The Association of CUZD1 and STAT5 in Adipocytes

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THE ASSOCIATION OF CUZD1 AND STAT5 IN ADIPOCYTES

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# TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................... iii

CHAPTER 1. GENERAL INTRODUCTION ......................................................................................... 1
  1.1. Adipocyte Biology ....................................................................................................................... 1
  1.2. Signal Transducers and Activators of Transcription (STATs) ................................................ 3
  1.3. CUB and Zona Pellucida-like Domain-containing protein 1 (CUZD1) .................. 4

CHAPTER 2. MATERIALS AND METHODS .................................................................................... 7
  2.1. Cell Culture ............................................................................................................................... 7
  2.2. Whole-Cell Extract Preparation ............................................................................................. 7
  2.3. Western Blotting ....................................................................................................................... 7
  2.4. Fractionation ............................................................................................................................ 8
  2.5. Immunoprecipitation ................................................................................................................ 8
  2.6. Antibodies ................................................................................................................................ 8
  2.7. Animals and Diets ..................................................................................................................... 9

CHAPTER 3. RESULTS ..................................................................................................................... 10
  3.1. CUZD1 is Present in the Adipocyte Nucleus ............................................................................. 10
  3.2. Regulation of CUZD1 in Adipocytes ......................................................................................... 12
  3.3. CUZD1 Interacts with STAT5A in the Adipocyte Nucleus ...................................................... 15

CHAPTER 4. DISCUSSION AND FUTURE DIRECTIONS ................................................................. 20

REFERENCES ..................................................................................................................................... 21

VITA .................................................................................................................................................... 26
ABSTRACT

Obesity is a metabolic disorder that has turned into a global epidemic over the past few decades. Adipocyte dysfunction brought on by obesity can lead to increased risk of metabolic diseases such as cardiovascular disease and type 2 diabetes mellitus (T2DM). Knowing how adipocytes function to regulate metabolic homeostasis is critical. CUZD1 (CUB and Zona Pellucida-like Domain-containing protein 1) has not been studied in the context of adipocyte biology but could play a role in several metabolic processes. CUZD1 has been suggested to be a biomarker for several inflammatory diseases and cancers but little functional data has been published. There is some evidence that CUZD1 can associate with STAT5 in mammary tissue. Novel research of CUZD1 in adipocytes could provide more insight into how adipocytes control whole-body homeostasis. To start understanding the role of CUZD1 in adipocytes, we examined its tissue localization along with its subcellular localization and observed that CUZD1 is expressed in the adipocyte nucleus. Using the cultured 3T3-L1 model system, we examined CUZD1 expression over a time course of adipogenesis as well as CUZD1 stability over a 2 hour period. Mouse models were used to explore CUZD1 expression patterns in different nutritional states. In mesenteric white adipose tissue (mWAT), CUZD1 expression was downregulated in the fasting state compared to the fed state. High-fat and low-fat feeding had no effect on CUZD1 expression. Immunoprecipitation was utilized to determine if STAT5 could be present in a protein complex with CUZD1. We observed that STAT5A interacted with CUZD1 following growth hormone (GH) stimulation. A mouse model lacking adipocyte STAT5 was used to examine if loss of adipocyte STAT5 had an effect on the expression of CUZD1. In inguinal white adipose tissue (iWAT) of female mice, increased expression of CUZD1 was observed in the STAT5 knockout model. Collectively, these studies suggest that CUZD1 may be involved in hormonal signaling by modulating the JAK/STAT pathway. Overall, the role CUZD1 plays in adipocyte biology remains unknown but this data provides the first steps toward its understanding.
CHAPTER 1. GENERAL INTRODUCTION

1.1. Adipocyte Biology

For a long period, the only function of adipocytes (fat cells) was thought to be storage of excess energy in the form of triglycerides. Today, it is understood that these cells play a much larger role in the body. Adipocytes not only store excess energy but serve as regulators of whole-body energy homeostasis through their insulin sensitivity and endocrine functions [1]. Disruption of normal adipocyte functions primarily leads to obesity, which is a major contributor to other diseases such as metabolic syndrome, type 2 diabetes mellitus (T2DM), and cardiovascular disease [2]. In the United States, the prevalence of obesity has increased by 11.4% over the past two decades [3]. In the same time frame, adult diagnosis of diabetes has doubled with at least 90% of diabetes diagnosis in the form of T2DM [4]. The significant increases in adult metabolic dysfunction along with the understanding that adipocytes are critical regulators of metabolic health has led to an increase in research studying what role adipocytes and adipose tissue play in metabolic health.

Adipose tissue exhibits a large amount of heterogeneity with adipocytes accounting for around 90% of the tissue's total volume but much less than 50% of its cellular content, depending on the specific adipose tissue depot. All other cell types are contained in the stromal vascular fraction which consists of preadipocytes, immune cells, fibroblasts, and endothelial cells [5]. Adipose tissue can generally be divided into white adipose tissue (WAT) or brown adipose tissue (BAT) based on its function. BAT is mainly associated with thermogenesis while WAT is mainly associated with energy storage [6-7]. More recently, beige adipocytes have been identified and are found in WAT but exhibit elevated levels of thermogenic markers found in BAT [7]. The exact role and function of these beige adipocytes within WAT continues to be controversial.

WAT’s main function is to store and release energy to maintain metabolic homeostasis. When energy is in surplus triacylglycerols (TAGs) are synthesized and stored within the adipocytes. When there is an energy deficit, adipocytes hydrolyze their stored TAGs to generate glycerol and free fatty acids (FFAs) [6]. White adipocytes are typically unilocular and store TAGs in their lipid droplet which occupies most of the cell and pushes the nucleus and other organelles closer to the plasma membrane [8]. In humans, WAT accounts for most of the body’s total adipose tissue volume and is generally characterized as subcutaneous WAT or visceral WAT. Subcutaneous WAT is located directly under the skin and makes up the majority of an individual's body fat, while visceral WAT primarily lines your internal organs and accounts for <20% of an individual’s body fat [9]. Increased accumulation of visceral WAT can cause tissue dysfunction and is closely associated with development of metabolic complications such as insulin resistance which can lead to T2DM [10]. Conversely, subcutaneous WAT
accumulation is not closely associated with obesity related metabolic risks such as insulin resistance and has been suggested to be metabolically protective [11]. Fat accumulation can have negative metabolic effects when lipid storage capacity is reached and lipid is ectopically deposited outside of adipose tissue [12].

BAT’s main function is to generate heat through non-shivering thermogenesis. In brown adipocytes thermogenesis requires the uncoupling of fatty acid oxidation from oxidative phosphorylation and ATP synthesis, via uncoupling protein 1 (UCP1), which allows the mitochondrial proton gradient to be used generate heat instead of ATP [13]. Brown adipocytes are multilocular and have a high mitochondrial content which contributes to their ability to produce heat and their brown color. In humans, BAT is abundant during infancy and decreases with age to account for only 1-2% of total body fat in adults [14]. In infancy, abundant BAT is necessary to maintain body temperature. Adults have little use for BAT thermogenesis. In adults, BAT can be thermogenically active but is now thought to play a role in energy metabolism [14,15].

To regulate whole-body metabolic homeostasis, through storage and release of energy, adipocytes must be able to sense and react to changes in energy availability. They accomplish this by responding to insulin and secreting several of their own hormones [6]. Insulin, released in the fed-state when energy is at a surplus, stimulates adipocytes to uptake glucose and synthesize TAGs for storage via lipogenesis. When energy is at a deficit adipocytes release their stored energy by breaking down TAGs and releasing FFAs and glycerol via lipolysis [16]. To further control metabolic homeostasis, adipocytes release hormones adiponectin, leptin, and resistin [17]. Adiponectin is thought to help maintain insulin sensitivity and has both endocrine and paracrine effects. It stimulates glucose uptake and fatty acid oxidation in both adipose tissue and skeletal muscle acts on the liver to suppress hepatic glucose output. It is also known to regulate adipogenesis, the formation of new adipocytes from precursor cells [18]. Leptin’s main action is to stimulate the hypothalamus to decrease food intake and increase energy expenditure. Leptin receptors are mainly found in the central nervous system (CNS) but have been found in other peripheral tissues as well, suggesting other possible roles for the hormone [19]. Resistin is thought to play a role in both metabolic and inflammatory pathways but the main function is debated and determining its role in metabolism is difficult because of the expression differences between mouse and human resistin. In mice, resistin is mainly secreted from adipocytes in WAT while in humans it is mainly secreted by immune cells such as macrophages in WAT [20]. Disrupting any adipocyte function can lead to an increased risk of obesity and associated metabolic diseases such as T2DM, cardiovascular disease, and stroke [2].

The formation of new adipocytes also plays an important role in maintaining metabolic health and disruption of adipogenesis can result in insulin resistance and metabolic syndrome [21]. Many molecular players and mechanisms such as transcription factors, signaling pathways, and epigenetic regulators control the
differentiation process. However, peroxisome proliferator-activated receptor gamma (PPARg) and CCAAT-enhancer-binding protein alpha (C/EBPα) are largely considered to be the master regulators of adipogenesis [22]. Knockouts of PPARg and C/EBPα in murine adipocytes have shown that PPARg is critical for adipocyte differentiation in the adult and embryonic stages, while C/EBPα is only important in the adult stage [23]. Signal transducers and activators of transcription 5A and 5B (STAT5A and B) also promote adipogenesis and their expression is induced by PPARg [24]. Although the main contributors are known, not all proteins that regulate the adipogenesis process and what their functions are have been identified.

Cultured 3T3-L1 adipocytes are widely used as a model system for studying adipogenesis and adipocyte function in vitro. 3T3-L1 cells originated from a line of mouse fibroblast cells (3T3 cells) and were isolated through selection for the ability to differentiate into cells with an adipocyte-like phenotype defined by lipid accumulation [25]. After growth arrest these cells can be stimulated to differentiate using a cocktail containing 3-isobutyl-methylxanthine, dexamethasone, and insulin resulting in 3T3-L1 cells with an adipocyte phenotype. Mature 3T3-L1 adipocytes are sensitive to insulin, produce adipokines, and act strikingly similar to adipocytes found in vivo. Being a homogeneous cell population this line does have limitations, such as lack of input from nerves other cell types found in adipose tissue [25]. Nonetheless, this culture cell model is used by thousands of scientists around the globe to study adipocyte development and function.

1.2. Signal Transducers and Activators of Transcription (STATs)

STAT proteins are a family of ubiquitously expressed transcriptional regulators. There are seven proteins in the STAT family designated STATs 1, 2, 3, 4, 5A, 5B, and 6. Each protein has a specific tissue distribution pattern and is responsible for regulating expression of tissue specific genes [26]. STATs primarily signal through the JAK/STAT pathway and are activated by cytokines and hormones such as interferon gamma, interleukins 6 and 11, leukemia inhibitory factor, oncostatin M, growth hormone and prolactin. Binding of a ligand to the appropriate receptor initiates a signaling cascade which activates STATs through tyrosine phosphorylation. Tyrosine phosphorylated STATs dimerize and are translocated to the nucleus where they modulate transcription [27]. Deletion of STAT genes in mice have shown unique functions for each protein in many cellular processes. Specific phenotypes were present in all transgenic mice, but normal development occurred except in STAT3 global knockouts which caused embryonic lethality [28].

STATs ability to regulate transcription is primarily controlled through the JAK/STAT signaling pathway. Janus kinase (JAK) proteins are tyrosine kinases which associate with cytokine receptors. When the ligand binds the receptor JAK becomes
active and phosphorylates tyrosine residues on the cytoplasmic domain of the receptor. The phosphorylation site on the receptor acts as a docking site for STATs and other signaling proteins [26]. Cytoplasmic STAT proteins are recruited to the receptor through the interaction of their SH2 domain with the receptor’s specific phosphorylated tyrosine residues. This interaction is important for determining which STAT is activated by the receptor [29]. After recruitment, JAK tyrosine phosphorylates the associated STAT causing dissociation of STAT from the receptor followed by dimerization with another phosphorylated STAT. Dimerization of STATs is mediated by interaction of each STATs SH2 domain with others phosphotyrosine [29]. The STAT dimer then translocates to the nucleus where it can bind DNA and activate or repress transcription of genes such as PPARg, C/EBPB, lipoprotein lipase, acyl CoA oxidase, fatty acid synthase, and pyruvate dehydrogenase kinase 4. In addition to binding gene promoters, STATs can bind co-activators and other proteins increasing its ability to regulate transcription [30].

In adipocytes, STATs play a significant role in adipocyte differentiation, lipid metabolism, and insulin action. STATs 1, 3, 5A, 5B, and 6 are expressed in adipocytes although hormonal activators have only been established for Stats 1, 3, 5A, and 5B. STAT1 and 3 have been found to be activated by a variety of cytokines while STAT5A and 5B are mainly activated by growth hormone and prolactin [31]. It is established that STAT5A and 5B are involved in adipogenesis but how exactly they contribute to the process is unclear. Early in the process, STAT5 proteins are phosphorylated and it has been shown that they can regulate PPARg promoter activity suggesting they have a role in regulating a crucial transcriptional regulator involved in adipogenesis [32]. In mature adipocytes, STAT5 proteins regulate de novo lipogenesis by repressing FAS transcription, and insulin action through transcription of pyruvate dehydrogenase kinase 4 (PDK4) in response to growth hormone (GH) or prolactin (PRL) [33,34].

1.3. CUB and Zona Pellucida-like Domain-containing protein 1 (CUZD1)

CUZD1 is a 607 amino acid protein that contains two CUB (complement subcomponent) motifs, a zona-pellucida like domain, and a transmembrane region. Very few studies have examined the exact function(s) of CUZD1 and there is currently very little known about how its domains contribute to its function. CUZD1 can be modified with N-acetyl-glucosamine at asparagines 394, 29, 419, 57, 67, and 271 but it is not known if it regulates its function. In several studies, CUZD1 has been identified as a biological marker for diseases like inflammatory bowel disease, Crohn’s disease, pancreatitis, and ovarian cancer but no functional information on how it is involved in these disease states has been elucidated [35,36,37,38]. Given the diseases it is associated with it could be suggested that it is somehow involved in the immune or inflammatory response, but this is not confirmed. One study involving Crohn’s disease
suggested that it could be released from the exocrine pancreas and play a role in the innate immune response inside the intestinal lumen [41].

Recent studies of CUZD1 in mammary glands have shed some light on the function of this protein. It is proposed that CUZD1 plays a critical role in mammary gland proliferation and differentiation during alveolar development associated with puberty, pregnancy, and lactation [39]. During pregnancy CUZD1 null mice showed significantly less mammary epithelial proliferation and a significant decrease in the expression of several genes in the epidermal growth factor (EGF) family in the mammary glands. The down regulated genes, Nrg1, Ereg, and Epng, produce ligands for ErbB receptors which start signaling cascades that control epithelial proliferation in mammary glands [39]. EGF gene transcription is controlled by the JAK/STAT5 pathway in response to PRL signaling and CUZD1 is thought to play a role in this signaling cascade. During pregnancy, CUZD1 null mice show a significant reduction of tyrosine phosphorylated STAT5 (pSTAT5) in the mammary epithelium but no overall reduction of STAT5 expression indicating that CUZD1 could play a role in the phosphorylation of STAT5 required for signaling [39]. In HC11 cells overexpressing CUZD1 (HC11-CUZD1), CUZD1 was shown to interact with both JAK proteins and pSTAT5 proteins. In these cells, treatment with PRL caused a significant increase in the levels of pSTAT5 compared to control HC11-LacZ cells with pSTAT5 localized primarily in the nucleus. In vivo, PRL treatment of control and CUZD1 null mice showed similar results. Treated control mice showed normal alveolar development, elevated expression and nuclear localization of CUZD1, and robust phosphorylation of and nuclear localization of STAT5 in the mammary epithelium. These results were absent in CUZD1 null mice [39].

Nuclear localization of CUZD1 along with pSTAT5 was also seen in HC11-CUZD1 cells treated with PRL and ChIP re-ChIP experiments showed that CUZD1 remains in a complex with STAT5 while STAT5 is bound to the DNA [39]. The same group later showed that overexpression of CUZD1 in the non-transformed HC11 mammary epithelial cells leads to increased proliferation and a cancerous phenotype. Injection of these cells into WT mice mammary glands lead to tumor growth. The isolated tumors showed increased CUZD1 expression, robust expression of STAT5 and pSTAT5, expression of EGF family targets of STAT5, and abundant active forms of ErbB receptors. These data suggest that overexpression of CUZD1 leads to increased proliferation and tumor growth by activating STAT5 and increasing expression of genes associated with mammary epithelial proliferation [40].

CUZD1 appears to play a role in hormonally activated JAK/STAT5 signaling associated with proliferation and differentiation in the mammary epithelium. Given the importance of the JAK/STAT5 pathway regulating key metabolic processes in adipocytes, including differentiation, studying CUZD1 in this context could help us better understand the regulation and mechanism of STAT5 signaling. No studies examining CUZD1 in adipocytes currently exist so I first looked into localization and expression
patterns of CUZD1 in adipocytes and adipose tissue. I then sought to establish a connection between STAT5A and CUZD1 and determine if this connection could be regulated by hormonal stimulation. Our lab was able to show that CUZD1 was expressed in all WAT depots and specifically in the adipocyte nucleus. CUZD1 protein expression was induced during the first 24 hours of adipogenesis and remained expressed at a stable level throughout the adipogenesis process. Protein expression of CUZD1 in iWAT and gonadal white adipose tissue (gWAT) was not regulated by nutritional state but expression in mWAT was decreased during the fasted state. In cultured adipocytes CUZD1 was found to be in a protein complex with STAT5A and this association was strengthened in the presence of GH. Together this suggests a potential role for CUZD1 in adipocyte biology, specifically in the processes of adipogenesis and hormonal regulation.
CHAPTER 2. MATERIALS AND METHODS

2.1. Cell Culture

Murine 3T3-L1 preadipocytes were grown in Dulbecco’s Modified Eagle’s Media (DMEM) (Sigma-Aldrich) containing 10% bovine calf serum (HyClone). Preadipocytes reached confluence and were subjected to differentiation 2 days later. Differentiation was induced using a standard protocol with a MDI induction cocktail containing 1% 3-isobutyl-methylxanthine, 0.1% dexamethasone, 1% insulin, and 10% characterized fetal bovine serum (FBS) (HyClone) in DMEM. Media was changed every 48-72 hours during growth and differentiation.

2.2. Whole-Cell Extract Preparation

Cell monolayers were scraped into non-denaturing immunoprecipitation (IP) buffer containing 10mM Tris (pH 7.4), 150mM NaCl, 1mM EGTA, 1mM EDTA, 1% Triton X-100, 0.5% IGEPAL CA-630, protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, 1 mM 1,10-phenanthroline), and phosphatase inhibitors (0.2mM sodium vanadate and 100 μM sodium fluoride). Whole-cell extracts used in adipogenesis, protein stability, and fractionation experiments were stored at -80°C, thawed, passed through a 20-gauge needle four times, and clarified by centrifugation at 10,000 x g for 10 minutes at 4°C. Whole-cell extracts used for immunoprecipitation experiments were stored on ice for two hours, passed through a 20-gauge needle four times, clarified by centrifugation at 10,000 x g for 10 minutes at 4°C.

2.3. Western Blotting

BCA assays were used to quantify protein content of all whole-cell extracts. Samples were separated on 7.5% or 10% sodium dodecyl sulfate (SDS) polyacrylamide gels (National Diagnostics) and transferred to nitrocellulose membranes (BioRad). Transfers took place in a transfer buffer (TB) containing 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were blocked in a 4% non-fat milk for one hour at room temperature then washed with Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Membrane strips were incubated with primary antibodies TBS-T containing 1% BSA overnight at 4°C. After incubation strips were washed three times with TBS-T for 15 minutes. After washing the strips were incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) for 1.5 hours. Strips were washed three times in TBS-T for 10 minutes each and visualized using enhanced chemiluminescence (Pierce/Thermo Scientific).
2.4. Fractionation

Cell monolayers were scraped into nuclear homogenization buffer (NHB) containing 20mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, 1 mM 1,10-phenanthroline), and phosphatase inhibitors (0.2mM sodium vanadate and 100 μM sodium fluoride). Nonyl phenoxypolyethoxylethanol 4-(NP-40) was added and whole-cell extracts were homogenized using a Dounce Homogenizer. Homogenized samples were centrifuged at 3000 rpm for five minutes at 4°C. Supernatant was extracted and served as the cytosolic fraction. Pellet was resuspended in NHB and centrifuged as previously mentioned. Supernatant was discarded and the pellet was resuspended in IP buffer to serve as the nuclear fraction.

2.5. Immunoprecipitation

Samples containing 300 μg protein from whole-cell extracts, determined by BCA assay, were equalized to 500 ul with IP buffer. Samples were incubated with 25 ul of protein A/G-agarose beads (Santa Cruz Biotechnology) on a rocker at 4°C for one hour to eliminate non-specific reactions (pre-clearing). Samples were centrifuged for one minute at 13,000 rpm and the supernatant was collected. Pre-cleared samples equalized to 500 ul with IP buffer and were incubated with 5 μg of primary antibody on a rocker at 4°C overnight. 50 ul of protein A/G-agarose beads was added to the samples and incubation continued for one hour. After incubation samples were centrifuged for one minute at 13,000 rpm to pellet beads. Supernatant was aspirated and beads were washed with 1 ml of IP buffer. This process was repeated four times. Washed samples were analyzed by western blotting.

2.6. Antibodies

Anti-CUZD1 (E-10; sc-514578; mouse monoclonal), anti-adipsin (M-120; sc-50419; rabbit polyclonal), and anti-PPARg (E-8; sc-7273; mouse monoclonal) antibodies were purchased from Santa Cruz Biotechnology. Anti-adiponectin (PA1-054; rabbit polyclonal) was purchased from Thermo Scientific. Anti-vinculin (#4650; rabbit polyclonal), anti-DBC1 (#5693; rabbit polyclonal), anti-ERKS ½ (#4695; rabbit monoclonal), and anti-FAS (#3180; rabbit monoclonal) antibodies were purchased from Cell Signaling Technology. Anti-STAT5γ (05-495; mouse monoclonal) antibody was purchased from Millipore. Anti-STAT5A (E289; ab32043; rabbit monoclonal) antibody was purchased from Abcam.
2.7. Animals and Diets

C57BL/6 mice were housed in temperature and humidity controlled rooms under a 12 hour light/dark cycle. For the whole tissue collection mice had *ad libitum* access to standard chow (13% kcal fat) and water and were fasted for four hours prior to sacrifice. For the high-fat or low-fat diet study mice began their twelve week diets at six weeks of age. The mice had *ad libitum* access to a high-fat diet containing 20% kcal protein, 20% kcal carbohydrate, and 60% kcal fat or a low-fat diet containing 20% kcal protein, 70% kcal carbohydrate, and 10% kcal fat (Research Diets). Mice were fasted for four hours prior to sacrifice. For the fasting a refeeding study mice had *ad libitum* access to standard chow and water. Prior to sacrifice treated mice were subjected to a 48 hour fast with or without an additional four hour refeeding period. For the STAT5 knockout study mice with STAT5A and STAT5B genes flanked by loxP sites (floxed) were bred to adiponectin-CRE mice. Offspring hemizygous for adiponectin-Cre were then crossed with floxed mice to create not expressing adipocyte STAT5 (STAT5<sup>AKO</sup>) and control littermates. Mice were allowed *ad libitum* access to standard chow and water for twelve weeks. Mice were fasted for four hours prior to sacrifice. All animal studies were approved by the Animal Care and Use Committee at Pennington Biomedical Research Center and all regulations were followed.
CHAPTER 3. RESULTS

3.1. CUZD1 is Present in the Adipocyte Nucleus

To examine the expression of CUZD1 in adipose tissue we harvested fat pads and several other tissues from 11 week old C57BL/6 mice. Each tissue was processed for protein analysis and was subjected to western blotting. Expression of CUZD1 was enriched in all white adipose tissue (WAT) depots as well as in the pancreas and was present at lower levels in BAT (Figure 1). Expression of adiponectin was used as positive control for adipose tissue and vinculin served as a measure of even protein loading. iWAT is a type of subcutaneous WAT and gWAT, mWAT, and rpWAT are types of visceral WAT depots.

![Western Blot Image]

Figure 1. CUZD1 is enriched WAT depots of female mice. Tissues from a female C57BL/6 WT mouse were harvested and were subject to protein extraction. Samples were examined using western blot analysis with 50 μg protein per sample. This is a representative experiment independently performed on 3 mice of the same genetic background.

After establishing the presence of CUZD1 in adipose tissue, we wanted to examine its cellular localization. In mammary epithelial cells CUZD1 was generally localized to the cytosol unless stimulated via PRL signaling to form a complex with STAT5 and translocate to the nucleus [38]. To fully understand the localization in adipocytes, we observed its localization in basal conditions and in the context for hormone signaling. To do this we treated fully differentiated 3T3-L1 adipocytes with growth hormone (GH) or a NaHCO₃ vehicle for 20 minutes, harvested the whole-cell...
extract (WCE), and subjected them to subcellular fractionation. Western blot analysis shows that CUZD1 was present only in the adipocyte nucleus regardless of the treatment (Figure 2). DBC1 and ERKs ½ serve as positive controls for nuclear and cytosolic localization respectively. STAT5A and STAT5\textsuperscript{py} translocate to the nucleus upon stimulation by GH so they serve as a positive treatment control.

![Image](image_url)

**Figure 2.** CUZD1 is present in the adipocyte nucleus. Fully differentiated 3T3-L1 adipocytes were serum deprived overnight media containing 0.3% BSA then treated with 10nM murine Growth Hormone or the same volume 10mM NaHCO\textsubscript{3}, for 20 minutes. Cells were collected and subjected to subcellular fractionation. Fractions examined using western blot analysis with 50μg protein per sample. This is a representative experiment performed independently three times.

To further establish the presence of CUZD1 in adipocytes, we examined the protein’s stability. We treated mature 3T3-L1 adipocytes with cycloheximide, a protein synthesis inhibitor, or an ethanol vehicle over the course of two hours. WCE was collected at multiple time points and subjected to western blotting. CUZD1 expression is stable for at least two hours after protein synthesis is inhibited (Figure 3). PPAR\textsubscript{g} is known to degrade quickly and serves as a positive control for protein synthesis inhibition. STAT5A is known to be stable for long periods and its presence demonstrates even protein loading. This data together suggests that CUZD1 is present
in adipocytes, but given its localization it could serve a different purpose in adipocytes than it does in mammary glands.

Figure 3: CUZD1 protein is stable for at least 2 hours. Fully differentiated 3T3-L1 adipocytes were treated with 5 μM cycloheximide (protein synthesis inhibitor), or the same volume of vehicle, ethanol, for two hours. Cells were collected at given time points and subjected to western blot analysis with 50 μg protein per sample. This is a representative experiment independently performed three times.

3.2. Regulation of CUZD1 in Adipocytes

As mentioned above, adipogenesis is a crucial process for adipocyte formation and disruptions in this developmental process can cause detrimental metabolic effects. Given that CUZD1 contributes to mammary gland proliferation and differentiation it could be inferred that it may play a similar role in adipocytes. In mammary glands CUZD1 regulates this process by interacting with STAT5 proteins and activating transcription. STAT5 proteins also play a critical role in adipocyte differentiation and are activated very early in the adipogenic process. In adipocytes, involvement of CUZD1 in this process has not been studied. To determine if CUZD1 expression was regulated during the process of adipogenesis, we induced 3T3-L1 pre-adipocytes to differentiate, a process that takes approximately seven days, and harvested WCE before induction and every 24 hours after. Expression of CUZD1 is highly induced during the first 24 hours of adipogenesis and then its expression is maintained as cells become mature adipocytes. The level of ERK expression was shown to demonstrate even protein loading and adiponectin expression was measured as a positive control for adipocyte
development (Figure 4). The early induction of CUZD1 could suggest that it plays a role in adipogenesis.

![Image of western blot analysis](image)

**Figure 4.** CUZD1 is highly induced during the first 24 hours of adipogenesis. 3T3-L1 pre-adipocytes were induced to differentiate using the 3-isobutyl-methylxanthine, dexamethasone, insulin (MDI) cocktail, and cells were collected every 24 hrs throughout the adipogenesis process. Cells were analyzed using western blot analysis with 50 μg protein per sample. This is a representative experiment independently performed three times.

Many proteins in adipocytes are regulated by nutritional states such as high-fat feeding. The development of obesity from high-fat feeding can result in gene expression changes that affect whole-body metabolism. To examine whether CUZD1 was regulated by nutritional state we fed C57BL/6 mice either a high-fat or low-fat diet for 8 weeks and harvested their adipose tissue. The tissues were processed for protein analysis and subjected to western blotting. CUZD1 expression in both gWAT (Figure 5A) and iWAT (Figure 5B) depots were not affected by diet. Adipsin expression is known to be decreased during high-fat feeding [42] and is used here as a positive control for nutritional state. This experiment was only performed with male mice. To determine whether CUZD1 is regulated by other nutritional states, we chose to examine expression in the fasted and refed states. 12 week old female C57BL/6 mice were allowed to eat freely, were subjected to a 48 hour fasting period, or were subjected to the same fasting period followed by a 4 hour refeeding period. Expression of CUZD1 remained unchanged in BAT, iWAT, gWAT, rpWAT, and liver but interestingly expression in mWAT changed based on the nutritional state (Figures 6A,B). CUZD1 expression in mWAT was reduced in the fasted and refed states when compared to the
free feeding state. This connection between regulation of CUZD1 in mWAT and different feeding states makes sense given that mWAT is the visceral fat that lines the intestines and would be the first fat depot exposed to changes in feeding. CUZD1 has also been implicated in inflammatory disease states associated with the intestines such as Crohn's disease and inflammatory bowel disease. What this connection means remains unclear.

Figure 5: CUZD1 protein expression is not affected by HFD feeding in WAT of male mice. 18 week-old male C57BL/6 mice were fed a low-fat or high-fat diet for 12 weeks and their fat pads were collected. gWAT (A) and iWAT (B) samples were subjected to protein extraction and analyzed by western blot analysis using 50 ug per sample.
Figure 6: CUZD1 expression is reduced in mWAT in the fasted and re-fed state. Female C57BL/6 mice were subjected to a 48 hour fasting period or a 48 hour fasting period followed by a 4 hour refeeding period. Fat pads and liver samples were collected from each group along with littermate controls fed freely. Samples were examined using western blot analysis with 50 μg protein per sample.

3.3. CUZD1 Interacts with STAT5A in the Adipocyte Nucleus

In mammary epithelial cells, CUZD1 regulates the JAK/STAT5 pathway by interacting with JAK proteins and pSTAT5 in the cytoplasm and translocating to the nucleus in a complex with pSTAT5 [39]. In cultured adipocytes, CUZD1 is expressed only in the nucleus indicating that it does not have the same localization in the two different cell types. Given the different localizations, we wanted to examine if CUZD1 still can be present in a complex with STAT5 proteins, specifically STAT5A, and if that interaction is regulated by hormonal stimulation. We treated mature 3T3-L1 adipocytes with GH or a NaHCO₃ vehicle for 20 minutes, collected WCE, and subjected them to immunoprecipitation. Immunoprecipitation was done using both CUZD1 and STAT5A antibodies to study the interaction. Immunoprecipitation samples were examined using western blot analysis. In both cases an interaction between CUZD1 (Figure 7A) and STAT5A (Figure 7B) was observed and the interaction was increased in the presence of GH. After establishing an interaction between the two proteins, we wanted to determine where the interaction was occurring. Since CUZD1 is expressed in the adipocyte nucleus we assumed this is where the interaction would take place. To verify this, we again treated mature 3T3-L1 adipocytes as previously mentioned and collected the WCE. After collection we subjected the samples to subcellular fractionation followed by immunoprecipitation. Western blot analysis shows that the interaction between CUZD1 and STAT5A only occurred in the adipocyte nucleus and that the association was increased upon translocation of STAT5A to the nucleus in response to GH stimulation (Figure 8A,B).
Figure 7: CUZD1 interacts with STAT5A and the interaction is increased in the presence of GH. Fully differentiated 3T3-L1 adipocytes were treated with 10nM murine Growth Hormone or the same volume 10mM NaHCO$_3$, for 20 minutes. Cells were collected and subjected to immunoprecipitation. This is a representative experiment performed individually three times. (A) 300 μg protein from whole cell extracts were incubated with 5 μg of CUZD1 antibody and precipitated using anti-IgG protein beads. Samples were examined by western blot analysis using a STAT5A antibody. (B) 300 μg protein from whole cell extracts were incubated with 5 μg of STAT5A antibody and precipitated using anti-IgG protein beads. Samples were examined by western blot analysis using a CUZD1 antibody.
Figure 8: The interaction of CUZD1 and STAT5A occurs in the nucleus and is stimulated by the presence of GH. Fully differentiated 3T3-L1 adipocytes were treated with 10nM murine Growth Hormone or the same volume 10mM NaHCO₃, for 20 minutes. Cells were collected and subjected to subcellular fractionation. Cytosolic (A) and nuclear (B) fraction were then subjected to immunoprecipitation. 300 μg of protein from each sample was incubated with 5 μg of CUZD1 antibody and precipitated using anti-IgG protein beads. Samples were examined by western blot analysis.

With a connection between CUZD1 and STAT5A established, we next examined if loss of STAT5A and B, specifically in adipocytes, would affect the expression of CUZD1 in adipose tissue. To do this we used transgenic mice, on a C57BL/6 background, that lack STAT5s in adiponectin expressing cells. Protein expression of CUZD1 and STAT5A was examined in iWAT from both females and males. In both females and males, STAT5A expression was significantly reduced but not completely ablated (Figures 9A, B, and C). Because of the heterogeneity of adipose tissue and STAT5A's expression in other cell types, we observe STAT5 expression in the adipose tissue of mice lacking STAT5 in adipocytes [5]. In females, loss of adipocyte STAT5s caused a significant increase in CUZD1 expression (Figures 9A and C). In males the increase in CUZD1 expression did not reach statistical significance (Figures 9B and C). Given the heterogeneity of adipose tissue and only correlative data we cannot definitely say that lack of adipocyte STAT5 caused the increase in CUZD1 expression. To further investigate this regulation, we examined BAT in the same context because it is known to be the least heterogeneous adipose tissue depot. As expected, in BAT there is
almost a complete ablation of STAT5A expression in both males and females (Figures 10A-C). Interestingly, there is no change in CUZD1 expression in any group (Figures 10A-C). Changes in CUZD1 expression between the tissues could be because of differences in baseline expression of the protein. Expression of CUZD1 is significantly higher in iWAT when compared to BAT (Figure 1). How exactly these expression patterns relate to each other remains unclear.

Figure 9: Expression of CUZD1 is increased in female iWAT from mice that lack adipocyte STAT5A expression. iWAT was collected from 12-week-old female (A) and male (B) mice lacking adipocyte STAT5 and floxed littermates. Tissues were subjected to protein extraction and examined by western blot analysis using 50 μg protein per sample. (C) Protein expression quantified, * p<.005.
Figure 10: CUZD1 expression is unaffected by the lack of adipocyte STAT5 in male and female BAT. BAT was collected from 12-week-old female (A) and male (B) mice lacking adipocyte STAT5A and floxed littermates. Tissues were subjected to protein extraction and examined by western blot analysis using 50 μg protein per sample. (C) Protein expression quantified, * p<.005.
CHAPTER 4. DISCUSSION AND FUTURE DIRECTIONS

While the function of CUZD1 in adipocytes remains unclear, our experiments have taken the first steps to examining CUZD1 in adipocytes and adipose tissue. We now know that CUZD1 is enriched in WAT depots and is located in the adipocyte nucleus in basal conditions and in the presence of growth hormone stimulation (Figure 1,2). In addition, CUZD1 is highly induced during the first 24 hours of adipogenesis (Figure 3). This could indicate CUZD1 plays a similar role in both mammary glands and adipocytes by regulating proliferation and differentiation. Conversely, the localization of CUZD1 to the adipocyte nucleus suggests that it could play a different role entirely.

Further evidence that CUZD1 could be involved in adipocyte differentiation is the fact CUZD1 interacts with STAT5A, a critical regulator of adipogenesis. CUZD1 also interacts with STAT5 proteins in the mammary epithelium to regulate differentiation. Further experiments are required to understand if CUZD1 does in fact play a role in adipogenesis and what that role is. RNAi experiments, diminishing the expression of CUZD1, during the adipogenesis process could help be the first step to understand if CUZD1 plays a role in adipogenesis.

We also demonstrated that CUZD1 can be regulated by nutritional state. In mWAT, the visceral WAT depot that lines the intestines, CUZD1 expression was decreased during fasting when compared to free fed littermates (Figure 6). This is interesting because CUZD1 has been shown to be a biomarker for multiple intestinal diseases such as inflammatory bowel disease and Crohn’s disease and has been suggested to be involved in the immune response. Adipose tissue is known to contain a large number of immune cells in its stromal vascular fraction. The presence of these immune cells could be part of the reason CUZD1 expression is altered in this situation. Fractionation of adipocytes from their stromal vascular fraction would be required to understand the full picture.

Most significantly, we have shown that CUZD1 is an interacting partner of STAT5A, a key regulator of several metabolic processes in adipocytes. It is evident that this interaction takes place in the adipocyte nucleus and that it is stimulated by the presence of GH (Figure 8). GH regulates several metabolic processes through the JAK/STAT5 pathway by stimulating STAT5 to translocate to the nucleus to regulate target gene transcription. Given that CUZD1 interacts with STAT5A in the presence of GH we can hypothesize that it could be involved in STAT5A’s ability to regulate transcription. Understanding if CUZD1 is involved and how it is involved in this process would take further experimentation. RNAi experiments knocking down CUZD1 expression in cells treated with GH could be a logical first step. qPCR analysis of the treated cells would allow us to determine if expression of STAT5 target genes are affected by the loss of CUZD1. Overall, these findings suggest a role for CUZD1 in adipocytes but more research is required.
REFERENCES


Zachary Thomas Wigger was born October 1993 in Zachary, LA where he attended elementary, junior high, and high school. After graduating from Zachary High School in 2012 he began his undergraduate studies majoring in microbiology at Louisiana State University, Baton Rouge. While an undergrad at LSU he worked at a plasma donation center and obtained teamwork and leadership skills. In 2017, he received his Bachelor of Science in microbiology. Unsure of what he wanted to do after graduation he continued to work at the plasma center before applying to graduate school at LSU. In 2019, Zach enrolled in the PhD program at LSU in the Department of Biological Sciences under the mentorship of Dr. Jacqueline M. Stephens. As he progressed through the program he fell in love with teaching and realized that pursuing a doctorate was not the path he wanted. Zach switched to the master’s program and anticipates graduating in the summer of 2022. After graduation he intends to pursue a career in teaching high school science.