180d). All data are shown as mean±SE. *, P<0.001 differences between genotypes in the same sex group; S P<0.05, significant differences between sexes within one genotype. Insert in A shows Aldh1 expression in SVF fraction in age-matched females on RC (same n=3/group as in fig. 3.2B).

(C&D) Relative expression of Aldh1 enzymes (C), and Cyp26A1 (D) enzymes was measured in VF isolated from sham-operated (sh, n=5) and ovariectomized (OVX, n=4) WT female mice (same study as Fig. 3B). Insert shows Aldh1 expression in WT male (M; n = 4) and female (F; n = 5) group on a chow diet (same as in Fig. 1, 0 time). Data are shown as mean ± SD. P value was determined by Mann-Whitney U test.

Weight (E) and VF mass (F) in sham-operated and ovariectomized WT (Sham, n = 10; OVX, n = 8) and Aldh1a12/2 (Sham, n = 9; OVX, n = 5) mice. Insert shows expression levels of Aldh1a2 and -a3 enzymes in sham and OVX Aldh1a1−/− mice (n = 5/group). Data are shown as percent of values seen in WT sham-operated mice (dashed line).

(G) Glucose tolerance test (GTT) in WT (open circles, sham-dashed and ovx-solid lines) and Aldh1a1−/− (closed circles, sham,- dashed and ovx,- solid lines) mice (n=4 in WT sham and n=5 in all other groups). *, P<0.001 differences between genotypes in sham or in ovx group; X, differences (P<0.04 or less) between sham and ovx mice within one genotype.

(H) Insulin levels were measured by ELISA in plasma isolated from sham-operated and OVX WT and Aldh1a12/2 mice (n = 4/group). Insulin levels were not significantly different (Mann-Whitney U test). n.s., N.s. not significantly different by two-way ANOVA.
Figure 3.4. Estrogen contributes to sex-specific expression of \textit{Aldh1} enzymes in VF [105].
Figure 3.5. Depot-and sex-specific RARE activation accompanies HF diet-induced fat formation in RARE-lacZ mice.

(A) mRNA β-galactosidase expression in VF explants from RARE-lacZ male mice treated with RA and Rald (both 10 nM) for 3h in 1% UV-treated FBS in DMEM. Immediately after excision explants were transferred to 1% UV-treated FBS in DMEM for 12h prior to stimulation with RA. P, significant induction by RA, but not Rald.

(B&C) mRNA Cyp25a1 (B) and Cyp26b1 (C) expression in VF explants from RARE-lacZ male mice treated with RA and Rald (both 10 nM) for 12h in 1% UV-treated FBS in DMEM. These RAR target genes are under control of a natural RARE promoter and required longer incubation time for expression upon induction by retinoids.

(D&E) Relative weight (fat pad mass per body mass) of subcutaneous (inguinal) (D) and visceral (perigonadal) (E) fat in male ‘M’ and female ‘F’ RARE-lacZ mice receiving regular chow (white bars) and HF diets for 5 months. Data are shown as mean±SE, n=5 in each of the four groups. Throughout this legend: P_HF indicates significant differences between mice (same sex group) receiving a chow or HF diet. P_S indicates significant differences between males and females receiving similar chow diets. Insert shows the significantly higher ratio of visceral to subcutaneous fat in RARE-lacZ female compared to male mice on a HF diet.

(F) Relative β-galactosidase expression in subcutaneous and visceral fat isolated from RARE-lacZ males and females on a regular chow diet. β-galactosidase expression was normalized to 18S expression by Ct method. P_S indicates significantly higher β-galactosidase expression in male compared to female VF.

(G) Increase in β-galactosidase expression (%) in adipose depots in RARE-lacZ mice receiving HF compared to chow diet (100%, dashed gray line). P_HF indicates significant differences between mice (same sex group) receiving chow (shown in C) or HF diet. P_S shows significant induction of β-galactosidase expression (%) in female compared to male VF.
Figure 3.5. HF diet-induced depot-and sex-specific RARE activation in RARE-lacZ mice [105].
Table 3.2. Biomorphic data of human subjects [105].

Differences in biomorphic data and serum levels of lipoproteins and inflammatory markers in lean and obese men and women. *,# versus same sex control, \( P<0.05 \) via; # Lean both sexes vs Obese both sexes, \( P <0.05 \). Statistical analysis was performed using 2 way ANOVA with Bonferroni post-test.

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Figure 3.6. Adiposity in humans is accompanied by increased Aldh1 expression in visceral SVF cells in obese vs. lean subjects and increased release of NEFA by Rald.

Schematic of hypothetical Aldh1 role in sexual dimorphism in visceral fat.

(A) Aldh1 expression was analyzed in stromal vascular cells (SVF) isolated from visceral (omentum) fat from lean (men, n=6 and women, n=4) and morbidly obese (men, n =3 and women, n=7) patients by Taqman assay and normalized to hypoxanthine phosphoribosyltransferase 1. P, significance levels determined by 2-way ANOVA with Bonferroni post-test.

(B) NEFA release from 13d-differentiated human SGBS adipocytes after stimulation with vehicle (Veh), Rald (100nM), RA (100nM), and E2 (10nM) for 3h in medium containing 2% delipidated BSA (n=3 per group). P, significant changes in NEFA released from Rald-stimulated SGBS adipocytes compared to both vehicle and RA-treated group.

(C) Males and females have different expression of Aldh1 enzymes, which regulate dissimilar Rald conversion to RA. Estrogen represses Aldh1a3 expression whereas HF diet consumption increases Aldh1a1 expression in women. RA has multiple genomic effects in adipose tissue and regulates expression of numerous transcription factors, culminating in the expression of PPARγ (reviewed in [165]), adipocyte differentiation and fat formation. RA also regulates non-genomic effects (dashed lines) through cytosolic RAR and/or mTOR in different tissues (32); however the relevance of these signaling pathways for adipose tissue has not been established. In the absence of Aldh1a1 and reduced expression of a2 and a3 in females, Rald is not converted to RA and supports ATGL-mediated lipolysis in VF, resulting in a resistance to HF diet-induced fat formation in this depot. Atgl-mediated lipolysis can play a causative role in the induction of thermogenesis through the generation of PPARα ligands, which activate PPARα and its target gene Ucp1 (184,185). Rald has also been shown to suppress PPARγ and RXR activation and fat formation (104). Here we show Rald’s role in the fast non-genomic regulation of ATGL protein levels, a triglyceride-hydrolyzing protein in adipose tissue.
Figure 3.6. Increased Aldh1 expression in visceral SVF cells in obese vs. lean subjects and increased release of NEFA by Rald. Schematic of hypothetical Aldh1 role in sexual dimorphism in visceral fat [105].
CHAPTER 4

Translational Regulation of ATGL by mTORC1

4.1 Introduction

Adipose tissue stores excess energy in the form of triglycerides (TG) and releases this free fatty acid from TG via activation of lipolysis in response to increased energy needs. Deregulation of these processes are associated with metabolic disorders, such as obesity and lipodystrophy [193]. Adipose tissue lipolysis is governed by three lipolytic enzymes, Atgl [180,181], HSL[130,194] and MGL[195]. ATGL cleaves the first ester bond of a triglyceride. ATGL is upregulated on a high-fat diet [182] and is activated by PKA and AMPK. In recent years, Atgl was identified as a PPARγ target gene [179,196]. In chapter 3, we found significantly higher ATGL protein levels in the visceral depots of female Aldh1a1^tm1Gdu mice contributing to thermogenic remodeling. However, the underlying mechanism of ATGL regulation by retinoids was not dissected. mTOR, a serine/threonine protein kinase, is known to regulate translation by phosphorylation of p70S6 kinase and elongation factor binding protein (4E-BP1) [197]. One of the complexes of mTOR - mTORC1, is a well-established nutrient sensor that plays a key role in adipogenesis and diet-induced fat formation in rodents [30,75,198] and humans [199]. Since we found that Rald regulated ATGL at the translational level (described in
chapter 2), we wanted to examine if this regulation is mediated by mTORC1. Although a recent study showed that stimulation of neurons with RA rapidly activated mTORC1 [32], whether vitamin A metabolites regulate mTORC1 activation in obesity still remains unknown. Here, in a pilot study, we investigated such regulation.

Understanding the regulatory mechanisms underlying Rald-mediated ATGL induction will provide better understanding of vitamin A role in adipose tissue as well as the pathophysiology of obesity and other metabolic disorders.

4.2 Materials & Methods

Reagents: We purchased reagents from Sigma-Aldrich and cell culture media from Invitrogen unless otherwise indicated; antibodies to phosphop70s6 kinase (Thr389) (p70S6K_P) were from Millipore, to total p70S6K, ATGL, and GAPDH were from Cell Signaling Technology, β-ACTIN and TUBULIN were from Abcam; infrared labeled secondary antibodies were from LI-COR. We used all-trans retinoid isomers.

Animal Study: As described in CHAPTER 3 (Materials & Methods section).

Ovariectomy Study: As described in CHAPTER 3 (Materials & Methods section).

Cell Culture: As described in CHAPTER 3 (Materials & Methods section).

Transfection studies: 3T3-L1 cells were stably transfected with lentivirus containing scrambled shRNA (scr control) or raptor shRNA (Raptor−/−) according to genecopoea protocol. Transfected cells were selected in 1µg/mL puromycin for 48 hours. Stable clones were selected and derived from the single cells selected by puromycin in three
weeks. (0.75-1.5mg/mL, Invitrogen, Grand Island, NY). Scr and Raptor\(^{-}\) fibroblasts were differentiated for 6 days and starved for additional 48h in a medium containing 1% UV treated FBS.

### 4.3 Results & Discussion

The etiology of visceral obesity is not fully understood, although it is evident that sex hormones govern fat distribution between subcutaneous and visceral depots [9]. Described in chapter 3, we showed that the Aldh1A1 enzyme generating RA is essential for the formation of visceral fat in female mice. Our conclusion is based on the comparison of C57BL/6J (WT) and Aldh1a1\(^{-/-}\)-tm1Gdu mice responses to a high-fat diet and to estrogen deprivation induced by ovariectomy. Aldh1a1 deficiency was sufficient to nearly prevent visceral fat formation in these two models [105]. This response was sex-specific, as Aldh1a1\(^{-/-}\)-tm1Gdu and WT males developed similar amounts of visceral fat mass on a high-fat diet. Proteomic analysis revealed sex-specific increase in ATGL, in the female Aldh1a1\(^{-/-}\)-tm1Gdu visceral fat. We were interested to examine the underlying mechanism and the participation of vitamin A metabolites regulating ATGL in this depot.

mTORC1 regulates many cellular processes, including cell growth, autophagy, nutrient transport, and protein synthesis [24, 74, 75]. It controls protein synthesis via its downstream targets S6k and 4E-BP [76, 77]. Rapamycin, a classic inhibitor of mTORC1, is known to inhibit adipocyte differentiation, implicating mTOR in adipogenesis [74].

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Our Aldh1a1\textsuperscript{-/-}\textsuperscript{tm1Gdu} mice has striking similarity with adipose-specific raptor null mice [105]. Raptor is the key regulatory protein of mTORC1, and raptor deficiency resulted in lean mice with significantly less adipose mass, improved insulin sensitivity, and browning of adipose tissue [30]. These mice were resistant to HF-diet induced obesity with increased thermogenesis, similar to what we see in Aldh1a1\textsuperscript{-/-}\textsuperscript{tm1Gdu} mice. However, the role of vitamin A metabolites in mTORC1 regulation is much less explored. A recent study showed that stimulation of neurons with RA rapidly activated mTORC1 [32], however, whether vitamin A metabolites regulate mTORC1 activation in obesity still remains unknown. Here, in a pilot study, we examined ATGL regulation by mTORC1.

To examine if mTOR inhibition affects ATGL protein levels, differentiated (8d) 3T3-L1 fibroblasts were stimulated with indicated concentrations of rapamycin (inhibitor of mTORC1) for 70 min (Figure 4A). Rapamycin treatment increased ATGL levels in a dose-dependent manner. 70 min incubation is sufficient to see kinase signaling. Studies have reported that 30 min incubation with RA can significantly induce MAP kinase [32]. We did not see any change in Atgl mRNA level. This result suggests that mTOR inhibition results in increased ATGL levels.

Since raptor is the major regulatory unit of mTORC1, to investigate direct regulation of ATGL by mTORC1, we created stable raptor knockdown and scrambled control using 3T3-L1 cell line. ATGL levels were significantly higher in differentiated raptor\textsuperscript{-/-} compared to Scr control (Figure 4B), which suggests a direct regulation of ATGL by mTORC1. Next, to test if retinoids exert their effects through mTORC1, we performed short-term stimulation of HEK293 cells and 3T3-L1 adipocytes with or without Rald and RA in the presence of insulin and subsequently analyzed these cells for p70S6K
phosphorylation by mTORC1. We have found that Rald acted in a rapamycin-like fashion and inhibited p70S6K phosphorylation in HEK293 cells in differentiated 3T3-L1 adipocytes (Figure 4C). In contrast, RA activated p70S6K phosphorylation in these experiments (Figure 4C). We examined if decreased RA production in the Aldh1a1−/− adipocytes contributes to mTORC1 inhibition and decreases p70S6K phosphorylation. We found that insulin treatment of WT and Aldh1a1−/− adipocytes increased p70S6K phosphorylation in WT adipocytes, but Aldh1a1−/− adipocytes had significantly reduced levels of phosphorylated vs. non-phosphorylated p70S6K (Figure 4D). Rapamycin significantly inhibited p70S6K phosphorylation in WT adipocytes, but did not have a statistically significant effect in Aldh1a1−/− adipocytes suggesting that mTORC1 activity was inhibited in Rald-generating Aldh1a1−/− adipocytes.

We also examined whether Aldh1a1 deficiency alters mTORC1 activation in vivo. Previously, we had found that only visceral fat of Aldh1a1−/−tm1Gdu female mice had impaired RA generation (Chapter 2). Consistent with the changes in RA production, we found significantly reduced p70S6K phosphorylation only in visceral fat of Aldh1a1−/−tm1Gdu females on a high-fat diet (Figure 4E). Of note, these differences were seen in starved (18h) mice without insulin treatment, and, represent ‘basal’ differences in mTORC1 activation. Previous genetic studies in mice with disrupted mTORC1 pathways highlighted increased thermogenesis as a key mechanism responsible for the resistance to diet-induced obesity seen in these mice [30,207]. Aldh1a1−/−tm1Gdu females were also resistant to HF-diet induced visceral obesity and had increased ATGL and UCP1
expression in visceral fat. However the contribution of mTORC1 to the thermogenic remodeling of visceral fat remain to be determined.

mTORC1 regulation was also impaired in a female model of obesity, in ovariectomized Aldh1al<sup>−/−</sup>tm1Gdu females. To distinguish between diet-induced and estrogen-dependent visceral obesity, ovariectomized mice were on a regular chow. WT ovariectomized mice had significantly up-regulated of Aldh1a3 expression (as previously described in chapter 3) and moderate increase in p70S6K phosphorylation that accompanied the increased visceral fat formation. On a chow diet, the deficiency in RA-generating enzymes in Aldh1al<sup>−/−</sup>tm1Gdu ovariectomized females resulted in decreased phosphorylated p70S6K and 4EBP1 in Aldh1al<sup>−/−</sup>tm1Gdu compared to WT ovariectomized mice (Figure 4F,4G). These Aldh1al<sup>−/−</sup>tm1Gdu mice maintained visceral fat levels identical to the sham-operated mice. In consonance with the suppression of these mTORC1 downstream targets, ATGL had increased protein levels in the visceral fat of Aldh1al<sup>−/−</sup>tm1Gdu versus WT ovariectomized mice (Chapter 3). Paradoxically, UCP1 and PPARγ expression was higher in WT than in Aldh1al<sup>−/−</sup>tm1Gdu ovariectomized mice. Probably, a primary mechanism for fat formation inhibition in Aldh1al<sup>−/−</sup>tm1Gdu ovariectomized mice was via mTORC1 dependent PPARγ inhibition by high Rald/RA ratio. A decrease in mTORC1 activation in adipocytes can change multiple adipose tissue responses and glucose tolerance [197]. The targeted disruption of the mTORC1 catalytic subunit, raptor, in adipose tissue renders these mice resistant to diet-induced obesity and glucose intolerance. [30]. We found that Aldh1a1 deficiency in mice decreases RA production and increases Rald levels only in females, resulting in sex-specific mTORC1
inhibition. Our \textit{in vivo} data, combined with our previous \textit{in vitro} observations, demonstrate that Rald and RA regulate mTORC1 in opposite fashions, with Rald inhibiting, and RA activating mTORC1. Moreover, we found that Rald-mediated induction of ATGL is regulated by mTORC1. Such finding unravels how vitamin A metabolites can regulate key lipolytic enzymes, such as ATGL via mTORC1. Further studies analyzing the influence of the types of nutrients (i.e. glucose, insulin, amino acid) on ATGL regulation through mTORC1 will lead to a much deeper understanding on the underlying mechanisms of mTOR regulation.
Figure 4.1. Inhibition of mTORC1 pathway increases ATGL protein levels in differentiated adipocytes and is mediated by Rald.

(A) ATGL levels in differentiated (8d) 3T3-L1 fibroblasts that were starved for 48h and then stimulated with indicated concentrations of rapamycin for 70 min.

(B) 3T3-L1 cells were stably transfected with lentivirus containing scrambled shRNA (scr control) or raptor shRNA (Raptor−/−). Transfected cells were selected in 1µg/mL puromycin for 48 hours. Clones were derived from a single cell. The deficient raptor mRNA expression in Raptor−/− fibroblasts were confirmed by TaqMan analysis (left panel). Scr and Raptor−/− fibroblasts were differentiated for 6 days and starved for additional 48h in a medium containing 1% UV treated FBS. Western blot shows increased ATGL protein levels in Raptor−/− compared to scr control adipocytes (right panel). Data shown as mean ±SD. P < 0.05.

(C) Phosphorylated (S6K_P, gray bars) and non-phosphorylated p70S6 (white bars) kinase levels in HEK293 cells (left panel) or differentiated for 6 d in 3T3-L1 adipocytes (right panels) measured by western blot. Cells were deprived from serum for 18h then stimulated with 100nM insulin/20%FBS (ins, 30min) with or without rapamycin (rapa), RA, and Rald (all 100nM, added 10min after insulin). *, P<0.05 (n=3, mean±SD) (right panels) shows a western blot example, S6K_P and S6K antibodies were from Cell Signaling).

(D) Phosphorylated (S6K_P) and total protein levels of p70S6 kinase (S6K) were measured in WT(FWT) and Aldh1a1−/− (FAldh1A1KO) differentiated fibroblasts (6d, starved for 48h) stimulated with 100nM insulin/20%FBS with or without rapamycin (100nM) for 30min using western blotting. Protein levels were quantified and normalized to tubulin levels. *, P<0.05 (n=3, mean±SD) difference compared to insulin-stimulated FWT.

(E) Western blot analysis of S6K_P (left panel), and p70S6 kinase levels in visceral fat from WT and Aldh1a1−/− male and female mice (all n=4, mean±SD) on a high-fat diet. Protein levels were normalized to Gapdh or tubulin for quantification. Right panel shows the ratio of S6K_P to p70S6 *, P<0.05 differences between genotypes; S, P<0.05 differences between sexes within one genotype. S6K_P antibody recognizes protein in insulin-stimulated control cell lysates.

(F&G) Western blot analysis of S6K_P and p70S6 kinase (F), 4Ebp1_P and total 4Ebp1, and (G) in WT and Aldh1a1−/− sham and ovariectomized female mice (all n=4, mean±SD). Insert shows a representative western blot in two ovariectomized WT and Aldh1a1−/−tm1Gdu mice. Protein levels were normalized to β-actin. p70S6 *, P<0.05 differences between genotypes; S, P<0.05 differences between sexes within one genotype.
Figure 4.1. Inhibition of mTORC1 pathway increases ATGL protein levels in differentiated adipocytes and is mediated by Rald.
CHAPTER 5

Epilogue

Visceral fat contributes to adverse metabolic effects and is considered a pathogenic fat depot. Understanding the diverse pathways regulating visceral fat formation can provide us information regarding the pathogenesis of metabolic disorders. This thesis identifies novel pathways involving endogenous vitamin A metabolites as regulators of sex-and-depot-specific fat formation. In chapter 2, the expression of vitamin A metabolizing enzymes was examined and offered insight into possible depot-specificity in RA production [168]. In vitro, adipogenesis in 3T3-L1 cells was accompanied by increased expression of Aldh1a1, which was responsible for approximately 70% of RA production. However, all three ALDH1 enzymes appeared to be redundant in RA production, and forced expression of Aldh1a1 or a3 enzymes could partially rescue adipogenesis in fibroblasts deficient in all ALDH1 enzymes [168]. This concerted autocrine action of the ALDH1 enzymes appears to establish varying levels of RA and transcription factor expression in visceral and subcutaneous depots in mice. In addition to their common feature in RA production, differential expression of ALDH1 enzymes appears to exert specific responses correspondent to their expression in different depots [168]. For instance, Aldh1a3-deficient fibroblasts expressed reduced levels of adipokine visfatin, and expression of Aldh1a1 could not compensate for this effect. These
observations could indicate some specific signaling mechanisms activated by ALDH1 enzymes or their products/substrates. The causal evidence of ALDH1A1’s participation in the formation of depot-specific fat depots came from observations in Aldh1a1−/−tm1Gdu mice. Deficiency in Aldh1a1 leads to impaired accumulation of fat in visceral more than in subcutaneous regions in Aldh1a1−/−tm1Gdu compared to WT female mice. Although Rald remains the preferential substrate for ALDH1A1 under physiologic conditions, it is known to utilize other aldehydes including 3-deoxyglucosone [99] and lipid aldehydes [222]. In the context of autocrine RA production, the signaling properties of other aldehydes and their respective acids have not been explored here.

Negative feedback is a common regulatory mechanism preventing enzymes from product overproduction. We showed that differentiated 3T3-L1 adipocytes respond to RA treatments by inhibition of predominantly expressed Aldh1a1 enzyme in a RA concentrations dependent manner [168]. In agreement, a recent study showed that treatment with 2 μM exogenous RA led to a 12-fold reduction in cellular RA levels in differentiated NIH-3T3-L1 cells [118]. Treatment with RA also decreases expression of its binding protein CRABP-II [223]. We used RA concentrations starting with 100nM, which falls in the saturation range and incubated differentiated adipocytes for 12 hours, which is commonly used time frame to study transcriptional responses. In our study described in chapter 2, we found that autocrine RA production in adipogenesis regulates PPARγ by alternative pathways. Aldh1 loss-of-function experiments revealed that ZFP423 expression was reduced by 99%. Both RA and forced expression of Aldh1 enzymes effectively recovered ZFP423 expression. Although precise mechanisms by
which RA regulates ZFP423 remain to be determined, the Aldh1-dependent induction of ZFP423 appears to play a major role in the depot-specific regulation of PPARγ and fat formation. Reduced expression of ALDH1 enzymes in visceral fat of Aldh1a1−/−tm1Gdu mice resulted in a marked 70% suppression of ZFP423 expression. In subcutaneous fat of Aldh1a1−/−tm1Gdu mice, ZFP423 levels were similar to those seen in WT mice, probably due to the remaining expression of Aldh1a3. We also considered C/EBPs as a possible pathway through which RA could regulate PPARγ. However, in our study, C/EBPα and C/EBPβ expression could not be conclusively linked to Aldh1 function in RA generation. The mechanisms by which RA induces ZFP423 remain to be elucidated; however, ZFP423 has been implicated in the activation of RARE in neuroblastoma cells potentiating RA effects [224]. Thus, important cross-regulation might exist between RAR and ZFP423 pathways that influence differentiation in many tissues. Future studies need to address autocrine mechanisms in human adipose tissues and their relevance in the regulation of the transcriptional network that is ultimately responsible for the endocrine and lipolytic differences between fat depots.

Interestingly, the regional differences in fat formation in humans are also associated with Aldh1 enzyme expression, although the pattern of Aldh1 expression is dissimilar to that of rodents [168]. In women, Aldh1a2 and a3 (also known as Raldh2, Raldh3) expression was significantly higher in visceral (omentum) than in subcutaneous fat, even though Aldh1a1 remained the predominantly expressed enzyme in both tissues. This difference, apart from having different progenitors and diet composition, could be due to hormonal influences, or even genetics. One important factor to note here is, stromal
vascular fraction (SVF) obtained from these human fat tissues were differentiated under cell culture conditions, which may also contribute to differences in enzyme expression. Our discovery of ALDH1A1 as a major contributor to visceral adiposity gives us the rationale to study the $Aldh1a1^{-/-}\text{tm1Gdu}$ mouse model further to understand how fat formation is regulated by vitamin A metabolites. More studies in larger patient cohorts will help us to understand whether $Aldh1$ expression and concentrations of dependent metabolites, RA and Rald, are linked to visceral obesity in humans and if these can predict propensity to visceral obesity in response to obesogenic diets in specific ethnic or disease populations.

Fat accumulation in visceral depots is associated with several metabolic disorders. Men and women differ with respect to preferential fat distribution. Men tend to accumulate more fat in visceral depots [162], whereas women store more fat in sc regions. However, this pattern of fat distribution is known to be altered once women reach menopause or consume a HF-diet, leading to more fat deposition in the abdominal region [163,164]. Our study described in chapter 3, examines how HF-diet and lack of estrogen, both can affect autocrine RA production and thus fat distribution, using various mouse models : C57BL/6J, $Aldh1a1^{-/-}\text{tm1Gdu}$, transgenic RARE and ovariectomized C57BL/6J mice [105]. We found that only female $Aldh1a1^{-/-}\text{tm1Gdu}$ mice were resistant to HF diet-induced visceral fat formation, while sc fat remained similar in both male and female groups. Such sex-specific differences in visceral fat accumulation was attributable to significantly higher protein levels of adipose triglyceride lipase (ATGL), a key enzyme involved in lipolysis.
RA is known to exert multiple effects on adipocyte biology. Its role as a pro-adipogenic or anti-adipogenic hormone still remains controversial. Various groups have reported that RA treatment (high dose) reduces body weight and adiposity [170, 227]. Berry et al. showed that RA, at pharmacological dosage, prevents obesity through mechanisms involving both RAR and PPARδ pathways [137]. However, since genetic deficiency in PPARδ is not associated with an obesity phenotype [225], further research will help us to understand RA involvement in the activation of PPARδ, RARs, and RXRs, or combinations of these receptors in vivo. CRBPI−/− and BCMO1−/−tm1Dnp mice were obese on a HF diet [228, 229]. CRBPI promotes retinol esterification; therefore, absence of this enzyme may induce obesity through mobilization of more retinol for the production of endogenous RA. Compared to wild type, CRBPI−/− mice had increased epididymal fat mass [228]. Consistent with this finding, PPARγ and its downstream target genes FABP4 (aP2), adiponectin, and LPL were found to be increased in WAT of CRBPI−/− compared to WT mice. Studies from BCMO1 knockout mice have demonstrated a close connection of vitamin A metabolism to the regulation of PPARγ expression [138, 229], however, it is not known how vitamin A metabolites mediate these responses. BCMO1 cleaves β-carotene into two molecules of Rald that could be further converted either to retinol or RA, leaving a possibility that these metabolites could also influence adipose tissue. Hormone sensitive lipase-null mice lack retinyl ester hydrolase activity in WAT and, consequently, have increased levels of retinyl esters and decreased levels of retinol, Rald, and all-trans RA. Impaired adipogenesis and increased thermogenesis in the WAT render HSL−/−tm1land mice resistant to HF-diet-induced obesity [230]. Together, these studies demonstrate that mobilization of retinol from retinyl esters
by HSL and its further metabolic conversion to RA is critical for the obesogenic responses of WAT to a high-fat diet; the absence of retinyl ester-hydrolyzing activity of HSL leads to the preferential formation of BAT [64,230]. Moreover, when fed a RA-supplemented HF diet, lean $HSL^{-/-}$tm1Land mice could partially recover white adipose tissue mass [230]. The puzzling similarity in phenotypes of $Aldh1a1^{-/-}$tm1Gdu mice, deficient in the major RA generating enzyme, and mice treated with RA that experience induction in thermogenesis and reduction of white fat depots warrants further examination [137, 149]. We speculate that RA produced in a regulated manner by an autocrine mechanism can exert more specific effects than RA treatments, especially those with high RA (µmol) concentrations. At these pharmacological levels, RA can potentially induce multiple transcription factors and also bind to proteins [151]. Detectable levels of retinoylated proteins in 3T3-L1 adipocytes were found after treatment with 100 nmol RA [151]. Further work needs to examine the contribution of retinoylation reactions to adipogenesis. Moreover, negative feedback loop suppressing $Aldh1a1$ expression could also contribute to reduced adipose mass, in addition to RA’s role to induce lipolysis. To date, very few literature has reported RA effects on visceral adiposity. Recently, Kiefer et al. reproduced many of our findings in adipose tissue [172]. They found similar thermogenic remodeling in visceral adipose tissue in $Aldh1a1^{-/-}$tm1Gdu female mice, however did not dissect sex-specific differences and only studied ALDH1A1.

In our study (chapter 3), we compared beta-galactosidase expression (indicating intrinsic RA production) in transgenic mice expressing RA receptor response element (RAREtg) which revealed HF diet-induced striking increase (860%) in RA generation in
female visceral fat, while visceral fat of RAREtg male mice lacked such association (we also found more visceral fat accumulation in WT female mice on a HF-diet). However, we did not find similar increase of beta-galactosidase in male visceral fat, despite increase in fat mass. This could be due to some RAR-independent mechanisms. Unliganded RAR, can impart effects through non-genomic mechanisms, also many different transcription factors involved in fat formation may also play a role. Amplified Glucocorticoid (GC) action can also lead to obesity [64]. Glucocorticoid excess produces cushing’s syndrome, which is characterized by visceral obesity, insulin resistance, and diabetes. Visceral fat is known to have higher glucocorticoid receptor density, therefore, this tissue is likely to be more affected by local GC action. 11-β-hydroxysteroid dehydrogenase 1(11βHSD1), expressed in the liver, adipose tissue and central nervous system, catalyzes the conversion of cortisone into cortisol. A transgenic male mouse model overexpressing 11βHSD1 (adipose-specific) develop visceral obesity and metabolic syndrome, suggesting GC role in the pathogenesis of obesity [64, 226].

Currently, the only available treatments utilizing retinoids are limited to acne/psoriasis treatments by 13-cis RA and acute promyelocytic leukemia by all-trans RA. However, these treatments are associated with serious side effects known as “RA syndrome” [169]. RA syndrome is characterized by dyslipidemia and obesity, supporting RA’s role in adipose tissue. Our novel findings described in this thesis could contribute to the formulation of therapy targeting ALDH1A1 inhibition. However inhibition of ALDH1A1 is not so simple, as it is an important enzyme in many tissues. We have adopted a different approach in overcoming this problem. We propose to encapsulate
Aldh1a1−/− fibroblasts into poly-L-lysine encoated microcapsules and inject these capsules in visceral fat depots in C57BL/6J mice. We reported increased UCP1 protein levels, reduced visceral adipose mass and triglyceride levels upon injection of Aldh1a1−/− containing microcapsules in the injected visceral depot [187]. Further studies evaluating the safety and inflammatory parameters would promote similar studies using larger animals.
REFERENCES


APPENDIX A: Additional data from chapter 1 & 2

Description of methods for chapter 2:

Human Subjects: Patients’ fasting plasma glucose concentrations did not exceed 120 mg/dL, they were not taking TZDs or steroids, and they had no reported malignancies. mRNA was isolated from visceral (omental) fat tissue, which was obtained from healthy women who underwent surgery as kidney donors and had given informed consent. All subjects had fasted at least 12 h. Abdominal subcutaneous (external to the fascia superficialis) and greater omental fat were obtained from each subject. Fat tissue was minced and then digested in phosphate buffered saline (PBS) containing 1 mg/ml collagenase in a shaking water bath (37°C). Digests were filtered and centrifuged at 800 g for 10 min. The digests were treated with an erythrocyte lysis buffer and then plated in alpha minimal essential medium (α-MEM) supplemented with 10% bovine serum and antibiotics. To eliminate endothelial cell and macrophage contamination, the adherent preadipocytes were replated after 12 h at a density of 4 ± 104 cells/cm2 in plating medium as described before [48]. The absence of macrophages in subcultured preadipocytes was confirmed by absence of macrophage markers assayed by quantitative RT-PCR. Preadipocytes were differentiated as described previously [48] using plating medium without serum supplemented with 100 nM dexamethasone, 500 nM human insulin, 200 pM triiodothyronine, 0.5 µM rosiglitazone, antibiotics, and 540 µM
isobutylmethylxanthine (removed after 2 days). Cells were differentiated for 21 days.
Medium was changed weekly.

**Transient Transfection:**

day 1: Cells were plated at a density of $1.5 \times 10^6$ per 24 well plate and incubated at 37 degree overnight.

day 2: 800µl of medium from each well were taken out and 80µl of transfection reagent was added to each well. At the end of the day, or 5/6hrs after transfection, 300µl of high glucose DMEM with 10% FBS was added to each well.

day 3: Stimulation reagents in 1% delipidated serum containing medium (DLP) was prepared and added to each well and kept in the incubator overnight.

day 4: Following 15/16hr incubation, the cells were harvested (follow elisa-style). Cells were washed with PBS twice. Then 100µl 1X Passive Lysis Buffer was added to each well and incubated in the shaker for 15 minutes.

day 5: 20µl from each lysate was transferred to a 96-well plate and luciferase induction measured using a luminometer ()

**PPAR structure:**

![Figure A.1. General Structure of PPAR](image)

All nuclear hormone receptors share some structural similarity. They have the 1) N-terminus : A/B domain, which has transactivation activity, termed, Activation function-1 (AF-1). The sequence and length of the A/B domain is highly variable, between receptors (i.e. GR and RXR) and also among receptor subtypes (i.e. RXRα and RXRβ), 2) C domain is the most conserved DNA binding domain, which is composed of two zinc
fingers, 3) D domain is the hinge domain. It is assumed to allow some conformational change to the protein structure following ligand binding. It may also have nuclear localization signals and protein-protein interaction sites, 4) E/F is the ligand-binding domain. X-ray crystal structure analyses show that PPARs have a larger ligand-binding pocket compared to other nuclear hormone receptors, which explains why PPARs can accommodate a wide range of natural and synthetic ligands. Activation of the ligand binding domain requires the presence (and activation) of Activation Function -2 (AF-2), located at the end of the C-terminus. AF-2 also participates in ligand-dependent degradation mediated by the ubiquitin-proteasome pathway. PPAR heterodimerizes with the RXR family members, through the E/F domain as well [221].
## APPENDIX B : Additional data from chapter 3

**Table B.1.** Gene expression in VF isolated from WT males (n=5), WT females (n=4), Aldh1a1<sup>−/−</sup> males and females (both, n=4) on a HF diet (180d) was examined by TaqMan quantitative PCR using 18S as normalization control. Significance was examined between males and females in each genetic group (columns ‘WT’ and ‘KO’) as well as between WT males/KO males, and WT females/KO females (Column ‘P value’) by nonparametric Mann-Whitney U test and Kruskal-Wallis rank test (for *Ucp1*).

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Figure B.1. Validation of responsiveness of RARE (Tg(RARE-Hspa1b/lacZ)12Jrt/J to nanomolar RA concentrations and β-galactosidase primer.

(A&B) Representative staining of β-galactosidase protein expression (brown staining) in hepatic and adipose tissues from RARE-lacZ injected with vehicle or RA (B), but no staining in C57/BL6J (WT) mice (A). WT or transgenic male mice (12-15 weeks old, regular chow) expressing β-galactosidase (lacZ) gene under the control of the RARE (Tg(RARE-Hspa1b/lacZ)12Jrt/J were injected 3 times (every 48 hours) with 1mL PBS with or without RA (500nM). Immediately after third injection mice were harvested, their hepatic and visceral adipose tissue were embedded in paraffin and stained with antibody to β-galactosidase (Abcam) according to manufacturer instructions. Brown staining indicates. 10x magnification.

(C) mRNA β-galactosidase expression measured using TaqMan assay in livers isolated from WT and RARE-lacZ mice. (12-15 weeks old, regular chow). n=3. Throughout this legend: P was determined by Mann-Whitney U test.
Figure B.1. Validation of responsiveness of RARE (Tg(RARE-Hspa1b/lacZ)12Jrt/J to nanomolar RA concentrations and β-galactosidase primer [168].
Figure B.2. Weight comparison between WT and Aldh1a1−/−tm1Gdu on HF/vitA 4IU versus HF/vitA 20IU.

A) & B) Body weight comparison between C57BL/6J (WT) and Aldh1a1−/−tm1Gdu male and female mice (n=5 for each group) fed a HF-diet with 4IU/g vitamin A vs. HF-diet with 20IU/g vitamin A content for 180 days. Data presented as mean±SD.

C) & D) Subcutaneous and visceral fat weight comparison between C57BL/6J (WT) and Aldh1a1−/−tm1Gdu female mice on the two diets for 180 days. Data presented as mean±SD.
Results:

Body weights of WT and Aldh1a1<sup>−/−</sup>tm1Gdu mice (both male and female) was similar after feeding HF-diets containing varying levels of vitamin A (4IU/g vs. 20IU/g, Research Diets, New Brunswick, NJ) for 180 days. Subcutaneous and visceral fat pads were dissected and weight compared between female mice of the two strains. Also, fat weight did not differ on the two diets, which suggests that moderate increase in vitamin A in the diet does not cause significant variation in weight.

Description of methods in chapter 3:

Immunohistochemistry:

Paraffin-embedded visceral fat tissue sections were de-paraffinized /hydrated using a series of washes: xylene, and then ethanol (various percentages). Then these sections were blocked with 3% BSA at room temperature, incubated with the primary antibody (UCP1, ATGL) overnight at 4°C, then incubated with a hydrogen peroxide-conjugated secondary antibody for 1h at room temperature. Finally, these tissue sections were stained with 3′3′-diaminobenedine+ (Dako, Carpinteria, CA) substrate. Hydrogen peroxide and BSA were obtained from Sigma Aldrich (St. Louis, MO). Secondary antibodies were obtained from Invitrogen. (Grand Island, NY). Primary antibodies against UCP1 and ATGL were purchased from (Abcam, Cambridge, MA) and (Cell Signaling, Denvers, MA).