Modeling partial lipodystrophy using human pluripotent stem-cell derived CIDE-C E186X mutant adipocytes

Audrey A. Tran
Wellesley College, atran2@wellesley.edu

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Modeling partial lipodystrophy using human pluripotent stem-cell derived $CIDE-C$ E186X mutant adipocytes

Audrey A. Tran
Wellesley College
Advisor: Adam G. Matthews
Harvard Stem Cell Institute Advisors: Chad Cowan and Carolyn Hudak

Thesis Committee Members: Chad Cowan, Carolyn Hudak, Adam G. Matthews, Megan Nunez, and Kimberly O’Donnell
Honors Visitor: Martin Brody

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ABSTRACT

Metabolic disorders, such as obesity and type II diabetes, are alarmingly prevalent in the world today. Because they rely on the same cellular machinery, metabolic disorders often share similar phenotypes, such as insulin resistance. These similarities provide motivation to characterize a broad range of metabolic disorders. One promising candidate for further study is familial partial lipodystrophy, a metabolic disorder in which a patient accumulates an abnormal distribution of adipose tissue along the body. Studies have recently implicated CIDE-C as a gene whose E186X nonsense mutation is associated with partial lipodystrophy. Previous studies in mouse and human models have demonstrated CIDE-C to be critical for large, unilocular lipid droplet formation in white adipocytes, which suggests that metabolic processes are linked to lipid droplet size.

In this study, we created and assessed a human pluripotent stem cell-derived adipocyte model of partial lipodystrophy. By comparing cell lines isogenic (genetically identical in every way except) for the CIDE-C gene E186X mutation, we demonstrated that this mutation alone is sufficient to induce the phenotype observed in the partial lipodystrophy patient. CIDE-C E186X mutant adipocytes, as compared to wildtype adipocytes, were observed to have extremely multilocular lipid droplets, a decreased ability to store triglycerides, and higher levels of basal lipolysis. E186X mutants also developed a preadipocyte-like, pro-lipolytic character. Possible mechanisms by which the truncated CIDE-C affects metabolic function, as well as future applications for the model, will be discussed. Modeling this rare disorder may prove useful in understanding the underlying mechanism of metabolic processes in white adipocytes.
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The senior thesis is a collegiate rite of passage whose notoriety is larger than life. As someone who can see the light at the end of the tunnel (almost there!), I think I can contribute to the pool of vague advice I was given as a rising senior: doing a thesis is definitely a commitment! But if you are really into what you're doing, all of it seems worth it.

My thesis experience at the Harvard Stem Cell Institute has been the highlight of my undergraduate career already filled with countless twinkles. In the process I have worked with brilliant scientists and have made countless, marvelous memories. Science is no longer a subject for me but a particular way of thinking.

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INTRODUCTION

Familial partial lipodystrophy, a metabolic disorder that involves an abnormal distribution of adipose

Metabolic disorders, or disorders that arise due to a disruption in the body’s normal metabolic processes, are alarmingly prevalent in the world today. Perhaps the most immediately obvious metabolic disorder is obesity. Obesity has been considered a worldwide epidemic, with 10% of all men and 14% of all women, aged 20 years or older, estimated to be obese in 2008.\(^1\) Prevalence is especially marked the United States; in 2012, the National Health and Nutrition Examination Survey estimated that over 35% of American adults are obese.\(^2\) Metabolic disorders are both personally and economically costly, with an estimated 300,000 deaths and $100 billion total health costs attributed to obesity-related disease in the United States.\(^1\)

Although difficult to precisely define due to its varying effects, obesity is medically defined as an excess accumulation of adipose (fat) tissue that exacerbates various health risks.\(^3\) Obesity is often determined and associated with an increase in body mass index (BMI), a crude proxy for the obese state of the patient. Obesity is complex due to a combination of factors, such as genetic predisposition, dietary habits and lifestyle choices. Obesity often leads to metabolic-related complications, including insulin resistant type II diabetes mellitus, and elevated blood pressure, coronary heart disease, and ischemic stroke, to name a few.\(^2\)

We may better understand obesity, and metabolism in general, by understanding and characterizing a broad range of metabolic disorders. One particularly interesting subset of metabolic disorders is familial partial lipodystrophy. Partial lipodystrophy refers to a subset of lipodystrophic disorders whose signature symptom is the abnormal distribution of subcutaneous adipose tissue along the body. Partial lipodystrophy is considered a rare disease, with only about...
historically 250 reported cases worldwide. The first landmark diagnoses were observed by Kobberling and Dunnigan in 1986, with the detailed study of two female patients with partial lipodystrophy symptoms. Most typically, patients with familial partial lipodystrophy experience a loss of fat in the arms, legs, and lower trunk and an accumulation of fat in the face and upper neck (Figure 1B). Onset of the disorder typically occurs in late childhood and early adulthood. This abnormal distribution of adipose tissue is associated with disrupted metabolic processes; symptoms associated with partial lipodystrophy include insulin resistant type II diabetes mellitus, a darkening around the armpit (acanthosis nigricans), and increased levels of lipoprotein, triglycerides, and other lipids in the blood (hyperlipoproteinaemia, hypertriglyceridemia, and hyperlipidaemia, respectively). Historically, partial lipodystrophy is most commonly attributed to autosomal dominant mutations in three proteins: LMNA, encoding for lamins A/C, nuclear lamina structural proteins important for nuclear stability and gene expression; AKT2, encoding for a kinase involved in the lipolysis signaling pathway, and PPARG, encoding for the nuclear receptor which is widely regarded as the master regulator of adipogenesis. These genes associated with partial lipodystrophy are highly expressed in adipose tissue.
Figure 1. Familial partial lipodystrophy is a subset of lipodystrophy disorders associated with an abnormal distribution of adipose tissue. Familial partial lipodystrophy is usually attributed to an autosomal dominant mutation in genes LMNA, PPARG2, and AKT2. Typically, a loss of fat is observed in the arms and legs, while an accumulation of fat is observed in the upper neck and head area, as demonstrated by the A) schematic and B) partial lipodystrophy patient with a PPARG2 mutation.

Interestingly, partial lipodystrophy and obesity – to recall, a deficiency as opposed to an excess of adipose tissue – share the insulin resistant phenotype. This commonality suggests that both disorders might arise due to similar cellular malfunctions. Therefore, studying partial lipodystrophy may also provide novel insights to the mechanisms that lead to obesity and metabolic disorders in general. Before delving into this study of partial lipodystrophy, a brief introduction to previous research in both the adipose and disease modeling fields may be useful.
**The white adipocyte: function and composition**

Adipose tissue, both subcutaneous and visceral, is an endocrine organ involved in energy homeostasis and reproduction. Adipose tissue can also offer protection to body parts that are delicate or experience routine mechanical stress (such as the eye, the heel, or the rump). Adipocytes are specialized cells within adipose tissue that function to store energy as triacylglycerides in the form of lipid droplet organelles. While the ability to store energy in the form of fat is a trait conserved and observed throughout all eukaryotes, only vertebrates seem to contain cells particularly specialized for this function.

![Image of adipocytes](image)

**Figure 2.** The three commonly accepted classifications of adipocytes: white, beige, and brown.

Currently, adipocytes are classified into three categories: white, brown, and “beige” adipocytes. The main function of white adipocytes is to store energy in the form of optimally sized, singular (unilocular) lipid droplets; often the lipid droplet is so large it displaces the nucleus within the cell (Figure 2). In contrast to white adipocytes, which function to store energy, brown adipocytes can burn energy off as heat by carrying out stress-induced thermogenesis via their mitochondria. Depending on the environmental conditions, beige adipocytes can function either like a white or brown adipocyte. White adipocytes are most directly associated in partial lipodystrophy, and we will focus primarily on understanding the white adipocyte.
On becoming an adipocyte

During development, three germ layers form: endoderm, mesoderm, and ectoderm. Adipocytes hail primarily from a mesoderm lineage. The commonly accepted precursor cell for white adipocytes is the mesenchymal progenitor cell, which can then differentiate into fat, bone, cartilage, bone marrow, tendons/ligaments, and other connective tissue (Figure 3A). One exception is the brown adipocyte, which is thought to share a common precursor with skeletal muscle. In the absence of the protein PR domain-containing 16 (PRDM16), brown adipocytes shift to a muscle cell-like morphology (Figure 3A).

The process of mesenchymal progenitor cells differentiating into white adipocytes has been well studied; differentiation begins with increased CCAAT/enhancer-binding protein alpha (CEBP-α) transcription factor expression, which arrests cell proliferation and encourages cell differentiation. This upsurge of CEBP-α is followed by expression of PPARG2, a nuclear receptor widely considered the master regulator of adipogenesis. Adipocyte differentiation is also marked by decreased expression of platelet-derived growth factor receptor alpha (PDGFR-α), a tyrosine kinase receptor that is marker of mesenchymal progenitor cells. Some characteristic markers of mature white adipocytes include adiponectin (ADIPOQ), a protein secreted by adipocytes that is involved in fatty acid oxidation and associated with increased insulin sensitivity, fatty acid-binding protein 4 (FABP4), involved in cytosolic shuttling of lipid ligands into lipid droplets, and PLIN1, a surface-associated lipid droplet protein.
Figure 3. The origins and composition of the white adipocyte. A) The commonly accepted lineage from a mesenchymal stem cell into adipocyte fate, as defined by characteristic markers. MPCs enter into a committed preadipocyte stage (not pictured) before differentiating into mature adipocytes. Dashed lines indicate a direct differentiation that can be achieved in vitro but does not necessarily occur in vivo. B) The most distinctive organelle in the adipocyte is the lipid droplet, which is comprised of a neutral lipid core, a phospholipid monolayer, and a coat of lipid droplet surface-associated proteins.

On lipid droplets, a dynamic store of energy

In white adipocytes, the organelle most important for function is the lipid droplet, the site of lipid (and thus energy) storage. Lipid droplets are comprised of neutral lipids, and most typically triacylglycerides and cholesterol esters, depending on the cell type.19 For white adipocytes, the lipid droplet is comprised of a triacylglyceride-rich core, an amphipathic phospholipid monolayer, and a coat of lipid droplet surface-associated proteins (Figure 3B).20
The lipid droplet membrane has a distinct membrane composition than membranes from endoplasmic reticulum (ER) or the sphingolipid-dense membrane microdomain.  

Lipid droplets are a dynamic energy reservoir, not simply a sink for lipid storage; lipid droplet synthesis and breakdown is constantly responding to the body’s needs. When the body receives excess energy in the form of glucose molecules, lipid synthesis and lipid droplet formation is activated. Lipid droplet formation is thought to originate in the smooth endoplasmic reticulum (ER). Vesicles can pinch off from the ER to form immature lipid droplets. Cytosolic lipid droplets increase in size with the aid of fatty acid-binding proteins, such as FABP4, which can help shuttle and incorporate hydrophobic molecules into existing lipid droplets.  

While the bulk of lipid vesicles are thought to bud off from the endoplasmic reticulum (ER), triacylglycerides (TAGs) can also be nascently synthesized from acyl transferase-catalyzed reaction and be incorporated into a nearby lipid droplet. TAGs can be regenerated from fatty acids released from lipid droplets. Before released free fatty acids can be reincorporated into lipid droplets, they must first be activated by coenzyme A via an acyl-coA synthetase-mediated process. It is thought that TAG synthesis may be activated in an attempt to sequester free fatty acid, which can be toxic in excess. Lastly, in both mouse and human adipocytes, lipid droplet transfer can occur between nascently formed and preformed lipid droplets, resulting in one larger, fused lipid droplet. It is observed that the direction of transfer has the smaller lipid droplet fuse into the larger lipid droplet.  

When the body signals for white adipocytes to release energy, triglycerides are hydrolyzed, via a process termed lipolysis, into their free glycerol and free fatty acid components; these free fatty acids can be mobilized into and metabolized in peripheral tissues to release energy in the form of ATP. Enzymes such as hormone-sensitive lipase (HSL) and
adipose triglyceride lipase (ATGL, or desnutrin) are both responsible for this TAG lipolysis.\textsuperscript{26, 27}

On the organelle level, upon lipolysis, large lipid droplets are reduced to smaller lipid droplets.\textsuperscript{24}

Brown and beige adipocytes also have high lipolytic activity, but in contrast to white adipocytes, they can burn energy off in the form of heat, not ATP. When induced by cold stress (i.e. low temperatures), the brown adipocyte-specific uncoupling protein 1 (UCP-1) disrupts the coupling of oxidative phosphorylation to ATP production, which results in the release of energy in the form of heat.\textsuperscript{21}

Lipolysis occurs both basally and through stimulation by different hormones, including but not limited to epinephrine, norepinephrine, glucagon, thyroid-stimulating hormone, growth hormone, glucocorticoids and catecholamines.\textsuperscript{28} Through these hormone signals, the body communicates with the adipocyte to release energy. Most lipolysis-stimulating hormones are involved in an adenylyl cyclase-mediated pathway.\textsuperscript{29} Adenylyl cyclase generates the secondary messenger, cyclic AMP (cAMP), which activates protein kinase A (PKA) to phosphorylate proteins and activate downstream lipases. Not all hormones stimulate lipolysis, however – one counterexample is insulin, which inhibits HSL activity to protect the lipid droplet.\textsuperscript{28}

**The many mechanisms regulating lipid droplet size**

Because the lipolytic pathway is comprised of many steps, many different factors can regulate lipolysis, and as a result, lipid droplet size. One factor that regulates lipolytic activity is the amount of exposed surface area of lipid droplets within an adipocyte. The presence of multiple small lipid droplets dramatically increases the lipid droplet surface area-to-volume ratio within an adipocyte, which gives lipases increased access and opportunity for lipolysis.
Consistent with this idea, it was found that smaller lipid droplets degrade more quickly than larger lipid droplets.\textsuperscript{24}

The abundance and localization of lipid droplet surface-associated proteins and lipases have also demonstrated to regulate lipid droplet size. Surface-associated proteins can regulate lipid droplet size by two general methods: \textit{actively}, by increasing lipid droplet size via lipid transfer; or \textit{passively}, by inhibiting both basal and stimulated lipolysis which decrease lipid droplet size.

Unphosphorylated PLIN1, for example, complexes with comparative gene identification-58 (CGI-58), a co-activator of ATGL. In this sense, PLIN1 not only restricts lipase access to the lipid droplet surface, but it inhibits ATGL by sequestering its co-activator. Upon protein kinase A-mediated phosphorylation of PLIN1, CGI-58 dissociates from the complex and is free to activate ATGL (Figure 5B).\textsuperscript{26} Hormone-sensitive lipase (HSL) is also recruited to the lipid droplet surface when phosphorylated by PKA.\textsuperscript{26,29} The reduction of large lipid droplets to small multilocular lipid droplets also was demonstrated to relocalize PLIN1, which would deter PLIN1 from shielding lipid from lipases.\textsuperscript{24}

In addition to PLIN1, the cell death-inducing DFFA effector (CIDE) protein 3, or CIDE-C, is another lipid droplet surface associated protein crucial for lipid droplet formation. Because of its relevance to the study, more background information on CIDE-C is needed.

The discovery, structure, and cellular function of CIDE-C

CIDE-C belongs to a family of CIDE proteins which, to date, consist of CIDE-C, CIDE-A, CIDE-B, DFF45, and DFF40.\textsuperscript{30} CIDE proteins were first characterized as proteins involved in the promotion of apoptosis, but have recently been primarily recognized to be involved in the
regulation of energy homeostasis.\textsuperscript{31} CIDE-A, CIDE-B, and CIDE-C all are lipid droplet surface-associated proteins that are expressed differentially across various cell types.\textsuperscript{32} CIDE-A is highly expressed in brown adipose tissue for mice, and both brown and white adipose tissue for humans, and localizes to the mitochondria.\textsuperscript{33} CIDE-A functions to inhibit the thermogenic activity of the brown adipocyte-specific uncoupling protein 1 (UCP-1).\textsuperscript{31} CIDE-B, meanwhile, is highly expressed in hepatocytes.\textsuperscript{31} CIDE-C, the CIDE protein of most interest to us, is most highly expressed in white adipocytes; during adipogenesis, the expression of CIDE-C is upregulated by over 50-fold.\textsuperscript{33}

In humans, CIDE-C is 238 amino acids in length and contains a highly conserved CIDE-N domain (amino acids 41-118) and a CIDE-C domain (amino acids 140-201) (Figure 4A).\textsuperscript{34} This CIDE-N domain, also generically termed the CIDE domain, is a protein interaction motif found in a wide variety of proteins across species.\textsuperscript{30} The CIDE-N domain is typically comprised of two alpha helices and five beta strands arranged in an alpha/beta roll fold. The crystal structure of the full length human CIDE-C has not yet been resolved; however, by similarity, the CIDE-N domain of the mouse ortholog Fsp27 may help provide structural insight. The CIDE-N domain in Fsp27 is quite typical, though it lacks the fourth beta strand, $\beta_4$ (Figure 4B).\textsuperscript{35} In CIDE-C, the CIDE-N domain is proposed to be the “interaction” domain, while the CIDE-C domain is proposed to be the domain important for both apoptosis and lipid droplet surface localization.\textsuperscript{13,31,33}
Figure 4. CIDE-C structure and proposed mechanism of interaction. A) Human CIDE-C is 238 amino acids long, with a CIDE-N (amino acids 41 to 118) and CIDE-C (amino acids 140-199) domain. B) The CIDE-N of Fsp27, the mouse ortholog, is conserved in CIDE-C, and has an α/β roll-like structure. The CIDE-N domain is thought to be important for CIDE-C:CIDE-C homodimerization, as evidenced by the multiple noncovalent interactions between CIDE-C chain A and B. Crystal structure obtained at 2.0 Angstrom resolution. Protein Data Bank (PDB) code for CIDE-N domain of Fsp27 is 4MAC.

Recent reports have identified that human CIDE-C, by similarity, can also form a homodimeric complex. This CIDE-CIDE complex is thought to be primarily due to key electrostatic interactions between two CIDE molecules. The basic residue on one CIDE-N domain, Arg46, forms multiple salt bridges with acidic residues Asp88 and Glu87 on the second CIDE-N domain (Figure 4C). Lys56 and Glu93 form another salt bridge. The CIDE-CIDE interface is thought to be further strengthened by the formation of two hydrogen bonds formed by the Gly57 and Ile91 on molecule one to Arg55 to Glu87 on molecule two, respectively (Figure 4C).

Previous in vitro studies on CIDE-C and orthologs have demonstrated CIDE-C to be crucial for lipid droplet formation and intracellular triglyceride storage in the context of adipocytes. The majority of studies have been developed in model systems, such as
mouse 3T3-L1 preadipocyte cell lines, COS monkey fibroblast-like kidney cell lines, and human non-adipocyte cells, such as human embryonic kidney (HEK 293T) cells. Fsp27-deficient mouse adipocytes were observed to have increased lipid droplet multilocularity and enhanced basal lipolysis.\textsuperscript{33,38} A significant increase in basal glycerol release was also observed in siRNA-mediated knockdown of Fsp27 and/or PLIN.\textsuperscript{33} When artificially expressed in non-adipocyte cells, Fsp27 was shown to increase triacylglyceride accumulation and overall lipid droplet size.\textsuperscript{26} While lipid droplet size increased in these non-adipocyte cell lines due to ectopic CIDE-C expression, no other adipocyte-specific gene was expressed, suggesting that CIDE-C is not sufficient to initiate adipogenesis.\textsuperscript{33}

There are many proposed mechanisms by which CIDE-C promotes unilocular lipid droplet growth. One mechanism through which CIDE-C/Fsp27 promotes lipid droplet growth is by mediating lipid transfer between droplets.\textsuperscript{13} It has been proposed that CIDE-C/Fsp27 is not spread uniformly across the lipid droplet surface but rather is enriched at certain regions on the surface. These regions are termed lipid droplet contact sites (LDCS). The LDCS act as a connecting point between two intracellular lipid droplets and is thought to be the site of lipid fusion (Figure 5A). Mechanistically, the close proximity of CIDE-C at LDCS allows for CIDE-C homodimerization, and the CIDE-C:CIDE-C complex is then activated to promote lipid transfer between droplets; CIDE-C is inactive in its monomeric state.\textsuperscript{13} Typically, net lipid transfer is unidirectional due to physical pressure gradients; smaller lipid droplets fuse to larger lipid droplets.\textsuperscript{24} PLIN1 specifically interacts with the CIDE-N domain of Fsp27, resulting in increased Fsp27-mediated lipid transfer and increased lipid droplet unilocularity (Figure 5A).\textsuperscript{13} Because the E186X mutation truncates a significant portion of the CIDE-C domain in CIDE-C, the localization of the protein to the lipid droplet surface may be compromised, which may
disrupt the how CIDE-C operates to promote lipid droplet transfer. This may explain for the smaller multilocular lipid droplets observed in CIDE-C-deficient adipocytes.

![Figure 5](image-url)

Figure 5. Proposed mechanisms of A) CIDE-C mediated lipid droplet transfer\(^{13}\), in which it was observed that the homodimerization of CIDE-C and PLIN1 associated with the homodimer most strongly promoted net lipid transfer from the smaller (lipid donor) into the larger (lipid acceptor) lipid droplet; and B) CIDE-C and PLIN1-mediated regulation of lipolysis\(^{26}\), in which it is observed that PLIN1 sequesters the ATGL co-activator CGI-58. Upon phosphorylation of PLIN1, the CGI-58 is released and free to activate ATGL, which can then be localized to the lipid droplet surface.

CIDE-C/Fsp27, along with other surface-associated proteins like PLIN1, is thought to also serve as a barrier to lipolysis by limiting lipase presence at the LD surface, though through different mechanisms\(^{26}\). Unphosphorylated PLIN1 regulates lipolysis by forming a complex with a CGI-58, the ATGL co-activator. Without its co-activator, ATGL fails to localize to the lipid droplet (Figure 5B)\(^{39}\). CIDE-C/Fsp27, on the other hand acts as a protective barrier against lipolysis by directly binding to and inactivating ATGL\(^{26}\). The absence of PLIN1 and CIDE-C, independently and together, results in elevated ATGL-mediated lipolysis.
The novel association of CIDE-C in partial lipodystrophy

Until recently, although the cellular role of CIDE-C was well defined, CIDE-C was not known to have an association with any disease. Interestingly, recent studies found a novel association of CIDE-C to partial lipodystrophy in human patients.\(^4\) In a 2009 study, a patient displayed symptoms highly reminiscent of partial lipodystrophy; namely, the accumulation of subcutaneous adipose in her upper neck and head, and a complete absence of femorogluteal adipose tissue. On a cellular level, a biopsy of the patient’s adipose tissue was observed to have small, multilocular lipid droplets, as compared to the adipose tissue of a BMI-matched healthy individual.\(^4\) Interestingly, the patient was wildtype for LMNA, AKT2, and PPARG, genes typically associated with the disorder. Further sequencing of the patient’s genome revealed a homozygous recessive nonsense mutation in the CIDE-C gene. The mutation, termed E186X was a transversion of a guanine to a thymine at nucleotide 558 (G558T), resulting in a premature truncation at the 186\(^{th}\) amino acid (E186X). Furthermore, a fat biopsy revealed that the adipocytes from the patient contained multilocular lipid droplets (Figure 6C, left).
Figure 6. The novel association of CIDE-C with familial partial lipodystrophy. A) The T1 weighted magnetic resonance image of the patient with symptoms of partial lipodystrophy, demonstrating the abnormal distribution of adipose tissue. Lower legs are observed to have almost no subcutaneous adipose, while upper neck and head seem to accumulate considerable adipose. B) Observation of the E186X mutation in the CIDE-C gene. C) A comparison of lipid droplet sizes between the patient (left) and BMI-matched healthy control (right). Brown outline is stain for PLIN1, the lipid droplet surface associated protein.

The association of this CIDE-C E186X mutation with partial lipodystrophy has generated interest in understanding if the CIDE-C mutation is causal or merely correlative. Furthermore, although the biological role of CIDE-C is well documented, it remains unclear as to how a mutation in CIDE-C leads to the partial lipodystrophy phenotype on a macroscopic level.

Many cell-based models, such as mouse 3T3-L1 preadipocyte lines and easily transflectable human embryonic kidney (HEK 293T) lines have been used to single out the biological functional role of CIDE-C. However, when attempting to recapitulate disease, there are obvious drawbacks to using models that do not accurately capture the full character of the human adipocyte. Although human primary adipocytes would be the “gold standard” system in
which to conduct experiments on the CIDE-C E186X mutation, their availability is limited. Primary adipocytes are challenging to collect, difficult to keep in culture, and do not have a readily available isogenic control due to the complex genetic variability amongst humans. Furthermore, as there may be other unknown genes that enhance and contribute to the patient’s diseased phenotype, further studies on primary adipocyte from the patient would be not be able to confirm that the E186X mutation is both necessary and sufficient to induce partial lipodystrophy.

To address these concerns, pluripotent human embryonic stem cells (hESCs) were chosen as the experimental system to model the disease. To be considered a pluripotent stem cell, a cell must both be able to self-renew indefinitely and have the potential to differentiate into all cell types from the three germ layers, endoderm, mesoderm, and ectoderm – including adipocytes. The stem cell, therefore, can theoretically generate a virtually endless supply of adipocytes for our specific experimental purposes. The creation of human embryonic stem cells through isolation of the inner cell mass of a human blastocysts was a particularly momentous breakthrough, which ultimately led to a stronger understanding of pluripotency and the establishment of the HuES9 human embryonic stem cell line (Harvard University, iPS Core).

The initial role of human embryonic stem cells was for transplantation into patients; however, stem cell transplantation faces multiple obstacles, such as risk of immunorejection, potential formation of teratoma tumors, and potential inability to graft transplanted cells into the body. In response, scientists also developed in vitro stem cell-based disease modeling. Charmingly described as a “disease in a dish,” disease models have proven to be an resource that could provide accurate insight to a disease at no expense to the patient. Disease models can be used as a tool to understand the pathogenesis of disease, to thoroughly characterize the effect that
one mutation has in a specific cell type. These models can also be used as a platform to find small molecule therapeutics that can restore the wildtype phenotype and/or slow the progression of the disease phenotype.\textsuperscript{42} Therefore, disease modeling is appealing for research on the basic, translation, and clinical level.

\textbf{On introducing the specific CIDE-C E186X mutation: CRISPR/Cas9 genome editing}

The next step in disease modeling is to accurately and efficiently introduce the mutation of interest at the level of the genome. Site-specific genome mutation has always been a goal in biological studies, as it would allow researchers to address multiple basic and clinical questions. Multiple early approaches have involved developments of small molecules, chemical moieties, peptides, and nucleic acids that can recognize DNA base pairs.\textsuperscript{43-46} While novel, these approaches have not been scalable or robust.

Two genome editing methods that showed promise are zinc finger nucleases (ZFNs) and transcription activator-like effort nucleases (TALENS).\textsuperscript{47} Both methods rely on nucleotide base pairing for specificity. The ZFN strategy is comprised of a customizable zinc finger DNA-binding protein joined to a FokI site-specific restriction endonuclease.\textsuperscript{48} Similarly, the TALEN system can induce a site-specific double strand break with the aid of a nonspecific DNA nuclease domain joined to a customizable DNA-binding domain.\textsuperscript{49,51} While both are relatively efficient methods for genome editing, ZFN and TALEN technology experienced multiple setbacks in designing, synthesis, and assessing the efficacy of DNA-binding proteins.

Most recently the CRISPR-Cas9 genome editing system has become the preferred method due to its high efficiency, scalability, minimized off-target effects, and ease of use.\textsuperscript{52} The term “CRISPR,” a frequent target of wordplay in the scientific media spheres, does not explain
the components involved in the genome engineering system. Rather, CRISPR, shorthand for “clustered regularly interspersed palindromic repeats” is a nod to the origins in which these components were first observed. The CRISPR-Cas9 system is a naturally occurring RNA-guided DNA-cleaving mechanism, first observed in *Escherichia coli*, that provides bacteria with adaptive immunity to foreign viruses and plasmids. While there are many types of CRISPR-Cas9 systems that employ different mechanisms to achieve the site-specific DNA cleavage, the type II CRISPR-Cas9 system is most useful for genome editing applications, as it only requires a single protein, instead of a protein complex, to induce the site-specific double strand break. The developed CRISPR/Cas9 genome editing system conveniently requires only two components: the single guide RNA (sgRNA) and the Cas9 nuclease (Figure 7B). The engineered sgRNA is a fusion of CRISPR RNA (crRNA), a 20 base pair-long, single-stranded oligoribonucleotide that directs Cas9 to genetic target site via Watson-Crick base pairing, and the trans-activating crRNA (tracrRNA) which provides a structural scaffold and is essential for crRNA maturation. The wildtype or D10A Cas9 nuclease induces the double or single strand break (DSB or SSB), respectively. Interestingly, a D10A/H840A double mutant Cas9, which mutates both the RuvC and HNH nuclease domains, transforms the nuclease into simply a DNA-binding protein. The sgRNA can target virtually any genomic DNA sequence, though some sequences result in higher cutting efficiency than others. For successful Cas9 binding and DNA cleavage, the protospacer-adjacent motif (PAM), a three base pair-long motif (NGG, where N represents any of the four typical nucleotides) must immediately follow the genomic target sequence. Truly, the CRISPR-Cas9 system is an extraordinary development that has led to efficient genome editing and facile creation of cell-based monogenic disease models.
Figure 7. The CRISPR-Cas9 genome editing system. A) Detailed mechanism of RNA-guided cleavage in the type II system from *Streptococcus pyogenes*. The location of the protospacer-adjacent motif (PAM) after the target genomic sequences directs the Cas9 enzyme-mediated cleavage. RuvC and HNH are putative nuclease domains within Cas9. The crRNA and the tracrRNA components are important for recognizing the target genomic sequence and stabilizing the Cas9 nuclease, respectively. B) The CRISPR-Cas9 genome editing system is comprised of a Cas9 nuclease responsible for the double strand break (DSB) and an engineered single guide RNA (sgRNA) that links together the crRNA and tracrRNA.

The last step in completing the partial lipodystrophy model is to differentiate the stem cells in the white adipocytes, the cell type in which *CIDE-C* is expressed. At this point, the mutant cells can be compared against the wildtype to observe any difference in phenotype. The Cowan lab was the first to publish a reproducible, highly efficient, and inducible protocol for differentiating pluripotent stem cells into mature white adipocytes.
Figure 8. A) CRISPR/Cas9 single guide strategy targeting CIDE-C. The protospacer-adjacent motif (PAM) is underlined, and the red triangle indicates where Cas9 is expected to cleave. B) Partial lipodystrophy disease model schematic\textsuperscript{15}. At Day 21, both WT and CIDE-C E186X adipocytes will be ready for assessment via microscopy and standard \textit{in vitro} adipocyte characterization assays.

**Thesis aims: modeling partial lipodystrophy with CIDE-C E186X hESC-derived adipocytes**

To reiterate, the utility of a stem cell-based disease model lies in the ability of the \textit{in vitro} system to accurately recapitulate the phenotype of the disease of interest. For example, a particularly convincing feature of a partial lipodystrophy model, specifically, might be expected to form multilocular lipid droplets. To our knowledge, no previous study has used a stem cell-based system to model partial lipodystrophy. As with any model, it is important to be acutely aware of the potential assumptions made in creating the model, such as the decision to grow cells
in a monolayer culture versus 3D culture, or the decision to not recapitulate the extracellular matrix and many cell types present in adipose tissue.

In this project, we aim to confirm that the CIDE-C E186X mutation observed in the partial lipodystrophy patient is necessary and sufficient to induce the partial lipodystrophy phenotype in vitro. In order to do so we carried out the following:

i. Differentiation of wildtype (WT) and CIDE-C E186X mutant (E186X) hESCs into white adipocytes, following an optimized protocol previously established in the Cowan Lab;\(^\text{15}\)

ii. Observation of morphological differences between WT and E186X adipocytes using confocal microscopy and immunostaining techniques;

iii. Assessing the biological function of the WT and E186X adipocytes with in vitro adipocyte characterization assays. Such characterization assays measured intracellular triglyceride content, relative basal lipolysis activity, and gene expression via quantitative real time PCR (RT-qPCR) between the isogenic cell lines.

Through a strategic combination of microscopy and adipocyte characterization studies, we have evolved better understanding of the distinct adipocyte-like phenotype that results from the CRISPR/Cas9-mediated E186X mutation of CIDE-C.
EXPERIMENTAL METHODS

Targeting CIDE-C via the CRISPR/Cas9 genome editing system

The HuES9 hESC cell line was maintained at passage 40 in stem cell media (mTesR1™ Basal Medium, Stemcell Technologies; 1% (v/v) penicillin/streptomycin, 1 ug/ml Plasmocin™, Invivogen; 5X Supplement, Stemcell Technologies) until fully confluent. The CIDE-C gene was targeted using a single guide strategy; stem cells were co-transfected via electroporation with plasmids containing the CIDE-C CRISPR guides and the Cas9-GFP (Figure 8A). GFP-positive cells, an indication of both successful transfection and Cas9 delivery, were positively sorted via a standard fluorescence-activated cell sorting (FACS). GFP-positive cells were plated at 1,000 cells/cm² and expanded into sizable colonies. Genomic DNA from healthy stem cell colonies was isolated and sequenced to screen for clones with the E186X mutation. Stem cell clones with the E186X mutation were expanded in preparation for differentiation into adipocytes.

Confirmation of CIDE-C E186X mutation via genomic DNA sequencing

A preliminary PCR was performed to determine the optimal annealing temperature for the CIDE-C-specific primer pair. The optimal annealing temperature was determined to be 67 °C (Supplemental Figure 1). The sequences of WT clone A-C5 and potential CIDE-C mutant clone E5, A2, and E6 were determined by amplifying the 250-bp CIDE-C amplicon via PCR, running the PCR products on a 1% agarose gel, gel-purifying the amplicon of interest according to manufacturer’s instruction (QIAEX II Gel Extraction Kit, Qiagen), and sending samples out for sequencing (Genewiz, Inc).
Directed differentiation of hESCs into mesenchymal progenitor cells (MPCs)\textsuperscript{15}

Stem cells were split at a very low density in stem cell media (mTESR1) with 4 uM rho-associated protein kinase (ROCK) inhibitor, allowing for easier attachment of cells to the dish by inhibiting the apoptotic pathway. On Day 0, mTESR1 was replaced with specialized MPC-forming media (MAC media: Dulbecco’s Modified Eagle Medium, 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin, 5 ng/mL basic fibroblast growth factor (bFGF), insulin-transferrin-selenium (ITS) liquid media supplement, Sigma I3146)). The MAC media was replaced daily for the first three days, then every other day for all days proceeding. At Day 14, cells were FACS sorted for CD73, a mesenchymal progenitor cell (MPC) marker. CD73\textsuperscript{+} cells were replated at a cell density of 10,000 cells/cm\textsuperscript{2} in MPC-mantaining media (DMEM10: Dulbecco’s Modified Eagle Medium (DMEM), 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin). MPCs can be passaged up to 8 to 10 times.

Creation of the PPARG lentivirus

Quality human embryonic kidney (HEK) 293T cells were grown to confluency in 15 cm dishes maintained in DMEM10. 3 viral packaging plasmids plus plasmid with PPARG gene were bubbled into calcium chloride solution and added to HEK 293T cells, which were then allowed to incubate at 5% CO\textsubscript{2} for 24 hours. Cells were replenished with fresh DMEM10, and allowed to incubate at 10% CO\textsubscript{2} for additional 24 hours. After this time, the virus particle-containing media from the cells were ready for infection.
Directed differentiation of MPCs into adipocytes

For even coverage, lentiviral infection was carried out in 10 or 15 cm dishes, as opposed to individual wells. DMEM10 media was replaced with virus media and allowed to incubate in 10% CO2 for 24 hours. Cells were replenished with a fresh round of virus and allowed to incubate in 5% O2 for at least 24 hours. After infection, cells were plated (50,000 cells/well for 24 well plate, 20,000 cells/well for 48 well plate) and allowed to reach confluency, at which point adipogenic differentiation medium supplemented with doxycycline (700 ng/ml) was introduced. At Day 16, cells were fed adipogenic differentiation medium in the absence of doxycycline (A2) to ensure that adipocytes differentiation were endogenously motivated. Day 0 is defined as the day adipogenic medium with doxycycline (A2 + dox) replaced DMEM10 media. Components of adipogenic differentiation (A2) media include 7.5% knockout serum replacement (KOSR; Invitrogen), 7.5% human plasma protein fraction, 0.5% non-essential amino acids (NEAA), 1% penicillin/streptomycin, 0.1 μM dexamethasone, 10 μg/ml insulin (Sigma) and 0.5 μM rosiglitazone in DMEM.

BODIPY Neutral Lipid Staining

At Day 21, cells were washed once in phosphate-buffered saline (PBS) then fixed with 4% paraformaldehyde in PBS. After 10 minutes, cells were washed in PBS and allowed to incubate in a staining solution of 1:5000 Hoechst and 1:5000 BODIPY, to stain nuclei and neutral lipids, respectively, in 0.025% Triton-100-X in PBS. After 10-15 minutes, the staining solution was replaced with mounting media (10% glycerol in PBS) and imaged. All images were acquired with a Nikon Digital Sight camera mounted to a Nikon Eclipse Ti-S microscope. NIS-Elements and Olympus DP2-BSW software packages were used for image analysis. A similar protocol
was followed to acquire images for the differentiation time course, except images were taken every two days up until Day 21.

**CIDE-C and PLIN1 Co-Immunostaining via Immunocytochemistry**

Cells were washed once in PBS then fixed with 4% (v/v) paraformaldehyde in PBS. After 10-15 minutes, cells were incubated in 0.25% Triton-100-X (PBST) for another 10 minutes. Cells were blocked in 1% (w/v) bovine serum albumin (BSA) in 0.25% (v/v) Triton-100-S for 30-90 minutes. Primary antibodies (rabbit anti-CIDE-C, Novus Biologicals and goat anti-PLIN1, Everest Biotech) were both applied, at a final concentration of 1 ug/ml, at 4 °C overnight. Donkey anti-rabbit and anti-guinea pig secondary antibodies were applied at a final concentration of 1 ug/ml at room temperature for 1 hour (Life Technologies). Samples were washed with PBST and stained for nuclei with 2ug/ml Hoechst. All images were acquired with a Nikon Digital Sight camera mounted to a Nikon Eclipse Ti-S microscope. NIS-Elements and Olympus DP2-BSW software packages were used for image analysis.

**Bradford Protein Concentration Determination Assay**

Whole cell lysate was obtained through incubating wells with 0.1% Triton-X-100 in PBS for 10-15 minutes. Protein concentration in cell lysate was determined with the Bio-Rad DC Protein Assay kit, following manufacturer’s instructions. Experiments were carried out in biological and technical triplicates.
**In vitro Triglyceride Quantification Colorimetric Assay**

Whole cell lysate was obtained by incubating wells with 0.1% Triton-X-100 in PBS for 10-15 minutes. Cell lysate sample was combined with triglyceride assay reagent in a 1:100 sample-to-reagent ratio (Infinity, ThermoScientific 2780-400H) Measurements were taken on a SpectraMax multiplate reader at 540 nm using SoftMaxPro data collection software. A free glycerol standard was used to quantify triglyceride levels. The amount of triglyceride per well was normalized to the amount of protein in the corresponding well (see Bradford Protein Assay). Experiments were carried out in biological and technical triplicates.

**In vitro Free Glycerol Release Quantification Colorimetric Assay**

Media from each well was combined with a free glycerol reagent (Sigma F6428) in a 1:80 ratio. Measurements were taken on a SpectraMax multiplate reader at 520 nm and compared to a free glycerol standard. The amount of free glycerol released per cell was normalized to the amount of protein in the corresponding well (see Bradford Protein Assay). Experiments were carried out in biological and technical triplicates.

**In vitro Free Fatty Acid (FFA) Quantification Colorimetric Assay**

Free fatty acid was quantified with a free fatty acid kit, following manufacturer’s instructions (Abcam). Measurements were taken on a SpectraMax multiplate reader at 570 nm and compared to a palmitic acid standard. The amount of free fatty acid released per cell was normalized to the amount of protein in the corresponding well (see Bradford Protein Assay). Experiments were carried out in biological and technical triplicates.
RNA extraction, cDNA synthesis and RT–qPCR.

Total RNA from human cell lines and human fat was extracted with Trizol (Invitrogen) and purified using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. The RNA yield was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Total RNA (350 ng) was converted to cDNA using the Superscript First-Strand kit (Invitrogen). RT–qPCR was carried out using a Applied Biosystems ViiA 7 Realplex Mastercycler (Eppendorf/Life Technologies) with the Quantifast-SYBR Green PCR mix (Qiagen) with 1 μl cDNA per reaction. Analysis of cycle times was mediated with accompanying ViiA 7 software. Primer sequences are listed in Supplementary Section (Supplemental Figure 3).
RESULTS

Confirmation of the *CIDE-C* E186X mutation in HuES9 pluripotent stem cells

Previous research in the lab designed a CRISPR/Cas9 genome editing strategy that specifically targeted a region near the 186\textsuperscript{th} codon in *CIDE-C* of the human embryonic stem cell line HuES9 resulting in the creation of many putative *CIDE-C* mutant clones. To identify if any of these putative *CIDE-C* mutant clones did indeed contain the E186X mutation, the genomic DNA of multiple putative mutants (A2, E5, and E6), were prepared for gene sequencing through the services of GENEwiz, Inc. For comparison, a wildtype clone (A-C5) was also prepared for sequencing. DNA primer sequences flanking the amplicon of interest, corresponding to the relevant region of *CIDE-C*, are listed in Supplemental Table 3. Through a standard annealing temperature optimization PCR, the optimal annealing temperature was determined to be 67 °C (Supplemental Fig 1A). Although the primer also picked up nonspecific bands, the bands were distinct enough such that the 250 base pair (bp) band corresponding to the *CIDE-C* amplicon could be isolated (Supplemental Fig 1B).

While the CRISPR/Cas9 genome editing system did not mimic the precise mutation observed naturally in the patient, both the natural and CRISPR-mediated mutations encoded for identical gene products (Figure 9A). The naturally occurring mutation featured a transversion (guanine to thymine) at base pair 558, and the CRISPR-mediated E5 clone featured an insertion of a thymine at base pair 559. Although different, both naturally occurring and CRISPR-mediated mutations resulted in a nonsense E186X mutation and premature truncation of the *CIDE-C* product. This confirms the creation of isogenic *CIDE-C* lines, legitimizing any future comparison between the wildtype and mutant lines. Other putative *CIDE-C* clones were confirmed to be *CIDE-C* mutants
that varied from the E186X mutation; while these mutants do not faithfully mimic the patient-observed mutation, they may be useful for further studies (Supplemental Figure 2).

**Figure 9.** The confirmed CIDE-C E186X mutation results in a putative adipocyte with multiple, small lipid droplet-like structures. A) Genomic DNA sequence comparison between wildtype CIDE-C, CIDE-C from patient, and CIDE-C from putative mutant stem cell clone E5. B) Brightfield images of Day 21 WT (top) and Day 21 E186X (bottom) adipocytes at various magnifications (4x, 10x, and 20x). A single adipocyte is outlined in red in the 20x magnification.

**E186X mutants exhibit a striking multilocular lipid droplet morphology**

In order to observe the effect of the CRISPR/Cas9-mediated CIDE-C mutation, both wildtype and mutant lines were differentiated into adipocytes, the cell type in which CIDE-C is most highly expressed. The morphology of WT adipocytes had what appeared to be prominent, large lipid droplets; at Day 21, only a few adipocytes were unilocular, which may suggest Day 21 adipocytes were not fully mature (Figure 9B, top panels). It was observed that the lipid
droplets often clustered in the general outline of a wheat chaff to fit inside the adipocyte. In some cases, the cluster of WT adipocytes would contain lipid droplets of uniform size, while other clusters contained a large, centrally located lipid droplet surrounded by microdroplets (Figure 9B, top right panel). In contrast to the WT adipocyte phenotype, the E186X phenotype featured what appeared to be extremely small, clustered multilocular lipid droplets; only at higher magnifications did the putative droplets become distinct (Figure 9B, bottom central and right panel). While each putative lipid droplet dramatically decreased in size, the relative number of lipid droplets per adipocyte increased, resulting in an adipocyte approximate five times larger in size.

To confirm that this observed morphology was an irregular composition of lipid droplets, a controlled differentiation was performed. WT and E186X pluripotent stem cells were differentiated to the mesenchymal progenitor cell (MPC) stage. As per protocol, both cell lines were evenly transduced with a lentiviral construct containing a doxycycline-inducible promoter driving *PPARG2* expression. When MPCs were introduced to doxycycline and adipogenic media, which contains crucial factors to facilitate adipocyte differentiation, expression of *PPARG2* would initiate adipogenesis. To assess if the differentiation was controlled, infected cells were plated under three conditions: in MPC media (DMEM10), adipogenic media (A2), or adipogenic media with doxycycline (A2 + dox) and cultured for at least twenty-one days. As expected, both WT and E186X cells kept in DMEM10 consistently retained an MPC-like character (Figure 10A, left). In addition to a striated, wheat chaff-like morphology, cells kept in DMEM10 did not stain positive for neutral lipids (BODIPY stain), which indicates an absence of triglyceride-filled lipid droplets (Figure 10A). In contrast, all cells kept in A2 media or A2 media + doxycycline stained positive and specifically for neutral lipids, confirming their adipocyte-like
character. The neutral lipid staining also emphasized the difference in lipid droplet distribution between WT and E186X mutant cells (Figure 10A, right). Consistently, at higher magnification, the spherical shape of the BODIPY-positive lipid droplets became more easily distinguishable (Figure 10B, right). Cells kept in A2 media or A2 media + doxycycline typically differentiated into adipocytes with an average efficiency of 50-60%, confirming the relatively successful control of adipocyte differentiation.

Figure 10. Neutral lipid (BODIPY) staining confirms the presence of small, multilocular lipid microdroplets in E186X mutant adipocytes. A) WT MPCs at passage number 13 (p13) and
CIDEC E186X MPCs at passage number 7 (p7) were transduced with a lentiviral construct containing a doxycycline-inducible promoter driving PPARG2 expression. Cells were kept in either MPC media (DMEM10), adipogenic media, or adipogenic media and doxycycline for 21 days (only cells in adipogenic media shown for clarity). Brightfield images (left panels) illustrate the difference between the MPC morphology of infected cells kept in DMEM10 and the prominent lipid droplets visible in infected cells kept in adipogenic media. Cell nuclei were stained for Hoechst (center panels), and neutral lipids were detected with BODIPY (right panels). All images taken at 10x magnification. B) The BODIPY staining pattern of Day 21 CIDEC E186X mutants at higher magnifications confirms that lipid microdroplets still form a spherical shape.

A qualitative observation of the differentiation time course of WT and CIDEC E186X mutant human embryonic stem cells into adipocytes provides insight into the timeline by which both wildtype and E186X MPCs differentiate. The E186X mutation is observed to affect the phenotype around Day 6 to Day 8. The first few lipid droplets in WT are detectable with brightfield and BODIPY staining around Day 4. Around Day 6 to Day 8, the BODIPY stain localizes to a unilocular lipid droplet for WT cells, but is more dispersed in the CIDEC cells (Figure 11).

Figure 11. Lipid droplet accumulation and adipocyte differentiation time course differ between E186X and WT cells. WT MPCs p11 (top rows) and CIDEC E186X MPCs p9 (bottom rows) were transduced with a lentiviral construct containing a doxycycline-inducible promoter driving PPARG2 expression. All cells were kept in adipogenic media and doxycycline and imaged every two days for 21 days. Cell nuclei were stained Hoechst (blue) and neutral lipids were stained with BODIPY (green). All images taken at 10x magnification.
In E186X mutants, truncated CIDE-C and full length PLIN1 may still colocalize to the lipid droplet surface

To confirm that the truncated CIDE-C gene product is ultimately responsible for the observed multilocularity in the E186X mutant adipocytes, a co-immunostaining experiment was designed to determine the localization of CIDE-C in E186X mutants. The full-length CIDE-C protein structure has yet to be solved, which provides challenges when determining which regions of CIDE-C are crucial for its function. Previous studies have proposed that the CIDE-C domain of CIDE-C is crucial for localization to the lipid droplet surface. Therefore, we might have expected E186X mutants, whose truncation disrupts the CIDE-C domain, to demonstrate punctate, cytosolic CIDE-C-specific staining.

To identify whether or not the CIDE-C truncation hinders localization of CIDE-C to the lipid droplet surface, WT and E186X Day 21 adipocytes were co-immunostained against CIDE-C and PLIN1, as a positive control. To identify both WT and truncated CIDE-C present, an antibody against the N-terminus of CIDE-C was used. In WT adipocytes PLIN1 and CIDE-C positively and definitively co-localized to the lipid droplet surface, as expected, though anti-CIDE-C staining was slightly nonspecific. The E186X mutants also tentatively demonstrated a co-localization around their poorly formed lipid droplets (Figure 12). It should be noted that the particular batch of E186X mutant cells available for co-immunostaining had difficulty differentiating, so results are inconclusive on this front.
Figure 12. CIDE-C and PLIN1 may colocalize to the lipid droplet surface in both WT and E186X adipocytes. At Day 21, CIDE-C p10 (top) and WT p11 (bottom) cells were fixed and immuno/stained for (from left to right): cell nuclei (Hoechst, blue), PLIN1 (anti-PLIN1 primary antibodies, red), and CIDE-C (anti-CIDE-C primary antibodies, green). Merged images (far right) generated using ImageJ software. All images taken at 10x magnification.

**E186X mutant adipocytes contain lower levels of triglyceride per cell and higher basal lipolytic activity, as expected.**

Although imaging provides useful qualitative information, *in vitro* adipocyte characterization assays may allow for quantitative comparisons between WT and E186X adipocytes. Three separate colorimetric assays were performed on both WT and E186X adipocytes to determine relative intracellular triglyceride content, free glycerol release, and free fatty acid release (Figure 13A, 14A, 14C).

The measure of intracellular triglyceride content is a metric to assess how well the adipocytes can store energy. To measure triglyceride content per cell, triglyceride levels must be normalized to respective protein levels in each biological sample. Normalized triglyceride levels in E186X adipocytes were significantly lower than those in WT adipocytes, suggesting that the
E186X mutant adipocytes are unable to store lipids properly. This observation is consistent with the hypertriglyceridemia phenotype associated with partial lipodystrophy.

Figure 13. E186X mutant adipocytes store less triacylglyceride, consistent with the hypertriglyceridemia phenotype associated with partial lipodystrophy. A) Schematic of the intracellular triacylglyceride colorimetric assay, where absorbance at 540 nm is proportional to concentration of triacylglyceride stored in cells. B) Comparison of intracellular triacylglyceride levels between WT MPCs, WT adipocytes, E186X MPCs, and E186X adipocytes. The quantity of triacylglyceride released, in micrograms, was normalized to the total amount of protein released, in milligrams. Experiments were carried out in biological triplicates. (p<0.05=*, p<0.01=**, p<0.001=***)

Measuring free glycerol and fatty acid release provides a metric to assess relative basal lipolysis levels of the adipocytes; because lipases can hydrolyze TAGs, diacylglycerols (DAGs), and monoacylglycerols (MAGs), the combination of both assays provides a more accurate picture of lipolytic activity. On average, E186X mutant adipocytes released higher levels of glycerol and significantly higher levels of fatty acid, normalized to respective protein levels (Figure 14B, 14D). Higher levels of free glycerol and fatty acid are indicative of higher levels of basal lipolysis. Although noisy, free glycerol levels corresponding to WT MPCs, WT adipocytes, E186X MPCs, and E186X adipocytes consistently match the trend observed with amongst free fatty acid levels.
Figure 14. E186X mutants have increased levels of basal lipolysis. A) Schematic of the free glycerol colorimetric assay, where absorbance at 520 nm is proportional to concentration of free glycerol released in supernatant of the cells. B) Comparison of free glycerol release between WT MPCs, WT adipocytes, E186X MPCs, and E186X adipocytes. The quantity of glycerol released, in micrograms, was normalized to the total amount of protein released, in milligrams. Experiments were carried out in biological triplicates. C) Schematic of the free fatty acid colorimetric assay, where absorbance at 570 nm is proportional to concentration of free glycerol released in cell media. D) Comparison of free fatty acid release between WT MPCs, WT adipocytes, E186X MPCs, and E186X adipocytes. The quantity of fatty released was normalized to the total amount of protein released, in milligrams. Experiments were carried out in biological triplicates. (p<0.05=*, p<0.01=**, p<0.001=***
**E186X mutant adipocytes present a contradictory, preadipocyte-like/pro-lipolytic expression profile.**

To determine if or how the E186X mutation affects the adipocyte character of the *CIDE-C* mutants, the distinct gene expression patterns exhibited by wildtype and E186X mutants were assessed. Levels of expression were standardized to the housekeeping gene *RPLP*, encoding for a ribosomal protein. In addition to surveying *CIDE-C*, other genes targeted included a variety of preadipocyte markers (*PPARG2, CEBP-α*, and *PDGFR-α*), mature adipocyte markers (*PLIN1, ADIPOQ, FABP4, and ATGL, and HSL*), and, as a negative control, the brown adipocyte-specific marker *UCP-1*. Most differences in gene expression were not statistically significant (p<0.05); only the expression of *PDGFR-α*, encoding for platelet-derived growth factor receptor alpha, was significantly upregulated, compared to the wildtype adipocyte profile. However, trends were present in the gene expression profile. There was consistently decreased expression of most mature adipocyte markers in E186X mutants and relatively increased expression of adipocyte progenitor markers (Figure 15). This data suggests that *CIDE-C* mutants may not be successfully differentiating into mature adipocytes. Unexpectedly, the expression of hormone-sensitive lipase, *HSL*, was upregulated in E186X mutants (Figure 15). Equally unexpectedly, the expression of *UCP-1*, a brown adipocyte-specific marker, was highly downregulated in E186X mutants. Additional trials may help increase significance in observed reduced gene expression in *CIDE-C* mutants.
Figure 15. E186X mutants demonstrate a preadipocyte-like, pro-lipolytic character. RT-qPCR assays were carried out on WT and E186X adipocytes. A variety of potentially informative adipocyte-related markers were surveyed (primers listed in Supplemental Figure 3). Relative expression levels shown are relative to RPLP (housekeeping gene) expression in each sample. All assays were performed in biological triplicate. (p<0.05 = *; p<0.01=**; p<0.001 = ***
DISCUSSION

In this study we aimed to model partial lipodystrophy with a human pluripotent stem cell-derived adipocytes with a *CIDE-C* E186X mutation. A thorough comparison of the adipocytes isogenic for *CIDE-C* confirmed that the E186X mutant adipocytes formed multilocular lipid droplets, stored lower amounts of triglyceride per cell, and experienced higher basal levels of lipolysis, as compared to the WT adipocytes. Therefore, the character of the E186X mutants is consistent with the partial lipodystrophy phenotype.

Assessing the partial lipodystrophy model on the cellular and morphological level

Although E186X mutants contained multiple, dramatically smaller lipid droplets, it is possible that collectively, the total concentration of lipid per mutant adipocyte was comparable to that per wildtype adipocyte. However, a comparison of normalized triglyceride levels demonstrated that the E186X mutants not only partition lipid droplets more inefficiently, but also contain lower levels of triglyceride per cell.

Due to their smaller lipid droplets, E186X mutant adipocytes also had relatively increased total surface area per adipocyte. It was predicted, then, E186X mutants should experience higher levels of basal lipolysis due to increased lipase access to the lipid droplet surface. Data from the free glycerol release and free fatty acid assays support this prediction; on average, E186X mutant adipocytes released higher levels of glycerol and significantly higher levels of fatty acid normalized to respective protein levels (Figure 14B, 14D). The varied distribution in size of lipid droplets in WT most likely serves as a mechanism to regulate lipolysis rates in the cell. Although the most efficient storage strategy would be to form the unilocalar lipid droplet, there may be some advantage in having lipid droplets of various sizes.
In the case of WT adipocytes, the cluster of lipid droplets seemed arranged such that the largest lipid droplet was often buried in the cluster, and thus less exposed to lipases (Figure 9). In the case of E186X mutants, however, with all lipid droplets inefficiently small, any strategy of metabolism regulation would be compromised. This lack of lipid droplet organization, and therefore lack of energy regulation, is likely to play a role in the disruption of metabolic processes.

**Assessing the partial lipodystrophy model on the molecular level**

Increased surface area is most likely not the sole reason for increased levels of basal lipolysis. As a lipid droplet surface-associated protein, CIDE-C is thought to indirectly and directly regulate lipolysis. Previous research has demonstrated that the direct binding of the CIDE-C domain to ATGL mediates ATGL-mediated lipolysis; therefore, ATGL activity may relatively increase in the absence of functional CIDE-C. A stimulated lipolysis assay comparing the WT, E186X, and another CIDE-C mutant truncated in the region thought to be responsible for ATGL binding might help confirm previous findings.

The mechanism by which the E186X disrupts the lipid droplet formation process is not yet clear. It was previously proposed that the CIDE-C is a lipid droplet surface-associated protein, and that the CIDE-C domain of CIDE-C is also responsible for localization to the lipid droplet surface. Because the E186X mutation prematurely truncates the CIDE-C domain, it was expected that CIDE-C would fail to localize to the lipid droplet surface. While not fully conclusive, the data suggest that PLIN1 and CIDE-C continue to co-localize at the lipid droplet in E186X adipocytes. Without the solved crystal structure of human CIDE-C protein, determining the function of various regions of CIDE-C through experimentation has not been precise. The entire
CIDE-C domain, for example, has been cited to be involved in apoptosis, lipid droplet surface localization, and lipolysis regulation. It is possible that one domain is responsible for a wide variety of functions, but without a specific designation of functional regions within the CIDE-C domain, it may be difficult to design rational experiments that tease out the CIDE-C mechanism on the molecular level. Assuming that the truncation did not completely disrupt the natural folding pattern of CIDE-C, the continued localization of CIDE-C to the droplet surface would suggest that a lipid droplet localization signal sequence is located prior to the E186 residue. Assessing the localization of CIDE-C, via co-immunostaining experiments, in hESC-derived adipocytes mutated at regions before E186 may help elucidate the region(s) within the CIDE-C domain that are important for droplet surface localization.

**Future developments of the CIDE-C E186X partial lipodystrophy model**

A few recommendations may help improve the future development of this partial lipodystrophy model. For initial rounds of adipocyte differentiation, cells kept solely in A2 media differentiated robustly into adipocytes, which was unexpected. We suspect that the virus was extremely powerful and PPARG2 expression was not under control of the doxycycline-inducible promoter. Over time, as the virus lost its potency, it was observed that differentiation did once again become doxycycline-inducible (data not shown). While this is not ideal, it does not necessarily detract from the goals of this study, which aims, in one way or another, to have WT and E186X adipocytes to compare. Nevertheless, it is recommended to check the potency of the virus before infecting MPCs, to ensure for complete control over the differentiation. A standard plaque-based assay can be used to quantify the potency of the viral media. In this assay, cells are divided into dishes and exposed to a range of dilutions of virus. The virus’ potency is
quantified by observing and calculating the number of plaque-forming units per milliliter per dish.\textsuperscript{59} It was also noted that adipocyte differentiation efficiency was highest when cells were plated on larger surface areas, so it is highly recommended to carry out differentiation on dishes with at least 1.9 cm\textsuperscript{2} of surface area or larger (Supplemental Figure 4).

**Applying the model to address the pathogenesis of partial lipodystrophy; future biological and clinical applications**

In summary, the combination of microscopy, immunostaining, *in vitro* adipocyte characterization assays, and gene expression profiling consistently and sufficiently confirm the partial lipodystrophy phenotype in the *CIDEC* E186X adipocytes. Still, the model would benefit from additional research that confirms that the model can accurately recapitulate insulin resistance, a characteristic symptom of partial lipodystrophy. Insulin resistance is strongly correlated with elevated levels of FFA released in the bloodstream.\textsuperscript{60} Because E186X adipocytes release relatively higher levels of FFA, they are also expected to exhibit an insulin resistant phenotype – that is to say, glucose uptake levels would not increase in the presence to insulin. Potentially informative insulin sensitivity assays might include an AKT2 phosphorylation assay. This assay qualitatively detects phosphorylated AKT2, a measure of insulin sensitivity, in the presence and absence of insulin. Unfortunately, unlike the colorimetric assays that measure TAG, free glycerol, and FFA, these current techniques to “measure” insulin sensitivity are not sensitive enough to detect a dose-dependent effect of insulin. Experiments investigating hormone-stimulated lipolysis may also be important to further characterizing the E186X mutation; it would be expected that E186X mutants have relatively higher hormone-stimulated lipolytic activity.
While future experiments further confirming the partial lipodystrophy phenotype would indeed be beneficial, the current model is in a strong position to address questions about the pathogenesis of partial lipodystrophy, the regulation of adipocyte differentiation, the necessary cellular parameters for normal metabolic function, and the singular role CIDE-C might play in the progression of the disorder. Although it is very clear that the E186X mutant adipocytes faithfully mimic the disease phenotype, it is not yet completely clear the mechanism by which the E186X mutation causes the cellular metabolic dysfunction and preadipocyte-like state. In combination with previous experiments, our data suggest that expression of CIDE-C is necessary to promote but not sufficient to initiate adipogenesis. The correlation between a lack of functional CIDE-C and a halted differentiation into mature adipocytes seems obvious on a macroscopic level; if CIDE-C is necessary for lipid droplet growth, but only mature adipocytes display unilocular, large lipid droplets, then a cell lacking functional CIDE-C can never reach a mature adipocyte stage.

But is it the solely the lack of efficient lipid storage that results in a distorted metabolic system and preadipocyte-like character? Would a mutation in any lipid droplet surface-associated protein result in a similar phenotype as the E186X mutation? Or is there a role unique to CIDE-C that is crucial in promoting both lipid droplet formation and encouraging adipogenesis? Previous research suggests that the maintenance of the lipid droplet is the most important factor in regulating metabolism. In 2011, clinicians identified patients with a novel subtype of partial lipodystrophy due to variants of a frameshift mutation in PLIN1. These patients had insulin resistance, hypertriglyceridemia, and type II diabetes, and their adipose tissue was also observed to have multilocular lipid droplets and fibrosis. Studies in mouse 3T3-L1 cell lines have confirmed that a loss-of-function PLIN1 (disrupting either Leu404 or Val398)
also results in a partial lipodystrophy phenotype in vitro, most likely because the PLIN1 fails to sequester CGI-58, the co-activator necessary for ATGL-mediated lipolysis. It might be interesting to confirm the partial lipodystrophy phenotype due to PLIN1 deficiencies in a human stem cell-based model. To do so, a PLIN1 frameshift mutation would need to be introduced into a hESC line, and differentiate PLIN1-mutants into adipocytes to compare against E186X and WT adipocytes. From these findings, it seems that further research into partial lipodystrophy may not necessarily need to focus on further characterizing CIDE-C function, than it would be to explore the variety of droplet surface-associated proteins that regulate and promote lipid droplet formation. Furthermore, a target therapeutic might focus on promoting lipid droplet transfer and lipolysis regulation broadly.

The gene expression profile of the E186X mutants also generates questions about how, either directly or indirectly, the CIDE-C mutation regulates expression of pre-adipocyte- or mature adipocyte-associated genes. CIDE-C expression is also reduced in E186X mutants, which suggests that the truncation of CIDE-C somehow inhibits further CIDE-C expression. Interestingly, hormone-sensitive lipase, HSL, was the only mature adipocyte marker upregulated in E186X mutants. Besides ATGL and monoacylglycerol (MGL), HSL is the rate-limiting lipase involved in hormone-stimulated lipolysis in adipocytes. HSL is stimulated by multiple hormones, such as epinephrine, glucagon, ACTH, and catecholamines, and it becomes activated via a cAMP-dependent protein kinase A (PKA) signaling pathway. Upon PKA-mediated phosphorylation, HSL is activated and translocated to the lipid droplet surface. HSL cleaves with broad specificity. Its hydrolytic targets are mainly di-, mono-, and tri-acylglycerides and cholesterol esters. The upregulation of HSL in E186X mutants, when coupled with the observation of increased basal lipolysis, is potentially interesting because it may suggest that
HSL is upregulated in response to the reduced size and number of lipid droplets within the adipocyte. Of course, it is not immediately clear the casual relationship between the CIDE-C mutation and increased HSL expression.

The increased HSL expression may be due to the multilocular lipid droplet composition within E186X adipocytes. This observation suggests, provocatively, that increased lipid droplet surface area within adipocytes may somehow be a signal to the cell to increase the production of lipases. In this sense, the E186X mutation would generate a positive feedback mechanism of sorts, in which lipase activity leads to smaller lipid droplets, which recruits lipase production and ultimately more robust lipase activity. To reconcile the observation that not all lipase-encoding genes were upregulated, it might be argued that lipid droplet formation somehow encourages an increase in HSL expression specifically. The specific upregulation in HSL may suggest that CIDE-C and HSL directly interact, and that the CIDE-C E186X mutation is directly causative of the increased HSL production. Previously not much research has been devoted to investigating CIDE-C and HSL interactions. To confirm this hypothesis, it might be useful to assess in vivo and in vitro protein-protein interaction studies, such as FRET or co-immunoprecipitation experiments, respectively, with CIDE-C and relevant lipases (HSL, ATGL) under basal and hormone-stimulated conditions in E186X and WT adipocytes. To isolate and determine the specific relationship between CIDE-C and HSL, it might be useful to carry out these protein-protein assays in non-adipocyte lines with ectopically expressed CIDE-C and HSL. It might also be interesting to express a variety of CIDE-C mutants with WT HSL to determine which regions are most important, if at all, for the CIDE-C:HSL interaction. If there was a correlation between smaller lipid droplets and the increased HSL expression, it might be expected that FABP4 expression would also increase. FABP4 is thought to attenuate fatty acid inhibition of HSL
activity by forming a complex with HSL, thereby increasing lipolytic activity.\textsuperscript{27} \textit{FABP4} expression was decreased in E186X mutants, which compels further investigation into the role and relationship, if any, between CIDE-C, HSL, and FABP4.

As previously mentioned, it is always important to realize both the potential and limitations of this partial lipodystrophy model. From a clinical perspective, the disease model is limited, since the model adipocytes are grown in a monolayer and not recapitulating adipose tissue fully. At this point, 3D culture of adipose tissue has been attempted but not necessarily well established, especially not for pluripotent stem cell-based systems.\textsuperscript{63} Certain symptoms associated with partial lipodystrophy, such as the darkening of the epithelial tissue in the armpit area, may not easily be explained with our cell-based model. Importantly, adipose tissue is an endocrine organ, and is actively interacting and exchanging with other cell types. Intercellular communication cannot be consistently recreated in our model. It may be argued, however, that the advantages of the stem cell-based model outweigh its limitations. Firstly, the cell-based model may be beneficial in identifying the independent contribution of one cell type to a complex metabolic disorder. Secondly, because the E186X adipocytes are derived from stem cells, the model is renewable, reproducible, and therefore primed for future investigations. The model may help design a rational high throughput drug screen that identifies compounds that perturb lipid droplet formation, resulting in a phenotype similar to the E186X mutation. Farther down the road, a separate drug screen may also attempt to restore lipid droplet formation within \textit{CIDE-C} mutant adipocytes, though further insight into the CIDE-C-mediated mechanism of lipid droplet formation may be needed to inform such a screen. With this \textit{in vitro} disease model, both the progression of partial lipodystrophy and the role of lipid droplet formation in regulating metabolic processes may soon be fully characterized.
Supplemental Figure 1. The optimal annealing temperature for the CIDE-C DNA amplicon was determined to be 67 °C, at which the primers had highest specificity to the region of interest. (A) The forward and reverse primers flanking the CIDE-C amplicon (250 bp) were nonspecific, as evidenced by the presence of multiple bands in a single lane. At 67 °C the CIDE-C amplicon was most clearly resolved from nonspecific bands. (B) Analysis of WT and multiple putative
*CIDE*-C mutant stem cell clones demonstrates a distinct amplification of the desired *CIDE*-C amplicon (250 bp), which was subsequently gel-purified and sent out for sequencing.

**CIDE-C wildtype A-C5:**

AGTAGAGACAGAGATTCCACATGGCCATGCTAGTCTCAAACAACCTGTACGTTACGCCCTCCCAAGTGCTAGGATTACA
GGCGTGAACGCATGGGCGACCTACTTCGTGGTTTGTTCACCTGGGCTCCCCTCTCATCACCAACCAGGGAAGCTTTCCGCTGGGGCTCCCTCTCAAGCAGCTG

**CIDE-C E5 (1 bp insertion, frame shift, nonsense mutation):**

AGTAGAGACAGAGATTCCACATGGCCATGCTAGTCTCAAACAACCTGTACGTTACGCCCTCCCAAGTGCTAGGATTACA
GGCGTGAACGCATGGGCGACCTACTTCGTGGTTTGTTCACCTGGGCTCCCCTCTCATCACCAACCAGGGAAGCTTTCCGCTGGGGCTCCCTCTCAAGCAGCTG

**CIDE-C A2 (3 bp deletion, not frame shift):**

AGTAGAGACAGAGATTCCACATGGCCATGCTAGTCTCAAACAACCTGTACGTTACGCCCTCCCAAGTGCTAGGATTACA
GGCGTGAACGCATGGGCGACCTACTTCGTGGTTTGTTCACCTGGGCTCCCCTCTCATCACCAACCAGGGAAGCTTTCCGCTGGGGCTCCCTCTCAAGCAGCTG

**CIDE-C E6 (R189L, 9 bp deletion, frame shift):**

AGTAGAGACAGAGATTCCACATGGCCATGCTAGTCTCAAACAACCTGTACGTTACGCCCTCCCAAGTGCTAGGATTACA
GGCGTGAACGCATGGGCGACCTACTTCGTGGTTTGTTCACCTGGGCTCCCCTCTCATCACCAACCAGGGAAGCTTTCCGCTGGGGCTCCCTCTCAAGCAGCTG

Supplemental Figure 2. The sequences of wildtype and various mutant *CIDE*-C clones generated from the *CIDE*-C CRISPR/Cas9 targeting system. Genomic DNA sequence was grouped by color (blue and dark blue) into the codons that would arise in the WT *CIDE*-C. The underlined and bold-faced codon is the 186th codon, which would normally encode for a glutamic acid. In the WT A-C5 sequence, the full underlined portion refers to the genomic sequence that would be targeted by the sgRNA.
Supplemental Figure 3. List of primers sequences used for genomic DNA sequencing and/or real time quantitative PCR (RT-qPCR). An asterisk (*) denotes that primer sequences obtained from Ahfeldt et al. (2010), while a cross (†) denotes that primer sequences were obtained from Qiagen.

<table>
<thead>
<tr>
<th>qPCR target</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
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<tr>
<td>rPLP†</td>
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Supplemental Figure 4. Increased surface area for plating infected MPCs seemed to improve adipocyte differentiation efficiency. Wildtype MPCs plated on A) 55 cm$^2$ total surface area, B) 1.9 cm$^2$ and C) 0.95 cm$^2$ differentiated into adipocytes at variable efficiencies.
REFERENCES


