Investigation of DNA Methylation in Obesity and its Underlying Insulin Resistance

by

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A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved April 2017 by the
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May 2017

ABSTRACT

Obesity and its underlying insulin resistance are caused by environmental and genetic factors. DNA methylation provides a mechanism by which environmental factors can regulate transcriptional activity. The overall goal of the work herein was to (1) identify alterations in DNA methylation in human skeletal muscle with obesity and its underlying insulin resistance, (2) to determine if these changes in methylation can be altered through weight-loss induced by bariatric surgery, and (3) to identify DNA methylation biomarkers in whole blood that can be used as a surrogate for skeletal muscle.

Assessment of DNA methylation was performed on human skeletal muscle and blood using reduced representation bisulfite sequencing (RRBS) for high-throughput identification and pyrosequencing for site-specific confirmation. Sorbin and SH3 homology domain 3 (*SORBS3*) was identified in skeletal muscle to be increased in methylation (+5.0 to +24.4 %) in the promoter and 5'untranslated region (UTR) in the obese participants (n= 10) compared to lean (n=12), and this finding corresponded with a decrease in gene expression (fold change: -1.9, P=0.0001). Furthermore, *SORBS3* was demonstrated in a separate cohort of morbidly obese participants (n=7) undergoing weight-loss induced by surgery, to decrease in methylation (-5.6 to -24.2%) and increase in gene expression (fold change: +1.7; P=0.05) post-surgery. Moreover, *SORBS3* promoter methylation was demonstrated *in vitro* to inhibit transcriptional activity (P=0.000003). The methylation and transcriptional changes for *SORBS3* were significantly ($P \leq 0.05$) correlated with obesity measures and fasting insulin levels. *SORBS3* was not identified in the blood methylation analysis of lean (n=10) and obese (n=10) participants suggesting that it is a muscle specific marker. However, solute carrier

family 19 member 1 (*SLC19A1)* was identified in blood and skeletal muscle to have decreased 5'UTR methylation in obese participants, and this was significantly ($P \leq 0.05$) predicted by insulin sensitivity.

These findings suggest *SLC19A1* as a potential blood-based biomarker for obese, insulin resistant states. The collective findings of *SORBS3* DNA methylation and gene expression present an exciting novel target in skeletal muscle for further understanding obesity and its underlying insulin resistance. Moreover, the dynamic changes to *SORBS3* in response to metabolic improvements and weight-loss induced by surgery.

DEDICATION

I am thankful for the support I have received from all of my family and friends throughout this process. My parents, Mike and Bridgette, for raising me to be the strong and determined person I am today, and my siblings, Ellen and Nick, for keeping me in check. My in-laws, Mike and Chris, for their encouragement and for raising their son to be the incredible husband I know today.

Sean, thank you for everything, I cannot imagine what this experience would have been like without you. You are an amazing partner, and I am excited for the next chapter in our journey through life.

ACKNOWLEDGMENTS

I would have never been able to successfully complete my doctoral program without the support and guidance of all my committee members. I can never truly express how grateful I am to my mentor, Dr. Dawn Coletta, for giving me the opportunity to be the first graduate student in her lab. All my achievements have come from her driving me, and spending copious amounts of time into shaping me into the professional I am today. I would like to thank Dr. Christos Katsanos for his open lab space, allowing me to grow from working with him and his lab members. Thank you to Dr. Lawrence Mandarino for your guidance in developing my writing and critical thinking skills. Thank you to Dr. Gabriel Shaibi for helping with my professional development towards my career goals. Moreover, for lending the expertise of Dr. Joon Young Kim during the beginning of my program, who is still a great colleague and friend I have today. Thank you to Dr. Valentin Dinu for helping form the bioinformatics infrastructure needed to complete my dissertation work, as well as lending the assistance of wonderful graduate students. Thank you Dr. Paul Langlais and Dr. Eleanna De Filippis, for you both were not on my committee, but still provided me with vital support and mentorship during this process. Lastly, I would like to thank Bruce and Jane Lawson for their support during the final year of my program through the ARCS Foundation, as well as their genuine interest and encouragement for my work.

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CHAPTER 1: GENERAL INTRODUCTION

Obesity is characterized by an excessive accumulation of body fat. The prevalence of obesity has drastically increased over the past few decades. In the United States, more than one-third of the adult population is considered obese (Flegal, Kruszon-Moran, Carroll, Fryar, & Ogden, 2016). One of the key underlying metabolic consequences of obesity is insulin resistance (Kahn, Hull, & Utzschneider, 2006). Insulin resistance is characterized by a reduced biological response of insulin on insulin-responsive tissues such as muscle, liver and adipose (Abdul-Ghani & DeFronzo, 2010). Under normal physiological conditions, skeletal muscle is the major site of insulin-stimulated total body glucose uptake (Abdul-Ghani & DeFronzo, 2010). As such, skeletal muscle is a primary tissue to study when deciphering the molecular basis of insulin resistance.

It has been proposed that the skeletal muscle insulin resistance observed in obesity is driven by an increased release of proinflammatory cytokines (i.e. interleukin 6, tumor necrosis factor-α) and plasma free fatty acids (FFA) from white adipose tissue (WAT) (Flatt, 1972; Maachi et al., 2004). The elevation of plasma FFA in the bloodstream from excess lipolysis of WAT has been shown to be deposited in tissues, such as skeletal muscle (Ferrannini, Barrett, Bevilacqua, & DeFronzo, 1983). This influx of FFA has been associated with mitochondrial dysfunction, by means of reduced mitochondrial biogenesis (Sparks et al., 2005) and impaired fatty acid oxidation (Pimenta et al., 2008). The accumulation of lipids stored in skeletal muscle inhibits insulin signaling, which decreases glucose uptake, thereby increasing blood glucose levels (Dresner et al., 1999; Goodyear et al., 1995). In addition, the action of proinflammatory cytokines on skeletal muscle receptors, such as Toll-like receptors (TLRs) can promote a cascade of

inflammatory kinase signaling (Reyna et al., 2008). These inflammatory kinases can reduce insulin signaling by insulin receptor substrate-1 (IRS-1) serine phosphorylation (Aguirre, Uchida, Yenush, Davis, & White, 2000). As such, a critical step for the prevention of obesity and insulin resistant skeletal muscle is understanding the genetic and environmental factors leading to the defective signaling cascade.

It is well known that genetic and environmental factors both contribute to the pathogenesis of obesity and its underlying insulin resistance (Qi & Cho, 2008). From an environmental perspective, obesity can be the result of increased fat storage from a combination of high caloric intake and physical inactivity (Johnson, Burke, & Mayer, 1956; Stefanik, Heald, & Mayer, 1959). In addition to the environmental component, there have been many studies providing evidence that there is a strong genetic basis of obesity and insulin resistance. Twin studies (Stunkard, Foch, & Hrubec, 1986; Stunkard, Harris, Pedersen, & McClearn, 1990), family history (Adams et al., 1993; Heller, Garrison, Havlik, Feinleib, & Padgett, 1984), and gene knockout studies in mice (Hummel, Dickie, & Coleman, 1966; Ingalls, Dickie, & Snell, 1950) have provided evidence for a genetic predisposition of obesity and its underlying insulin resistance. More recently, we have observed an increase in high-throughput or 'omic' technology, which has allowed for the probing of thousands of variants in genetic studies. In 2007, following this increase in 'omics' technology, a single nucleotide polymorphism (SNP) associated with increased body mass index (BMI) was identified in the fat mass and obesity associated (FTO) gene (Frayling et al., 2007). Since that paper, genome-wide association studies (GWAS) have identified many obesity related polymorphisms identified in genes that are associated with appetite (Willer et al., 2009), fat distribution

(Lindgren et al., 2009), and insulin resistance (Meigs et al., 2007). Collectively, obesity and its underlying insulin resistance have been recognized as a complex interplay between genetic and environmental factors. In this respect, identifying the role of epigenetics, which is influenced by both the environmental and genetic factors, has become important for understanding obesity and insulin resistance.

Epigenetics was first introduced in 1942 by Conrad Waddington (Waddington, 2012). Epigenetic modifications can be described as a heritable change in gene function that occurs without changes in the nucleotide sequence (Berger, Kouzarides, Shiekhattar, & Shilatifard, 2009). The most studied epigenetic mark is deoxyribonucleic acid (DNA) methylation, which is the addition of a methyl group to the carbon-5 position of a cytosine residue preceding a guanine, termed CpG dinucleotide (Ronn & Ling, 2015). The enzymes, DNA methyltransferases (DNMTs), catalyze the methyl addition using S-adenosylmethionine (SAM) as the methyl group donor (Z. X. Chen & Riggs, 2005). Specifically, *de novo* methylation is established by DNMT3A and DNMT3B (Yokochi & Robertson, 2002), and DNMT1 is for maintenance (Pradhan, Bacolla, Wells, & Roberts, 1999). Identifying these methylation marks is crucial for furthering our understanding of diseases such as obesity and its underlying insulin resistance, because it can regulate gene transcription.

Two distinct mechanisms have been proposed for differential DNA methylation in transcript regulation. Promoter and untranslated region methylation has been associated with gene silencing, by inhibiting the binding of transcription factors or the transcriptional machinery (Ling & Groop, 2009; Merkenschlager & Odom, 2013). In contrast, methylation altered in the gene body has been proposed to contribute to

regulating alternative splicing (Lev Maor, Yearim, & Ast, 2015). In addition, the placement of methylation in the genome can be influenced by genetic variation, such as SNPs. Studies assessing SNP-CpG sites have shown that a single base pair mutation may introduce a CpG site for methylation, and increase the risk for disease (Volkov et al., 2016). Another means of affecting the placement of DNA methylation can be through DNMT activity. DNA methylation is considered to be a stable covalent addition (Mikeska & Craig, 2014). However, these additions can be plastic in response to environmental cues, such as changes in nutrition (Dunn & Bale, 2009; Jacobsen et al., 2012) or physical activity (Barres et al., 2012; Nitert et al., 2012). From the aforementioned studies, it could be postulated that DNMTs function may be repressed, or enzymes responsible for demethylation may be activated (Branco, Ficz, & Reik, 2011). Therefore, it has become important to focus on the effects of environmental change on DNA methylation in obesity and insulin resistance. The work herein has used weight loss to improve metabolic outcomes by bariatric surgery to investigate its impact on DNA methylation.

Bariatric surgery is a treatment option for individuals that are morbidly obese or obese with comorbidities, such as type 2 diabetes (T2D), hyperlipidemia, and heart disease (Buchwald et al., 2004). Bariatric surgery has become the most effective treatment for sustained weight loss (Genser, Casella Mariolo, Castagneto-Gissey, Panagiotopoulos, & Rubino, 2016). Roux-en-Y gastric bypass (RYGB) is one of the most common surgeries performed and combines restrictive and malabsorptive techniques (Catoi, Parvu, Muresan, & Busetto, 2015). The RYGB has been shown to be effective at restoring glycemic control (Mingrone et al., 2012; Rubino et al., 2004). This has been proposed by

an increased release of incretin hormones, which promotes insulin production (Rubino et al., 2004). The overall health benefits associated with RYGB have been improvements in dyslipidemia, hypertension, glucose homeostasis, insulin sensitivity and secretion (Catoi et al., 2015; Genser et al., 2016). However, it is not fully understood how these surgical interventions improve health at the molecular level. To date, there have been a limited number of studies that have been trying to decipher the epigenetic changes in response to surgery (Barres et al., 2013; Nilsson et al., 2015). For example, Barres *et al*. found promoter methylation of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) and pyruvate dehydrogenase kinase, isozyme 4 (PDK4) was altered with obesity, and restored to non-obese levels after Roux-en-Y gastric bypass (Barres et al., 2013). However, this work has only provided a foundation and warrants further investigation. Therefore, we have chosen to continue our understanding of key epigenetic components of obesity and it underlying insulin resistance in response to surgery. The work presented herein has used the latest sequencing technology to identify novel epigenetic targets with obesity and insulin resistance, and then pursued those findings in response to surgery.

Advancements in omic technologies have been made over the past 15 years. In 2003, there was a marked increase in 'genomics' research, which started with the International Human Genome Sequencing Consortium release of the human genome (Yan et al., 2015). The increase in 'omics' data has led to the utilization of high-throughput technology and bioinformatics for global analyses of biological states (Yan et al., 2015). Previous studies from our laboratory have used 'omic' approaches to understand the molecular basis of obesity and insulin resistance. Richardson *et al.* showed that a 48 hour lipid infusion in

healthy individuals revealed a decreased expression in nuclear encoded mitochondrial genes, and an increased expression of extracellular matrix genes using transcriptomics in skeletal muscle (Richardson et al., 2005). In the study by Tangen *et al.*, transcriptomic analysis of whole blood identified gene expression changes in pathways including ribosome, oxidative phosphorylation and mitogen-activated protein kinases (MAPK) signaling in individuals with and without metabolic syndrome (Tangen et al., 2013). Proteomic analyses of skeletal muscle from lean, obese, and type 2 diabetics, revealed decreased abundance of mitochondrial proteins and altered abundance of cytoskeletal structure proteins (Hwang et al., 2010). By using these 'omic' technologies we have uncovered novel targets for deciphering the insulin resistance underlying obesity and type 2 diabetes (T2D). To explore the environmental and genetic basis of obesity and insulin resistance, we have utilized an epigenomic approach to identify methylation changes and relate our present findings to our previously published transcriptomic and proteomic studies.

The overall goal of this dissertation was to (1) determine if previous 'omic' findings in insulin resistant states of obesity and T2D could be explained by changes in DNA methylation, and (2) to identify novel DNA methylation markers for obesity and its underlying insulin resistance, and for the improvements from weight-loss induced by surgery. The work included herein involves the assessment of DNA methylation from skeletal muscle of lean, insulin sensitive, obese, insulin resistant, and morbidly obese, bariatric surgery participants. In addition to skeletal muscle, the highly accessible tissue, whole blood has also been collected. Specific aims for this dissertation are outlined as follows:

Aim 1: To determine how obesity and its underlying insulin resistance alters DNA methylation globally in human skeletal muscle. We hypothesize that:

(1) DNA methylation will be altered in the promoter of genes involved in mitochondrial biogenesis, oxidative phosphorylation, extracellular matrix and cytoskeletal in the obese, insulin resistant participants compared to the lean, insulin sensitive; and

(2) The differences in methylation will negatively correspond to gene expression and protein abundance.

Aim 2: To determine if the changes in DNA methylation identified in obesity and its underlying insulin resistance can be altered by the RYGB surgery. We hypothesize that:

(1) DNA methylation post-surgery will reflect levels detected in lean, insulin sensitive participants; and

(2) The changes in methylation post-surgery will negatively correspond to gene expression and protein abundance.

Aim 3: To identify DNA methylation biomarkers in whole blood that reflect skeletal muscle. We hypothesize that:

(1) DNA methylation biomarkers in blood will be associated with genes involved in inflammation and MAPK signaling; and

(2) Overall methylation levels will be altered between the lean and obese participants, and that this difference in methylation will be reflected in both tissues types.

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CHAPTER 2: NEXT-GENERATION SEQUENCING METHYLATION PROFILING OF SUBJECTS WITH OBESITY IDENTIFIES NOVEL GENE CHANGES

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Abstract

Obesity is a metabolic disease caused by environmental and genetic factors. However, the epigenetic mechanisms of obesity are incompletely understood. The aim of our study was to investigate the role of skeletal muscle DNA methylation in combination with transcriptomic changes in obesity. Muscle biopsies were obtained basally from lean (n=12; BMI= 23.4±0.7 kg/m^2) and obese (n=10; BMI= 32.9±0.7 kg/m^2) participants in combination with euglycemic hyperinsulinemic clamps to assess insulin sensitivity. We performed reduced representation bisulfite sequencing (RRBS) next generation methylation and microarray analyses on DNA and RNA isolated from *vastus lateralis* muscle biopsies. There were 13,130 differentially methylated cytosines (DMC; uncorrected $P<0.05$) that were altered in the promoter and untranslated (5' and 3'UTR) regions in the obese versus lean analysis. Microarray analysis revealed 99 probes that were significantly (corrected $P<0.05$) altered. Of these, 12 genes (encompassing 22 methylation sites) demonstrated a negative relationship between gene expression and DNA methylation. Specifically, sorbin and SH3 domain containing 3 (*SORBS3*) which codes for the adapter protein vinexin, was significantly decreased in gene expression

(fold change -1.9) and had 9 DMCs that were significantly increased in methylation in obesity (methylation differences ranged from 5.0 to 24.4%). Moreover, differentially methylated region (DMR) analysis identified a region in the 5'UTR (Chr.8:22,423,530-22,423,569) of *SORBS3* that was increased in methylation by 11.2% in the obese group. The negative relationship observed between DNA methylation and gene expression for *SORBS3* was validated by a site specific sequencing approach, pyrosequencing, and qRT-PCR. Additionally, we performed transcription factor binding analysis and identified a number of transcription factors whose binding to the differentially methylated sites or region may contribute to obesity. These results demonstrate that obesity alters the epigenome through DNA methylation, and highlights novel transcriptomic changes in *SORBS3* in skeletal muscle.

Introduction

Obesity is a condition that affects about one-third of the United States adult population (Ogden, Carroll, Kit, & Flegal, 2014). It is a major disease associated with other co-morbidities, including type 2 diabetes, metabolic syndrome and cardiovascular disease (Guh et al., 2009). An underlying feature of obesity is insulin resistance. Insulin resistance is a reduced biological response of insulin on peripheral tissues including skeletal muscle, liver, and fat (Kahn et al., 2006). Under normal physiological conditions, skeletal muscle accounts for approximately 80% of insulin-stimulated total body glucose uptake (Abdul-Ghani & DeFronzo, 2010). Previous studies from our laboratory have investigated the molecular mechanisms of insulin resistance in skeletal muscle. We have previously shown that insulin resistance in skeletal muscle is in part due to mitochondrial dysfunction (Patti et al., 2003). In experimentally induced insulin

resistance we have shown a low grade inflammatory response, with increases in extracellular matrix (ECM) turnover (Richardson et al., 2005). Furthermore, by using a proteomic approach on insulin resistant muscle, we identified alterations in the abundance of protein involved in cytoskeletal structure and assembly (Hwang et al., 2010). Our findings, to date, demonstrate a cross talk relationship between inflammation, extracellular remodeling, cytoskeletal interactions, mitochondrial function, and insulin resistance in human skeletal muscle (Coletta & Mandarino, 2011).

The pathogenesis of obesity associated insulin resistance is due to environmental and genetic factors (Fernandez, Pearson, Kell, & Bohan Brown, 2013; T. Wang, Jia, & Hu, 2014). However, the role of epigenetic factors, which may provide a potential link between the genetic and environmental factors observed in obesity, is poorly understood. Epigenetics can be described as heritable changes in gene function that occur without a change in nucleotide sequence (Egger, Liang, Aparicio, & Jones, 2004). DNA methylation is an epigenetic modification and is generally observed as a methyl addition to the carbon 5 position of cytosines and more commonly on cytosines preceding guanines, called CpG dinucleotides (Huidobro, Fernandez, & Fraga, 2013). DNA methylation patterns are established during early development and are maintained in differentiated tissue by DNA methyltransferases (Jeltsch & Jurkowska, 2014). Changes in DNA methylation are a potential mechanism by which the expression of a gene may be regulated (Huidobro et al., 2013). For example, it is generally accepted that gene expression is often reduced when DNA methylation is present at a promoter or untranslated region of a gene (Ling & Groop, 2009; Maussion et al., 2014; Yu et al., 2015).

There have been a number of studies that have focused on the epigenetic basis of obesity (Alibegovic et al., 2010; Barres et al., 2013). However, the majority of the DNA methylation studies performed to date have either used a candidate gene approach or the array based technology that probes 450K methylation sites simultaneously. Therefore, our study is unique in that we performed reduced representation bisulfite sequencing (RBBS), which has the ability to capture millions of methylation sites in the human genome. Moreover, we performed transcriptomic analyses, which allowed us to measure global mRNA expression levels in genes altered in people with obesity. Furthermore, we combined epigenetic and transcriptomic analyses to identify associations between the datasets. Based on our previous findings in skeletal muscle, we hypothesize that there will be alterations in the methylation of genes involved in mitochondrial function, inflammation and extracellular matrix remodeling.

Methods

Participants

Ten insulin resistant participants with obesity and twelve insulin sensitive participants without obesity were recruited. Insulin sensitivity was assessed by the euglycemic-hyperinsulinemic clamp (DeFronzo, Tobin, & Andres, 1979). Demographic, medical history, anthropometric, metabolic, and screening blood tests were obtained on all participants. Percent body fat was assessed by body impedance analysis. Normal glucose tolerance was assessed by a 75-g oral glucose tolerance test following a 10-12 hour overnight fast. No subject was taking any medication known to affect glucose metabolism. All subjects gave informed written consent to participate in the study, which

was approved by the Institutional Review Boards of the Mayo Clinic in Arizona and Arizona State University.

Study design

Following an overnight fast, participants reported to the Clinical Studies Infusion Unit at the Mayo Clinic in Arizona. A two hour euglycemic-hyperinsulinemic clamp (80 mU.m-2.min-1) was performed (DeFronzo et al., 1979). A primed infusion of 6,6 di-deuterated glucose was begun at -120 minutes to determine the basal rate of glucose metabolism. Sixty minutes after the start of deuterated glucose infusion, a resting, basal *vastus lateralis* muscle biopsy was performed percutaneously, under local anesthesia, as previously described (Cusi et al., 2000; DeFronzo et al., 1979). After resting for one hour, a primed continuous infusion of insulin was started. The constant infusion of deuterated glucose was discontinued at time 15 minutes after the start of the insulin infusion, and a variable infusion of 20% dextrose that was enriched with 6,6 di-deuterated glucose was used to maintain euglycemia and a constant enrichment of the tracer. Enrichment of plasma glucose with 6,6 di-deuterated glucose was assayed using GC/MS in the Center for Clinical and Translational Science (CCaTS) Metabolomics Core at the Mayo Clinic in Rochester. The rates of glucose appearance and disappearance were calculated using steady state equations to derive insulin sensitivity levels, termed the M value (Debodo, Steele, Altszuler, Dunn, & Bishop, 1963).

Substrate and hormone determinations

Plasma glucose concentration was determined by the glucose oxidase method on an YSI 2300 STAT plus (YSI INC., Yellow Springs, OH, USA). Plasma insulin was measured by a two-site immunoenzymatic assay performed on the DxI 800 automated

immunoassay system (Beckman Instruments, Chaska, MN, USA). Inter-assay C.V.'s were 6.2% at 5.3 uU/mL, 6.5% at 46.1 uU/mL, and 7.7% at120.4 uU/mL. A comprehensive metabolic panel, lipid panel and hemogram panel were performed by the Biospecimens Accessioning and Processing (BAP) Core at the Mayo Clinic in Scottsdale.

Muscle biopsy processing

For genomic DNA analyses, homogenization of the muscle biopsy (25 mg) was performed in 1X PBS with the Bullet Blender (Integrated Scientific Solutions, San Diego, CA). DNA was isolated using QIAamp DNA mini kit, as per the manufacturer's instructions (Qiagen, Valencia, CA). For mRNA analyses, muscle biopsy specimens (50 mg) were homogenized in TRIzol solution (Invitrogen, Carlsbad, CA) using a Polytron (Brinkmann Instruments Westbury, NY). Total RNA was purified with RNeasy MinElute Cleanup Kit (Qiagen, Chatsworth, CA). DNA and RNA quality and quantity were determined using gel electrophoresis and A260/A280 values.

Reduced Representation Bisulfite Sequencing (RRBS)

RRBS was performed at the Mayo Clinic Genotyping Shared Resource facility as previously described (Gu et al., 2011). DNA (250ng) was digested with Msp1 (New England Biolabs, Ipswich, MA) and purified using QIAquick Nucleotide Removal Kit (Qiagen, Valencia, CA). End-repair A tailing was performed (New England Biolabs, Ipswich, MA) and TruSeq methylated indexed adaptors (Illumina, San Diego, CA) were ligated with T4 DNA ligase (New England Biolabs, Ipswich, MA). Size selection was performed with Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN). Bisulfite conversion was performed using EZ-DNA Methylation Kit (Zymo Research, Irvine, CA) as recommended by the manufacturer with the exception that an incubation

was performed using 55 cycles of 95 °C for 30 seconds and 50°C for 15 minutes. Following bisulfite treatment, the DNA was purified as directed and amplified using Pfu Turbo C Hotstart DNA Polymerase (Agilent Technologies, Santa Clara, CA). Library quantification was performed using Qubits dsDNA HS Assay Kit (Life Technologies, Grand Island, NY) and the Bioanalyzer DNA 1000 Kit (Agilent Technologies Santa Clara, CA). The final libraries from RRBS were placed onto seven lanes of a paired-end flow cell at concentrations of 7-8 pM and the control sample, PhiX, was placed in the eighth lane to allow the sequencer to account for the unbalanced representation of cytosine bases. The flow cell was then loaded into the Illumina cBot for generation of cluster densities. After cluster generation, the flow cells were sequenced as 51 x 2 paired end reads using Illumina HiSeq 2000 with TruSeq SBS sequencing kit version 3. Data was collected using HiSeq data collection version 1.5.15.1 software, and the bases were called using Illumina's RTA version 1.13.48.

RRBS data analysis

RRBS data was analyzed using a streamlined analysis and annotation pipeline for reduced representation bisulfite sequencing, SAAP-RRBS (Sun et al., 2012). FASTQ were trimmed to remove adaptor sequences, and any reads with less than 15 base pair (bp) were discarded. Trimmed Fastqs were then aligned against the reference genome Hg19 using BSMAP (Xi & Li, 2009); which converts the reference genome to align the bisulfite treated reads. Samtools was used to get mpileup and PERL scripts as described elsewhere (Sun et al., 2012), were used to determine CpG methylation and non-CpG methylation to estimate the bisulfite conversion efficiency (H. Li et al., 2009). Methylation ratios were reported along with custom CpG annotation. The methylation

dataset supporting the conclusions of this article are available in the Gene Expression Omnibus repository, GSE73304 (http://www.ncbi.nlm.nih.gov/geo/). Additionally, bigwig files were used to create a custom track on the UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgTracks?hgS_doOtherUser=submit&hgS_otherUserName=rlcolett&hgS_otherUserSessionName=testnoinitial).

Differentially methylated cytosines (DMC) analysis

To determine differences in methylation between groups, the aligned data was imported into the free open source R package, methylSig. A minimum of five reads and the recovery of the site in at least eight participants from each group were required for the inclusion of a cytosine in downstream analyses. The mean methylation differences (%) between the groups with and without obesity were adjusted by a beta binomial approach to account for biological variation among the groups being compared (Park, Figueroa, Rozek, & Sartor, 2014). A comparison of the DNA methylation between groupings at each site was based on a likelihood ratio test (nominal P value), and a Benjamini-Hochberg multiple testing correction was applied. Benjamin-Hochberg correction yielded no significant sites, therefore for subsequent analyses, an uncorrected $P<0.05$ was used. The RefSeq Genes and CpG Island tracks from the University of California, Santa Cruz (UCSC) Genome Browser were imported for additional region annotations. When applying regional annotation to each DMC, priority was given to annotating the site as a promoter or untranslated region if that site was in another transcript of the gene or in a different gene.

Differentially methylated region (DMR) analysis

DMRs were identified using the open source R package dispersion shrinkage for sequencing data (DSS) (Wu et al., 2015). The BSmooth algorithm was applied to the entire data set to determine the level of methylation in a region for each sample and to account for biological variation. The following criteria were used for the analysis: each region contained two CpGs supported with a read coverage of 5X, the recovery of the site in at least eight participants from each group, and significance of $P<0.05$ from the DMC analysis. DMRs were created based on a t-statistic cutoff of 2.5 and a sliding-window of 500 bp. The significance of a DMR was weighted by the Area Stat, which is the sum of t-statistic values in each DMR. Additional region annotations were included by importing RefSeq Genes and CpG Island tracks from the UCSC Genome Browser into the R package, Genomic Ranges. When applying regional annotation to each region, priority was given to annotating the region as a promoter or untranslated region if the sites were in another transcript of the gene or in a different gene.

Microarray processing

Total RNA (100 ng) was amplified and labeled using the Low Input Quick Amp Labeling Kit, One-Color, as per manufacturer's instructions (Agilent Technologies, Santa Clara, CA). After labeling, complimentary RNA (cRNA) was fragmented using Agilent Gene Expression Hybridization Kit (Agilent Technologies, Santa Clara, CA), as per instructions. The fragmented cRNA was hybridized to the SurePrint G3 Human Gene Expression 8x60K v2 Microarray (Agilent Technologies, Santa Clara, CA) using a SureHyb DNA Microarray Hybridization Chamber at 65°C, for 17 hours in a rotating incubator. After hybridization, slides were washed in Gene Expression wash buffers 1, 2,

and acetonitrile as per instructions, and then scanned with an Agilent DNA microarray scanner (Agilent Technologies, Santa Clara, CA).

Microarray analysis

Feature Extraction Software version 12.0.1.1 (Agilent Technologies, Santa Clara, CA), was used for the array image analysis. The microarray dataset supporting the conclusions of this article are available in the Gene Expression Omnibus repository, GSE73078 (http://www.ncbi.nlm.nih.gov/geo/). The data files were imported into the free open source R package, Linear Models for Microarray Data (Limma) version 3.22.0 (http://www.bioconductor.org/packages/release/bioc/html/limma.html). Data were background corrected using normal exponent, quantile normalized, and an unweighted linear model was performed to generate fold changes between groups. The fold changes were log transformed. Expression values obtained were evaluated by a moderated t-statistic (nominal P value), and adjusted using the Benjamini-Hochberg multiple testing correction.

***SORBS3* DMC site specific validation**

DNA methylation was assessed using a site specific sodium bisulfite sequencing method. DNA (500ng) was treated with sodium bisulfite using the EZ DNA Methylation-Lightening kit (Zymo Research, Irvine, CA). Chromosome 8 (Chr.8) positions 22,422,428-22,422,868 proximal to the transcription start site for *SORBS3* was amplified by PCR using the following primers: forward 5'-AGAGATATAATTTGGTAGAAATTGGTAGGATTG-3', reverse 5'AATTACCCGCAAATCCTTATCCAAC-3' (342 bp). The cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 30s, 56°C for 40s, and 72°C for 1 min

with touchdown annealing temperatures for the first 10 cycles, and a final extension at 72°C for 7 mins. The products were run on a 1% agarose gel with ethidium bromide and ultraviolet detection. The 342bp product bands were purified using Zymoclean Gel DNA Recovery Kit, per the manufacturer's instructions (Zymo Research, Irvine, CA). Sanger Sequencing was performed on the bisulfite converted forward DNA strands at Arizona State University's Sequencing Core. The proportion of methylation on each CpG site was detected using the Epigenetic Sequencing Methylation analysis software (ESME).

***SORBS3* DMR pyrosequencing validation**

To confirm DNA methylation of the chromosome 8 region 22,423,530-22,423,569, pyrosequencing PCR and sequencing primers were designed using the PyroMark Assay design Software 2.0 (Qiagen, Valencia, CA). The forward and reverse primers were biotinylated at the 5′ end. Bisulfite conversion of 500 ng genomic DNA was performed using the EZ DNA Methylation-Lightening kit according to the manufacturer's instructions (Zymo Research, Irvine, CA). To assess the forward strand, bisulfite-converted DNA was amplified by PCR using the following primers: forward 5'-AGTAGGGGGAGGAAGGAA-3' and biotinylated reverse 5'-ACTCTCCACAAAATATCCTACTTC-3'. To assess the reverse strand, bisulfite-converted DNA was amplified by PCR using the following primers: biotinylated forward 5'-AGTAGGGGGAGGAAGGAA-3 and reverse 5'-ACCCCCATCCTCTACTAAAAATTAACTACC-3'. Pyrosequencing was performed using the PyroMark Q96 MD system and the Gold Q96 kit with sequencing primers: 5'-GTGTTAGGGAGGGAT-3' (forward strand assessment) and 5'-CTACTAAAAATTAACTACCCTC-3' (reverse strand assessment) according to the

manufacturer's instructions (Qiagen, Valencia, CA). Data analysis was performed using the PyroMark CpG SW 1.0 software (Qiagen, Valencia, CA).

SORBS3 qRT-PCR validation

Skeletal muscle gene expression for *SORBS3* was detected using quantitative real-time PCR on the ABI PRISM 7900HT sequence detection system (Life Technologies, Carlsbad, CA). TaqMan Universal Fast PCR master mix reagents and the Assay-On-Demand gene expression primer pair and probes (Life Technologies, Carlsbad, CA) were added to 20 ng cDNA, which was synthesized using the ABI High Capacity cDNA Reverse Transcription Kit, as per manufacturer's instructions. The quantity of *SORBS3* (Hs00195059_m1) in each sample was normalized to *18S* (Hs99999901_s1) using the comparative (2-ΔΔCT) method (Livak & Schmittgen, 2001).

SORBS3 Predicted Transcription Factor Binding Analysis

Transcription factor binding sites analysis was performed using PROMO version 3.0.2 (Messeguer et al., 2002). The sequences were analyzed with a 5% maximum matrix dissimilarity rate using TRANSFAC version 8.3 database. Analysis of the nine *SORBS3* DMCs was assessed as three separate sequences: Chr.8: 22,409,277-22,409,317; Chr.8: 22,422,628-22,423,112; and Chr.8: 22,423,280-22,423,363. Furthermore, the *SORBS3* DMR sequence Chr.8:22,423,530-22,423,569 was assessed for transcription factor binding sites.

Statistical Analysis

Participant characteristic data was presented as a mean ± SEM, and comparisons between the groups with and without obesity were based on an independent sample t-test. Non-normally distributed data for the 2 hour insulin were log10 transformed; however,

untransformed data are presented for ease of interpretation. Analysis of covariance (ANCOVA) was used to adjust for the effects of age, sex, and the interaction between age and sex. PASW version 22.0 was used for the characteristic data analyses with the significance set at $P \leq 0.05$. Pearson correlation was used for all correlations presented. See above for the statistical analysis of the methylation and microarray data.

Results

Participants

Table 2-1 shows the phenotypic characteristics for participants with and without obesity. There was a significant age difference between groups whereby, individuals with obesity were older. By design, the lean participants had significantly lower body mass index (BMI), body fat, and waist circumference. The participants with obesity were significantly more insulin resistant compared to the lean group, determined by the M value. These differences remained significant after adjusting for potential covariates including age, sex, and the interaction between age and sex.

Global methylation analysis in human skeletal muscle

Prior to the quality control of the sequence data, 5,421,504 sites were captured using the RRBS technology. For our RRBS analysis, we set a threshold of greater than 80% call rate and a minimum of 5X coverage for the sequencing data. Of the 22 participants sequencing data, 20 (11 lean and 9 obese) met this threshold criteria and were used for subsequent downstream analyses. For the sequencing data, we only included methylation sites that were captured in at least 8 participants in each group. In total, we captured 2,586,085 methylation sites using these criteria. The distribution of the methylation sites was defined by genic regions (Figure 2-1A) and CpG island features (Figure 2-1B). We

demonstrated that the majority of the methylation sites were in intronic regions (Figure 2-1A). However, the sites in the promoter and 5' untranslated regions (UTR) dominantly overlapped with CpG islands (Figure 2-1B).

Table 2-1. Characteristics of study participants (n=22) classified by body mass index.

Characteristics	Lean	Obese	P value	P value (Age, Sex, Age*Sex)
Sex	7F/5M	4F/6M	NS*	-
Age (years)	28.8 ± 2.0	40.3 ± 2.5	< 0.01	-
Body mass index (kg/m^2)	23.4 ± 0.7	32.9 ± 0.7	< 0.001	< 0.001
Body fat (%)‡	25.2 ± 1.4	35.2 ± 2.2	< 0.001	< 0.001
Waist circumference (cm)	82.0 ± 3.0	104.4 ± 2.5	< 0.001	< 0.01
Systolic blood pressure (mmHg)	119.8 ± 2.4	123.9 ± 3.1	NS	NS
Diastolic blood pressure (mmHg)	72.6 ± 1.5	78.2 ± 1.3	< 0.05	NS
Triglycerides (mg/dL)	96.5 ± 13.3	114.7 ± 15.2	NS	NS
Cholesterol (mg/dL)	176.2 ± 9.2	186.1 ± 11.4	NS	NS
High density lipoproteins (mg/dL)	57.1 ± 5.2	50.2 ± 3.4	NS	NS
Low density lipoproteins (mg/dL)	99.9 ± 7.3	113.0 ± 10.3	NS	NS
Hemoglobin A1c (%)	5.2 ± 0.04	5.4 ± 0.1	NS	NS
Fasting plasma glucose (mg/dL)	86.7 ± 1.8	89.5 ± 1.7	NS	NS
2 hour plasma glucose (mg/dL)	101.9 ± 5.2	111.2 ± 7.0	NS	NS
Fasting plasma insulin (µU/mL)	6.3 ± 1.1	11.1 ± 0.9	< 0.01	NS
2 hour plasma insulin (µU/mL)	43.2 ± 5.3	93.3 ± 16.4	< 0.01	≤ 0.05
M value (mg/kg·min)	7.3 ± 0.6	4.5 ± 0.7	< 0.01	< 0.01

Data presented as mean ± SEM, based on independent sample t-tests and two-tailed P values. Adjustment for age, sex, and the interaction of age*sex using ANCOVA. Calculated by Chi-Square Test. ‡Body fat determined by biometric impedance analysis (BIA).

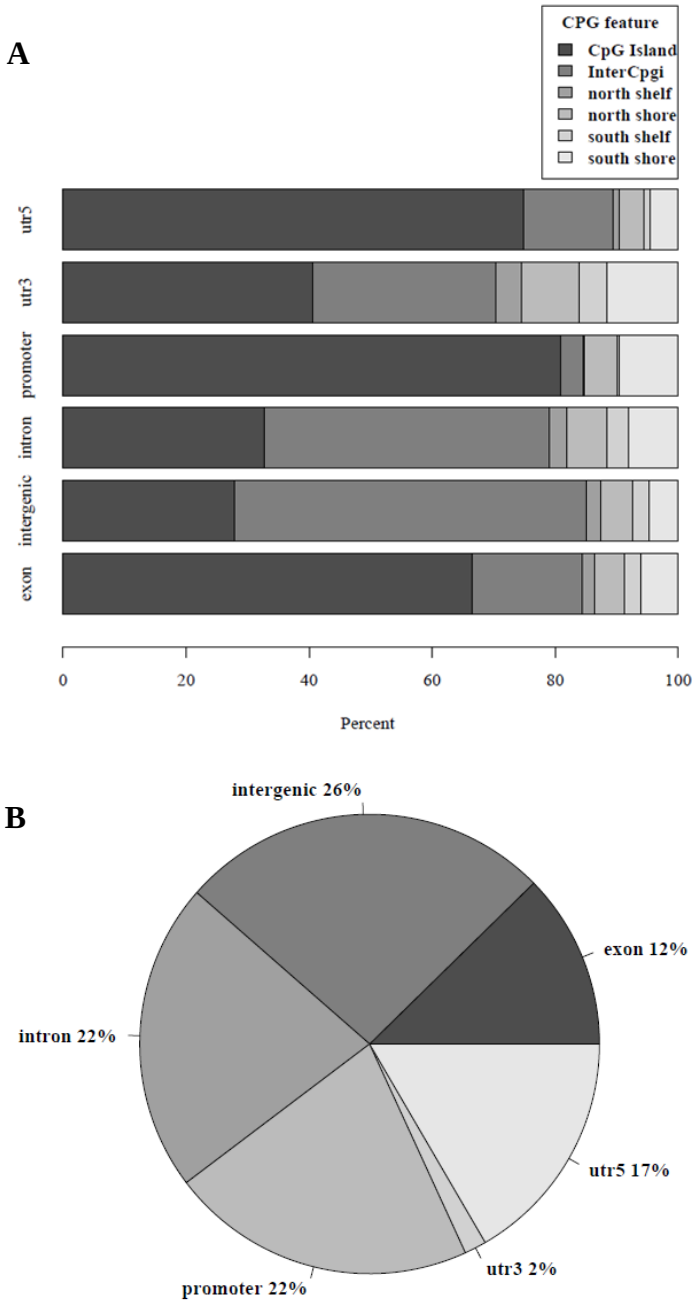

Figure 2-1. The methylation sites captured in our skeletal muscle samples using reduced representation bisulfite sequencing technology were mapped (A) in the context of both gene regions and (B) CpG island features. The regions were defined using UCSC browser refGene and CpG island tracks (see methods). The promoter region was defined as 1000 bp (basepairs) upstream of the transcription start site (TSS); untranslated region (UTR); CpG island is 200-3000 bp stretch of DNA with a C+G content of 50% and observed CpG/expected CpG in excess of 0.6; North (N) and South (S) shores flank the CpG island by 0-2000 bp; the North (N) and South (S) shelf flank the shores by 2000 bp (2000-4000 bp from the island).

Differentially methylated cytosine (DMC) analysis in promoter, 5'UTR, and 3'UTR regions

To investigate the sites that may generate the greatest changes in mRNA expression based on proximity, we sought sites in untranslated regions (5' and 3'UTR) and assigned our promoter region as 1,000 base pairs from the transcription start site region (0 to -1,000 base pairs). Of the 2,586,085 methylation sites captured, 710,981 sites were located in our defined proximal regions and 13,130 of those sites were significantly altered (Appendix A) between our groupings. Differentially methylated cytosines (DMCs) between the groupings were assessed for false discoveries. There were no sites that met the criteria of a false discovery rate $P<0.05$. As such, we used nominal P value cutoffs, which have been accepted in other studies (Hall et al., 2014; Yu et al., 2015).

Overlying changes between DNA methylation and gene expression

Transcriptomic analysis identified 99 probes that were significantly (false discovery rate $P<0.05$) altered in the group with obesity (Appendix B). We compared the significant genes identified from our microarray analysis with the significant DMCs that were found in the promoter, 5'UTR, and 3'UTR ($n=13,130$; $P<0.05$; Figure 2-2). We identified 12 genes (encompassing 22 methylation sites) that demonstrated a negative relationship between gene expression and DNA methylation. Of these, Sorbin and SH3 Domain Containing 3 (*SORBS3*) had increased methylation (9 DMCs) and was associated with a decrease in gene expression. The 11 remaining genes had an increase in gene expression that correlated with a decrease in methylation (Table 2-2).

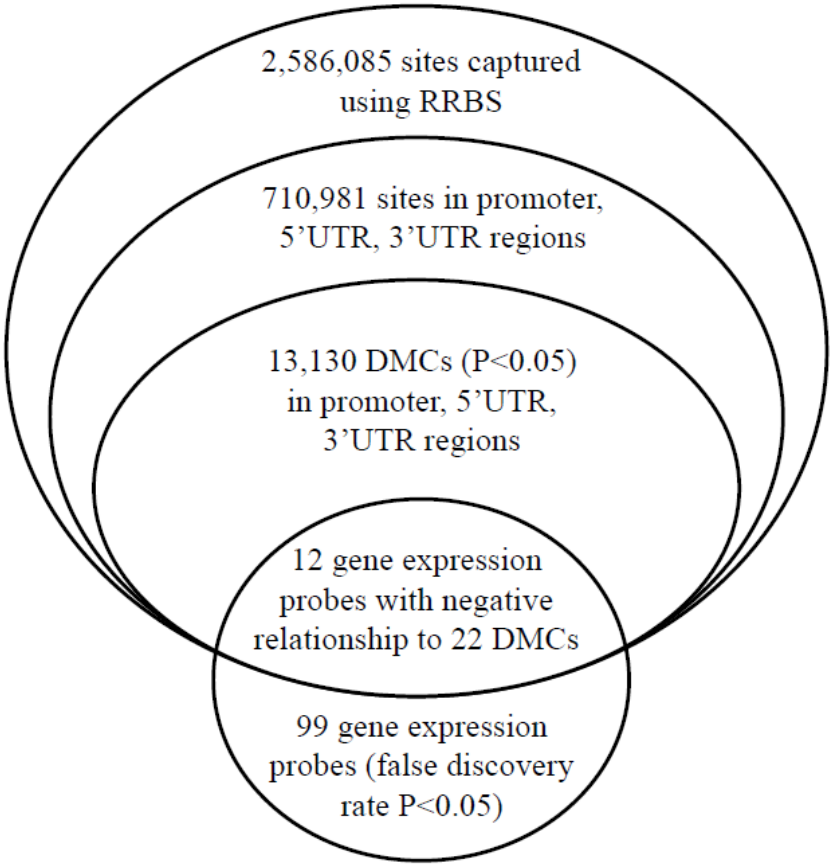

Figure 2-2. Diagram of the analysis for differentially methylated cytosines (DMCs) localized in a promoter, 5' UTR, or 3' UTR region overlapping with transcriptomic changes.

Differentially methylated region (DMR) analysis in the promoter, 5'UTR, and 3'UTR regions

To further interrogate changes in methylation, a regional analysis was performed and identified 700 DMRs. Of these, 170 were located in our defined proximal regions (Appendix C). The 170 DMRs were compared with the 99 probes identified from the microarray analysis. We identified one DMR (Chr.8:22,423,530-22,423,569) in the 5'UTR of *SORBS3* that demonstrated a negative relationship with gene expression. The DMR was increased by 11.2% in the obese group.

Table 2-2. Differentially methylated cytosines (DMCs; P<0.05) that had a negative relationship with gene expression (FDR P<0.05).

Chr. Position	Gene	Methyl Difference (%)	log Fold Change	Fold Change	Gene Region	CpG Island Region
chr11.64670967	ATG2A	-6.8	0.62	1.5	Promoter	InterCpG
chrX.107334934	ATG4A	-11.1	0.59	1.5	Promoter	CpG island
chrX.107334999	ATG4A	-5.3	0.59	1.5	Promoter	CpG island
chr21.45749947	C21orf2	-6.3	0.43	1.3	3'UTR	North shelf
chrX.30671522	GK	-31.4	0.42	1.3	5'UTR	CpG island
chrX.30671506	GK	-14.1	0.42	1.3	5'UTR	CpG island
chr19.5153271	KDM4B	-20.7	0.39	1.3	3'UTR	South shore
chr9.34381797	KIAA1161	-13.1	0.60	1.5	Promoter	South shore
chr4.6641531	MRFAP1	-11.2	0.70	1.6	Promoter	North shore
chr1.145609911	POLR3C	-19.7	0.47	1.4	Promoter	North shore
chrX.20286470	RPS6KA3	-15.3	0.46	1.4	Promoter	CpG island
chr9.135231749	SETX	-16.2	0.55	1.5	Promoter	South shore
chr8.22409297	SORBS3	3.9	-0.91	-1.9	5'UTR	CpG island
chr8.22422648	SORBS3	5.0	-0.91	-1.9	Promoter	CpG island
chr8.22423300	SORBS3	10.6	-0.91	-1.9	5'UTR	CpG island
chr8.224233343	SORBS3	16.4	-0.91	-1.9	5'UTR	CpG island
chr8.22422936	SORBS3	16.6	-0.91	-1.9	Promoter	CpG island
chr8.22422959	SORBS3	17.1	-0.91	-1.9	Promoter	CpG island
chr8.22422927	SORBS3	17.7	-0.91	-1.9	Promoter	CpG island
chr8.22423332	SORBS3	20.3	-0.91	-1.9	5'UTR	CpG island
chr8.22423092	SORBS3	24.4	-0.91	-1.9	Promoter	CpG island
chr6.56972737	ZNF451	-4.7	0.55	1.5	3'UTR	InterCpG

SORBS3 Validation

SORBS3 has two transcript variants (variant 1: NM_005775 and variant 2: NM_001018003) as shown in Figure 2-3. We used a site specific sequencing approach to validate a promoter site of variant 2 (Chr.8:22,422,648). The RRBS data had shown a 5% increase in methylation in the obese compared to the lean participants (Appendix A). Validation using site specific sequencing demonstrated an increase in methylation in the participants with obesity (lean 0.078±0.01 vs obese 0.14±0.03 methylation ratio; P=0.03; Figure 2-4). Pyrosequencing of the *SORBS3* DMR (Chr.8:22,423,530-22,423,569) in the 5'UTR of variant 2 resulted in an overall increase in methylation, as shown in Figure 2-5. Three sites on the forward strand and three on the reverse strand were significantly different (P<0.05) with obesity using the pyrosequencing analysis, which further validated the RRBS findings (Figure 2-5). The qRT-PCR confirmed the microarray results (Table 2-2) demonstrating a decrease in gene expression of *SORBS3* in the participants with obesity (fold change -1.4; P=0.01).

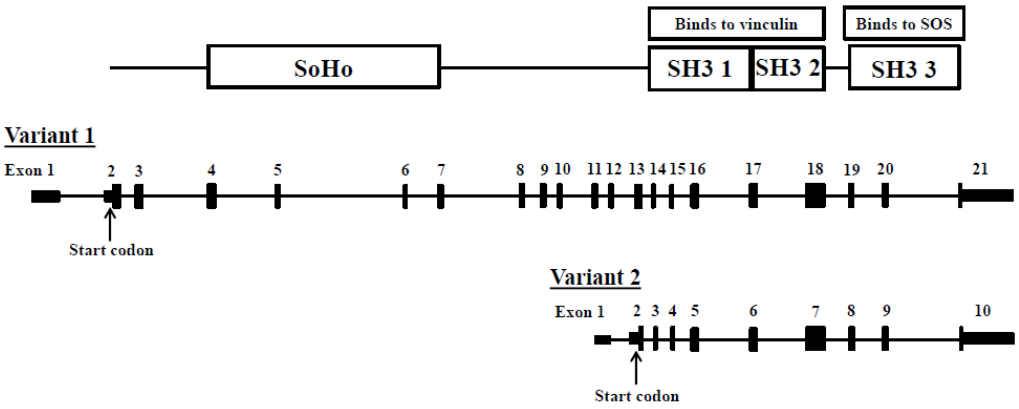

Figure 2-3. Sorbin and SH3 domain containing 3 (SORBS3) consists of two transcript variants that code for two protein isoforms, vinexin alpha and beta respectively. Variant 2 (vinexin beta) exons 3-10 are consistent with variant 1 exons 14-21, containing all three SH3 domains. Variant 2 differs by lacking the coding regions for the N terminal end SoHo domain.

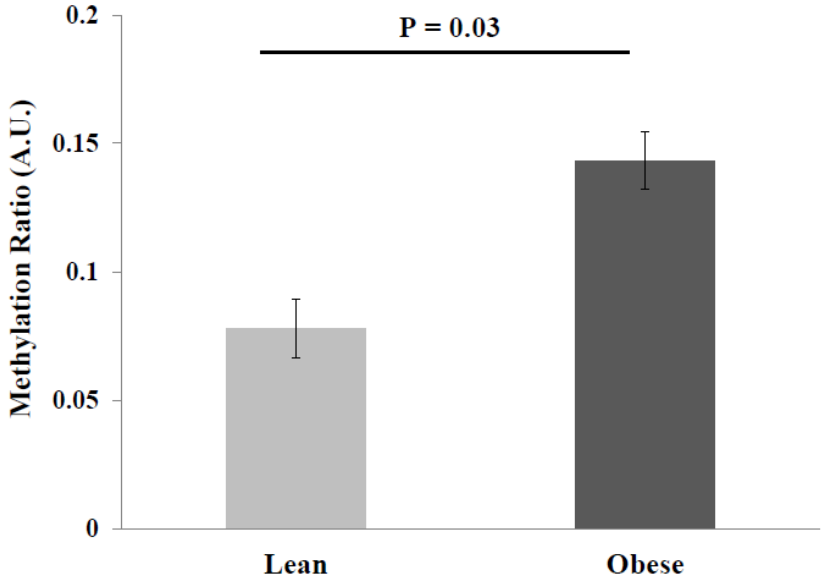

Figure 2-4. DNA methylation in the promoter of *SORBS3* was validated with the site specific sequencing approach.

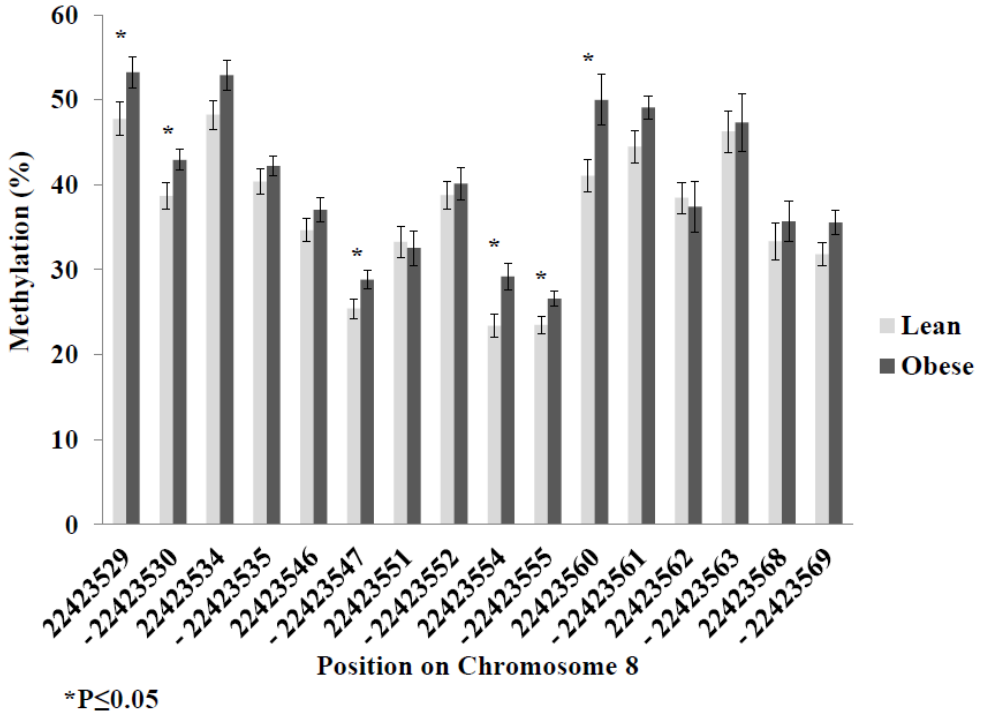

Figure 2-5. DNA methylation detected using pyrosequencing in the differentially methylated region (DMR) of sorbin and SH3 domain containing 3 (*SORBS3*) on both the forward and reverse (-) strands.

Predicted Transcription Factor Binding Analysis

To further understand the regulatory role of *SORBS3* methylation on transcription, we analyzed the sequences containing DMCs and the DMR using the program PROMO (Messeguer et al., 2002). Transcription factor binding motifs were identified for the following DMC positions: Chr.8:22,409,297- Sp1 (Figure 2-6A); Chr.8:22,422,648-p53, Chr.8:22,422,648-PAX5 and Chr.8:22,422,936-AP-2alpha (Figure 2-6B); Chr.8:22,423,300-RXRalpha, Chr.8:22,423,332-GCF and 22423343-GCF (Figure 2-6C). The transcription factor binding motifs identified within the DMR for *SORBS3* on Chr.8:22,423,530-22,423,569 were: ENKTF-1, STAT4, E2F-1, and GCF (Figure 2-6D).

Correlation analysis

To identify whether the methylation and transcriptomic findings for *SORBS3* were driven by body mass index (BMI) or age, Pearson correlation analysis was performed. Of the nine DMCs, five were significantly correlated with BMI and one was significantly correlated with age (Table 2-3). When comparing the normalized gene expression data with BMI there was a significant correlation (R^2=0.288; P=0.022); whereas with age, there was no correlation (R^2=0.034; P=0.464).

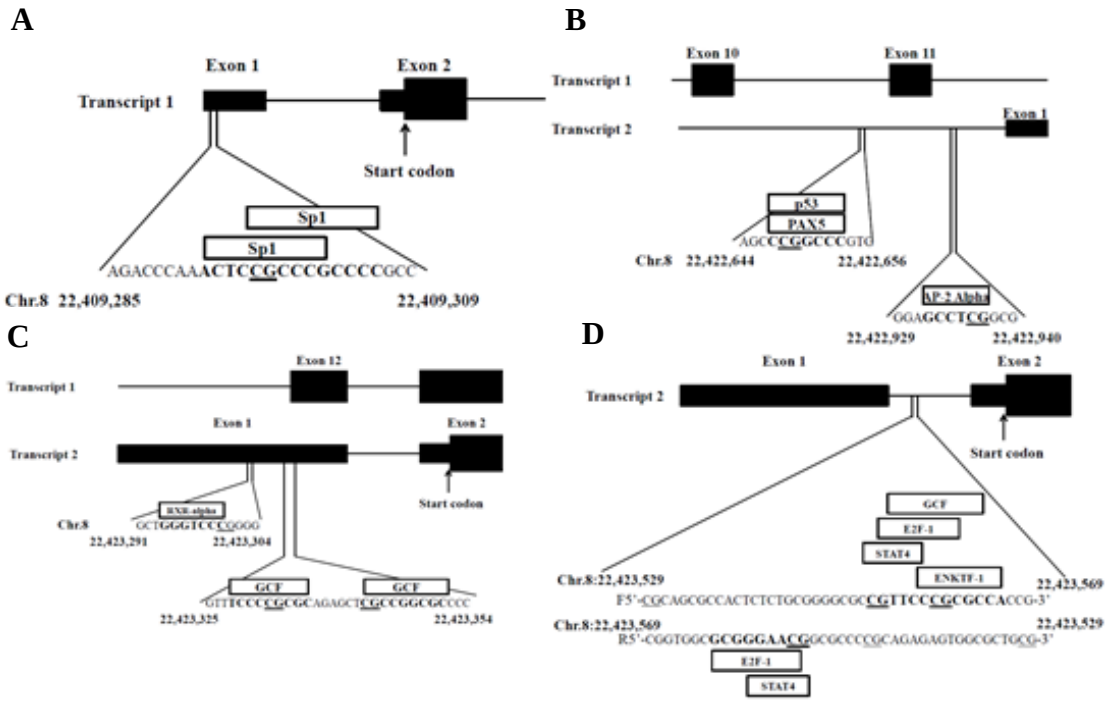

Figure 2-6. Transcription factor binding analysis. (A) Differentially methylated cytosine (DMC) at chromosome 8 position 22,409,297 is in the 5' untranslated region of sorbin and SH3 domain containing 3 (*SORBS3*) variant 1. This DMC is within two binding motifs for the transcription factor specificity protein 1 (Sp1). (B) DMCs at chromosome 8 positions 22,422,648 and 22,422,936 are in the promoter region of *SORBS3* variant 2. The DMC position 22,422,648 is within a two binding motifs for the transcription factors paired box 5 (PAX5) and tumor protein p53 (p53). The DMC position 22,422,936 is within a binding motif for the transcription factor activating enhancer-binding protein 2-alpha (AP-2 Alpha). (C) DMCs at chromosome 8 positions 22,423,300, 22,423,332, and 22,423,343 are in the 5' untranslated region of SORBS3 variant 2. The DMC position 22,423,300 is within the binding motif for the transcription factor retinoid X receptor, alpha (RXR-alpha). The DMC positions 22,423,332 and 22,423,343 are both within binding motifs for the transcription factor GC binding factor (GCF). (D) The *SORBS3* differentially methylated region (DMR) is located at chromosome 8 position 22,423,529-22,423,569 is in the 5' untranslated region of variant 2. On the forward strand, position 22,423,554 is within a binding motif for signal transducer and activator of transcription 4 (STAT4) and positon 22,423,560 is within the binding motif of enkephalin transcription factor 1 (ENKTF-1), E2F transcription factor 1 (E2F-1), and GC binding factor (GCF). On the reverse strand, position 22,423,555 is within the binding motif of E2F-1 and STAT4.

Table 2-3. Correlation analysis of differentially methylated cytosines (DMCs) sorbin and SH3 domain containing 3 (*SORBS3*) with body mass index (BMI) and age.

		BMI		Age	
Chr.	Position	R^2	P Value	R^2	P Value
8	22,409,297	0.092	NS	0.009	NS
8	22,422,648	0.329	0.013	0.209	NS
8	22,422,927	0.243	0.038	0.169	NS
8	22,422,936	0.264	0.029	0.238	0.040
8	22,422,959	0.169	NS	0.078	NS
8	22,423,092	0.254	0.033	0.017	NS
8	22,423,300	0.199	NS	0.202	NS
8	22,423,332	0.135	NS	0.144	NS
8	22,423,343	0.261	0.036	0.043	NS

R^2 and P value generated using Pearson correlation.

Discussion

The present study was undertaken to decipher the epigenetic basis of obesity and its associated insulin resistance. DNA methylation in the promoter and untranslated regions (5' and 3' UTR) have been noted to have regulatory effects on transcription (Ling & Groop, 2009; Maussion et al., 2014; Yu et al., 2015). This regulation can be mediated by a single CpG or by a group of CpGs in close proximity to each other (S. Li et al., 2013). Therefore, in our study we performed a comprehensive analysis of the sequencing data using both a DMC and DMR approach. To identify obesity-related alterations in gene expression that may be associated with DNA methylation, our study also utilized a transcriptomic approach. Merging across our omic datasets identified sorbin and SH3 domain containing 3 (*SORBS3*) as a novel obesity gene. *SORBS3* is decreased in expression in obesity, and this in part may be due to increased methylation. Moreover, we detected a number of transcription factors whose binding to the differentially methylated sites or regions may contribute to these findings (Attwood, Yung, & Richardson, 2002).

SORBS3 has two transcript variants that code for the adapter protein vinexin α and β, respectively. Both isoforms have a common C-terminal sequence containing three SRC homology 3 (SH3) domains, but differ at the N-terminal where vinexin α contains a sorbin homology (SoHo) domain. Vinexin α and β play roles in cell signaling and the cytoskeletal structure (Kioka, Ueda, & Amachi, 2002). The first two SH3 domains (SH3 1 and SH3 2) are important binding partners for vinculin, which is an actin-binding cytoskeletal protein localized at cell-extracellular matrix (ECM) and cell-cell adhesion sites (Kioka et al., 1999). It has been shown elsewhere that the upregulation of vinexin α promotes actin stress fiber formation and vinexin β enhanced cell spreading (Kioka et al., 1999). Our obesity associated decrease in gene expression may suggest a reduced plasticity of cytoskeleton organization. The third SH3 domain (SH3 3) is an important binding partner for the son of sevenless (SOS), a guanine nucleotide exchange factor for Ras and Rac (Kioka et al., 2002). Vinexin's interaction with SOS has been implicated to regulate growth-factor induced signal transduction (Kioka et al., 2002). For example, a knockdown model of vinexin has been shown to play a key role in the cell's migratory response during wound healing (Kioka et al., 2010). The reduction in *SORBS3* gene expression seen in our group with obesity may lead to a delayed response in growth-factor signaling.

Additional studies have evaluated vinexin under diseased states. A study using immunohistochemical analyses of vinexin in Otsuka Long Evans Tokushima Fatty (OLETF) rats with hyperinsulinemia and hyperglycemia, demonstrated a disorganized pancreatic islet structure (Yamauchi et al., 2013). Although abundance of vinexin was not discussed in that study, these findings infer that an obese environment can disrupt typical

localization of vinexin within a cell. We have previously shown alterations in cytoskeletal proteins in insulin resistant states (Hwang et al., 2010). Therefore, we hypothesize that a change in expression of *SORBS3* in obesity could be contributing to altered skeletal muscle structure. However, further investigation would be required. Chen *et al.* found that left ventricles of failing human hearts had a decrease in mRNA for vinexin β, and the disruption of vinexin expression in C57BL/6 mice exaggerated pathological cardiac remodeling and fibrosis (K. Chen et al., 2013). Obesity can lead to cardiovascular changes such as left-ventricular hypertrophy (Cuspidi, Rescaldani, Sala, & Grassi, 2014; Vasan, 2003). Although our study found reduced expression of *SORBS3* in the *vastus lateralis* of individuals with obesity, it is tempting to speculate that there may be a similar remodeling and fibrotic affect due to vinexin β.

The findings from our previous studies had led to a proposed model of a relationship between inflammation and insulin resistance in skeletal muscle (Coletta & Mandarino, 2011). In this model, chronic inflammation from obesity may induce changes to the extracellular matrix that are reminiscent of fibrosis and alter mechanosignal transduction mediated by cytoskeletal elements (Coletta & Mandarino, 2011). The changes in obesity with *SORBS3* expression coding for vinexin may be connected to our proposed model by regulating the plasticity of cytoskeletal elements. Interestingly, if vinexin is a key component to this model, we have identified possible regulation at the level of DNA by differentially methylated sites and regions. Moreover, the mechanism for this regulation could be due to the interaction of these methylation sites with the transcription factors identified in our analyses.

To our knowledge, this was the first study to examine obesity related differential DNA methylation in skeletal muscle using RRBS. The design of our epigenomic study not only allowed us to test our specific hypotheses, but also generated a novel methylation and transcriptional finding for further investigation. Furthermore our RRBS data can serve as a reference methylome for human skeletal muscle tissue. Despite these strengths, we acknowledged potential limitations that should be considered. There is a difference in age between our groupings that could be a confounding factor in the results presented. We did attempt to reduce this concern by running correlation analysis of age with *SORBS3* gene expression and each associated methylation site. Future age matched studies could elucidate any findings that may have been influenced by this variable. In addition, the potential for false discoveries may be at higher risk since our methylation data remained uncorrected. However, our chances of detecting true biological effects may be increased by the use both DMC and DMR analyses.

Overall, our study identified possible epigenetic influence on differential gene expression in *SORBS3* under obese conditions. We identified potential transcriptional regulators; however, follow up studies of their protein interactions with DNA methylation are necessary to refine the mechanism. Furthermore, the previously mentioned functional studies of vinexin under diseased states have been conducted in rodent models and should be further assessed in humans.

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CHAPTER 3: ALTERATIONS OF SORBIN AND SH3 DOMAIN CONTAINING 3 (SORBS3) IN HUMAN SKELETAL MUSCLE FOLLOWING ROUX-EN-Y GASTRIC BYPASS SURGERY

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Abstract

DNA methylation is known as a transcriptional regulator that can be influenced by environmental factors, and contribute to conditions such as obesity and insulin resistance. The aim of our study was to investigate the role of weight-loss induced by Roux-en-Y gastric bypass (RYGB) on skeletal muscle methylation associated with sorbin and SH3 domain containing 3 (*SORBS3*). We previously had shown increased methylation (methylation differences ranged from: 5.0 to 24.4 %) and decreased gene expression (fold change: −1.9) of *SORBS3* with obesity ($n = 10$; BMI $= 32.9 \pm 0.7$ kg/m^2) compared to lean controls ($n = 12$; BMI $= 23.4 \pm 0.7$ kg/m^2). In the present study, basal muscle biopsies were obtained from seven obese (BMI >40 kg/m^2) female subjects (45.1 ± 3.6 years) before and 3 months after RYGB surgery, in combination with euglycemic-hyperinsulinemic clamps to assess insulin sensitivity. Promoter and 5' untranslated region (UTR) *SORBS3* methylation detected with reduced representation bisulfite sequencing (RRBS) and pyrosequencing found a decrease (-5.6 to -24.2%) post-surgery. This decrease in DNA methylation was associated with an increase in *SORBS3* gene

expression (fold change: +1.7; P=0.05) post-surgery. Moreover, the relationship between *SORBS3* DNA methylation and decreased gene expression was achieved *in vitro* through the detection of luciferase activity (P=0.000003). The alterations in *SORBS3* methylation and gene expression post-surgery were significantly (P≤0.05) associated with obesity measures (BMI: Pre 42.1 ± 2.2 kg/m^2 vs Post 35.3 ± 1.8 kg/m^2; percent body fat: Pre 46.4 ± 1.2 vs Post 40.6 ± 1.3) and fasting insulin levels (Pre 18.2 ± 2.7 µU/mL vs Post 7.5 ± 1.0 µU/mL). These results demonstrate that *SORBS3* methylation and gene expression are dynamic and can be influenced by obesity, and restored to normal levels through weight-loss induced by RYGB surgery.

Introduction

One-third of the United States adult population is obese (body mass index [BMI] >30 kg/m^2), and the number of individuals entering into morbid obesity (BMI > 40 kg/m^2) is on the rise (Flegal, Carroll, Ogden, & Curtin, 2010; Sturm & Hattori, 2013). In 2010, it was estimated that 6.6 percent of the adult population in the United States were morbidly obese (Sturm & Hattori, 2013). In order to combat the obesity epidemic, various lifestyle interventions including diet and exercise have been utilized. However, difficulty with compliance to these lifestyle changes has been a substantial barrier to improving obesity and the co-morbidities associated with it (Mauro, Taylor, Wharton, & Sharma, 2008). The most effective treatment for providing sustained weight loss in morbid obesity or obesity with comorbidities is bariatric surgery (Genser et al., 2016). Roux-en-Y gastric bypass (RYGB) is one of the most common surgeries performed and combines restrictive and malabsorptive techniques (Catoi et al., 2015). Besides weight loss, other benefits of surgical intervention have included improved blood glucose levels, insulin sensitivity and

secretion (Catoi et al., 2015). These improvements to glycemia have been attributed to the increased incretin effect of glucagon-like peptide 1 (GLP-1) and glucose-dependent insulintropic peptide (GIP) on reducing glucagon and normalizing insulin secretion (Laferrere, 2009). However, these studies do not completely explain the molecular basis of these metabolic improvements.

A limited number of studies have shown gene expression changes in response to RYGB and gastric banding in blood (Edwards, Hindle, Fu, & Brody, 2011; Moran-Atkin, Brody, Fu, & Rojkind, 2013) or adipose tissue (Leyvraz et al., 2012). Three months post-RYGB and gastric banding, decreased expression in leptin and resistin (Edwards et al., 2011) and increased GIP expression (Moran-Atkin et al., 2013) has been observed in blood. In adipose tissue, decreases in leptin and 11-hydroxysteroid dehydrogenase type 1 and increases in peroxisome proliferator-activated receptor-1 expression were identified immediately after RYGB surgery (Leyvraz et al., 2012). Given that skeletal muscle is recognized as the primary tissue for insulin-stimulated glucose disposal (Abdul-Ghani & DeFronzo, 2010), investigation of skeletal muscle gene expression changes in response to surgical weight loss is important but lacking. We previously identified a novel decrease in cytosolic ribosomal genes and protein abundance with obesity that was normalized in skeletal muscle 3 months after RYGB surgery, using both transcriptomic and proteomic approaches (Campbell et al., 2016). These changes were accompanied with weight loss and metabolic improvements such as fasting insulin and glucose 3 months post-surgery (Campbell et al., 2016). The studies performed to date have assisted in understanding the metabolic improvements following surgery, but still remain incomplete. The metabolic

improvements observed in our participants post-surgery may be further explained by epigenetics, specifically DNA methylation.

One of the most studied epigenetic marks is DNA methylation, which is the addition of a methyl group to the fifth carbon of a cytosine, typically preceding a guanine, termed CpG dinucleotide (Ronn & Ling, 2015). The addition or removal of these marks has regulatory influence on gene expression (Huidobro et al., 2013). Barres *et al.* has shown epigenetic changes in response to surgery induced weight loss (Barres et al., 2013). Differential skeletal muscle promoter methylation was identified in peroxisome proliferator-activated receptor γ coactivator-1 α (*PGC-1α*) and pyruvate dehydrogenase kinase, isoenzyme 4 (*PDK4*) and restored to levels of healthy controls 6 months post-RYGB (Barres et al., 2013). Our previously published study assessed DNA methylation differences between lean and obese participants, and identified a novel gene, Sorbin and SH3 Domain Containing 3 (*SORBS3*) that was differentially methylated with obesity (Day et al., 2016). Specifically, we had shown an increase in skeletal muscle promoter methylation and a decrease in mRNA expression of *SORBS3* with obesity (Day et al., 2016). The *SORBS3* gene codes for the adapter protein vinexin, and has been shown to play roles in growth-factor-induced signal transduction and cytoskeleton structure (Kioka et al., 2002). Moreover, it has been shown by others that vinexin may play a role in cardiac hypertrophy (K. Chen et al., 2013). One frequent complication for obese individuals is cardiac hypertrophy (Cuspidi et al., 2014). Chen *et al*. found decreased expression of vinexin in failing human hearts and hypertrophic mouse hearts, and knockdown of vinexin exaggerated pathological cardiac remodeling and fibrosis (K. Chen et al., 2013). The decreased expression seems to be a consistent feature for both

obesity and cardiac hypertrophy disease states. However, it is unclear whether these changes can be rescued through surgical weight loss interventions such as RYGB.

Here we set out to determine if the changes in DNA methylation identified in obesity and its underlying insulin resistance can be altered by the RYGB surgery. Specifically, to identify alterations with the gene, *SORBS3*, in response to weight loss using our previous RYGB surgery cohort (Campbell et al., 2016). We hypothesized that 3 months post-surgery, *SORBS3* methylation will decrease and gene expression will increase and be normalized to levels of lean controls.

Methods

Study Design

Seven (one of which was diabetic, treated with metformin), morbidly obese (BMI > 40 kg/m^2) females (ages: 33-59 years) participated in this study before and 3 months post-RYGB surgery (Campbell et al., 2016). Medical history, anthropometric, body impedance analysis, 75g oral glucose tolerance test (OGTT) were obtained on all participants. Metabolic and screening blood tests were performed by the Biospecimens Accessioning and Processing (BAP) Core at the Mayo Clinic in Scottsdale. Insulin sensitivity was determined by the euglycemic-hyperinsulinemic clamp (DeFronzo et al., 1979), before and 3 months post-surgery. All plasma glucose and serum insulin were measured by the Center for Clinical and Translational Science Metabolomics Core at the Mayo Clinic in Rochester, as described in Chapter 2. All participants gave informed written consent for this study, which was approved by the institutional review boards at the Mayo Clinic in Arizona and Arizona State University.

Each participant reported to the Bariatric Surgery Program at the Mayo Clinic in Arizona. The program provided 8 weeks of behavioral modification classes on nutrition, exercise, and behavioral aspects of lifestyle change. Each participant met twice with a dietitian and had three sessions with psychologist to work on behavior goals. The pre-surgery diet focused on portion control for modest weight loss prior to surgery. The post-surgery diet was a transition from liquids for 3 weeks, soft food for 3 weeks, and then progressed to normal textures. Daily multivitamin, calcium with vitamin D, B12 monthly injections, iron supplements, and protein supplementation were maintained for all participants.

Muscle biopsy processing

Genomic DNA was extracted by homogenizing muscle biopsies (25 mg) in 1X PBS with the Bullet Blender (Integrated Scientific Solutions, San Diego, CA). The tissue was further processed using QIAamp DNA mini kit, as per the manufacturer's instructions (Qiagen, Valencia, CA). To isolate RNA, muscle biopsies (50 mg) in TRIzol solution (Invitrogen, Carlsbad, CA) were homogenized using a Polytron (Brinkmann Instruments Westbury, NY). The RNA was isolated with RNeasy MinElute Cleanup Kit (Qiagen, Chatsworth, CA). DNA and RNA quality and quantity were determined using gel electrophoresis and spectrophotometric A260/A280 values.

Reduced representation bisulfite sequencing (RRBS)

RRBS sample preparation was performed on skeletal muscle genomic DNA from before and 3 months post-surgery at the Mayo Clinic Genotyping Shared Resource facility, as described in Chapter 2. Sequence data was processed using the streamlined analysis and annotation pipeline for reduced representation bisulfite sequencing, SAAP-

RRBS (Day et al., 2016; Sun et al., 2012). The pipeline includes removal of adapter sequences and genomic segments under 15 base pair (bp), and aligns the remaining sequence against the human reference genome Hg19.

Differentially methylated cytosines (DMCs) analysis pre- and post-surgery

Differences in methylation sites were assessed in participants before and after surgery. The aligned (Hg19) sequencing data was imported into the free open source R package, methylSig (Park et al., 2014). A minimum of five reads and the recovery of the site in all seven participants from before and after surgery were required for the inclusion of a cytosine in subsequent analyses. The mean methylation differences (%) were determined and annotations were applied, as described in Chapter 2.

***SORBS3* pyrosequencing**

DNA methylation sites detected in skeletal muscle from RRBS were confirmed using pyrosequencing. To assess *SORBS3* DMCs at positions Chr.8:22,423,519 and Chr.8:22,423,529 on the sense strand, bisulfite-converted DNA was amplified by PCR using the following primers: forward 5'- AGTAGGGGGAGGAAGGAA-3' and biotinylated reverse 5'- ACCCCCATCCTCTACTAAAAATTAAC-3'. For the DMCs at positions Chr.8:22,423,690 and Chr.8:22,423,702 on the antisense strand, bisulfite-converted DNA was amplified by PCR using the following primers: forward 5'-GGGTTTTGGGTTTTTTATAGGATG-3' and biotinylated reverse 5'-CCACCCAAAACAACTAACTCCTAAC-3'. Pyrosequencing was performed using the PyroMark Q96 MD system and the Gold Q96 kit with sequencing primers for the sense 5'-GGGGGAGGAAGGAAT-3' and antisense 5'- TGGGTTTTTTATAGGATGT-3' strands according to the manufacturer's instructions (Qiagen, Valencia, CA). Sequence

analysis was performed using the PyroMark CpG SW 1.0 software (Qiagen, Valencia, CA).

SORBS3 quantitative real-time PCR (qRT-PCR)

Skeletal muscle gene expression for *SORBS3* pre- and post-surgery was detected using qRT-PCR on the ABI PRISM 7900HT sequence detection system (Life Technologies, Carlsbad, CA). Synthesis of cDNA was performed with ABI High Capacity cDNA Reverse Transcription Kit, as per manufacturer's instructions. TaqMan Universal Fast PCR master mix reagents and the Assay-On-Demand gene expression primer pair and probes (Life Technologies, Carlsbad, CA) were added to 20 ng cDNA. The quantity of *SORBS3* (Hs00195059_m1) in each sample was normalized to *18S* (Hs99999901_s1) using the comparative (2-ΔΔCT) method (Livak & Schmittgen, 2001).

Luciferase Assay

An 811 bp fragment of the human *SORBS3* promoter (Chr8:22,422,247-22,423,057) was cloned into a CpG-free luciferase reporter vector (pCpGL-Basic), kindly provided by Dr. Maja Klug and Dr. Michael Rheli (Department of Hematology and Oncology, University Hospital Regensberg, Regensburg, Germany). The *SORBS3* construct was either mock methylated or methylated using 1600 µM S-adenosylmethionine (SAM) and two different DNA methyltransferases: SssI that methylates all cytosines of CG sites, and HhaI that methylates only the internal cytosine in the CGCG sequence (New England Biolabs, Frankfurt, Germany). Mouse muscle cell lines C2C12 were cultured in DMEM, supplemented with 10% serum and 1% of an antibiotic/antimycotic mixture. C2C12 cells were seeded onto a 96 well plate in 100 µl of medium ($2 \cdot 10^4$ cells per well) and incubated for 24 hours. Cells were then co-transfected with 100 ng of pCpGL-basic with

the *SORBS3* promoter insert or without (control) and 2 ng of pRL renilla luciferase control reporter vector using the Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, firefly luciferase activity was measured and normalized against the measured renilla luciferase activity using the Dual Luciferase Reporter Assay System (Promega, Madison, WI). The results presented are a mean of 4 independent experiments, containing the mean of 5 replicates in each experiment.

***SORBS3* comparative DMC analysis**

The skeletal muscle RRBS data from Chapter 2 was used for comparative analysis. The data comprised of 11 lean (ages: 21–43 years; 7 females/4 males; BMI 23.4 ± 0.7 kg/m^2) and 9 obese (ages: 32–52 years; 4 females/5 males; BMI 32.9 ± 0.7 kg/m^2) participants. The DMC analysis from Chapter 2 and the DMC analysis for the RYGB surgery cohort were performed using the program MethylSig (Park et al., 2014).

Predictive transcription factor binding analysis

PROMO version 3.0.2 was used to perform transcription factor binding site analysis (Messeguer et al., 2002). Sequences were analyzed with a 5% maximum matrix dissimilarity rate using TRANSFAC version 8.3 database. Analysis of the 30 *SORBS3* DMCs was assessed as 10 separate sequences: Chr.8: 22,411,723-22,411,734; Chr.8: 22,422,932-22,422,973; Chr.8: 22,423,009-22,423,025; Chr.8: 22,423,086-22,423,116; Chr.8: 22,423,181-22,423,215; Chr.8: 22,423,219-22,423,256; Chr.8: 22,423,514-22,423,573; Chr.8: 22,423,684-22,423,695; Chr.8: 22,423,697-22,423,741; and Chr.8: 22,423,769-22,423,857.

Statistical analysis

Comparisons between data from pre- and post-surgery were based on a paired Student's t-test. All characteristic data was normally distributed data, and are presented as a mean ± standard error of the mean (SEM). Pearson correlation analysis was performed to determine the relationship between DNA methylation or gene expression and characteristic data. See above for the statistical analysis of the methylation and qRT-PCR data.

Results

Participants

Table 3-1 shows the phenotypic characteristics for participants pre- and post-surgery. At three months post-surgery, significant improvements were observed in BMI, body fat percentage, cholesterol, low density lipoprotein (LDL), fasting plasma glucose (FPG), fasting serum insulin (FSI) and homeostatic model assessment for insulin resistance (HOMA-IR). However, there were no significant improvements observed in blood pressure, triglycerides, high density lipoprotein (HDL), hemoglobin A1c (HbA1c), endogenous glucose production (EGP) and insulin-stimulated glucose disposal (M-value).

SORBS3 differentially methylated cytosines (DMCs)

Methylation sites within the promoter (0 to −1000 base pairs from transcription start site) and untranslated regions (5' and 3'UTR) were used to detect sites that may lead to a change in *SORBS3* mRNA expression. Using this criteria, there were 20 DMCs in the sense strand and 10 DMCs in the antisense strand identified (Table 3-2). Of the 30 DMCs, 29 sites were decreased in methylation post-surgery compared to pre-surgery.

Table 3-1. Characteristics before and 3 months after Roux-en-Y gastric bypass surgery.

	Pre-surgery Obese	**Post-surgery Obese**	***P* value**
			Pre vs. Post
Sex	7 Female	7 Female	-
Age (years)	45.1 ± 3.6	45.3 ± 3.5	NS
Body mass index (kg/m^2)	42.1 ± 2.2	35.3 ± 1.8	<0.001
Body fat (%)	46.4 ± 1.2	40.6 ± 1.3	<0.01
Systolic blood pressure (mmHg)	125.1 ± 3.9	119.1 ± 4.6	NS
Diastolic blood pressure (mmHg)	71.7 ± 2.0	75.1 ± 1.7	NS
Triglycerides (mg/dL)	121.9 ± 17.5	107.7 ± 11.2	NS
Cholesterol (mg/dL)	181.4 ± 13.2	151.5 ± 11.2	<0.01
High density lipoprotein (mg/dL)	45.0 ± 2.7	45.0 ± 2.5	NS
Low density lipoprotein (mg/dL)	112.1 ± 11.9	84.8 ± 10.5	<0.01
Hemoglobin A1c (%)	6.0 ± 0.2	5.7 ± 0.1	NS
Fasting plasma glucose (mg/dL)	104.2 ± 7.8	86.7 ± 3.1	<0.05
Fasting plasma insulin (μU/mL)	18.2 ± 2.7	7.5 ± 1.0	<0.01
EGP (mg/kg/min)	1.5 ± 0.1	1.5 ± 0.1	NS
M-value (mg/kg/min)	2.4 ± 0.3	2.9 ± 0.4	NS
M-value (mg/kgFFM/min)	4.4 ± 0.6	4.9 ± 0.6	NS
HOMA IR	4.4 ± 0.8	1.6 ± 0.3	<0.05

Data presented as mean ± SEM, significance based on independent sample t-tests. Endogenous glucose production (EGP). Homeostatic model assessment for insulin resistance (HOMA-IR).

SORBS3 Validation

From the 30 *SORBS3* DMCs identified using RRBS, pyrosequencing was used for confirmation of sites where primers could be designed. Sequencing captured the DMCs at positions Chr.8:22,423,519 and Chr.8:22,423,529 on the sense strand, as well as four additional CpG sites. All six sites were decreased in methylation post-surgery; however, none were significantly changing (Figure3-1a). The sequence that encompassed the DMCs at positions Chr.8:22,423,690 and Chr.8:22,423,702 on the antisense strand included two additional CpG sites. All four sites were decreased in methylation post-

surgery, and changes in three of these sites were statistically significant (P<0.05; Figure3-1b).

Table 3-2. Differentially methylated cytosines (DMCs; $P < 0.05$) post-surgery associated with *SORBS3*.

Position	Methyl Difference (%)	P value	Strand	Gene Region	CpG Island Region
Chr8.22411728	-8.8	0.03	Sense	5'UTR	South Shelf
Chr8.22423014	-13.7	0.003	Sense	Promoter	CpG Island
Chr8.22423020	-9.6	0.04	Sense	Promoter	CpG Island
Chr8.22423091	-12.7	0.006	Sense	Promoter	CpG Island
Chr8.22423100	-9.3	0.03	Sense	Promoter	CpG Island
Chr8.22423111	-8.9	0.02	Sense	Promoter	CpG Island
Chr8.22423186	+14.1	0.04	Sense	5'UTR	CpG Island
Chr8.22423198	-7.4	0.03	Sense	5'UTR	CpG Island
Chr8.22423202	-10.4	0.01	Sense	5'UTR	CpG Island
Chr8.22423204	-7.7	0.0001	Sense	5'UTR	CpG Island
Chr8.22423206	-7.7	0.03	Sense	5'UTR	CpG Island
Chr8.22423210	-9.9	0.03	Sense	5'UTR	CpG Island
Chr8.22423224	-9.9	0.001	Sense	5'UTR	CpG Island
Chr8.22423235	-6.4	0.04	Sense	5'UTR	CpG Island
Chr8.22423251	-8.2	0.04	Sense	5'UTR	CpG Island
Chr8.22423519	-12.6	0.002	Sense	5'UTR	CpG Island
Chr8.22423529	-12.0	0.005	Sense	5'UTR	CpG Island
Chr8.22423568	-11.1	0.02	Sense	5'UTR	CpG Island
Chr8.22423689	-16.0	0.01	Sense	5'UTR	CpG Island
Chr8.22423736	-7.1	0.007	Sense	5'UTR	South Shore
Chr8.22411729	-5.6	0.005	Antisense	5'UTR	South Shelf
Chr8.22422937	-12.4	0.04	Antisense	Promoter	CpG Island
Chr8.22422940	-17.8	0.02	Antisense	Promoter	CpG Island
Chr8.22422953	-24.2	0.003	Antisense	Promoter	CpG Island
Chr8.22422968	-13.3	0.007	Antisense	Promoter	CpG Island
Chr8.22423205	-6.4	0.001	Antisense	5'UTR	CpG Island
Chr8.22423690	-13.2	0.04	Antisense	5'UTR	CpG Island
Chr8.22423702	-19.8	0.007	Antisense	5'UTR	CpG Island
Chr8.22423774	-20.6	0.03	Antisense	5'UTR	South Shore
Chr8.22423852	-14.8	0.03	Antisense	5'UTR	South Shore

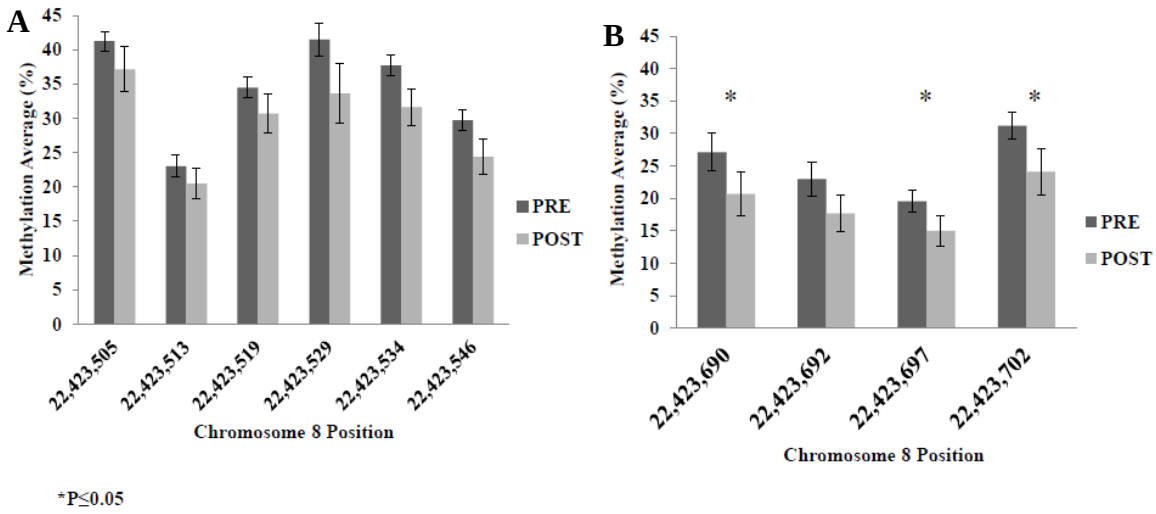

Figure 3-1. Differentially methylated cytosines (DMCs) associated with *SORBS3* detected using pyrosequencing on the sense strand (a) and antisense strand (b) pre- and post-surgery.

SORBS3 gene expression

The qRT-PCR results demonstrated an increase in gene expression of *SORBS3* three months post-surgery compared to pre-surgery (fold change +1.7; P=0.05).

SORBS3 correlation analysis

Pearson correlation analysis was performed to determine the relationship between characteristic data and *SORBS3* DNA methylation and gene expression changes observed post-surgery. Of the 30 DMCs, 20 were significantly (P≤0.05) associated with at least one characteristic (Table 3-3). *SORBS3* methylation level was positively correlated with BMI, percent body fat, triglycerides, cholesterol, LDL, FPG, FSI, and HOMA-IR. Negative correlations were observed between methylation levels and HDL. The only DMC opposing the observed associations was Chr.8: 22,423,186, which was the one site to be increased in methylation post-surgery. Association of *SORBS3* gene expression with characteristic data was performed using the Ct values from qRT-PCR. Pearson's

correlation analysis identified a significant relationship between gene expression and BMI (r=0.8, P=0.00040), percent body fat (r=0.6, P=0.02), and FSI (r=0.5, P=0.04). Furthermore, an association between *SORBS3* gene expression and methylation was identified at DMCs Chr.8:22,423,519 (r=0.7, P=0.004), Chr.8:22,423,689 (r=0.5, P=0.05), and Chr.8:22,423,702 (r=0.6, P=0.03).

***SORBS3* promoter methylation in vitro alters reporter gene expression**

An 811 bp human *SORBS3* promoter was inserted into a luciferase expression plasmid that was free of CpG dinucleotides. The *SORBS3* construct was created to test the effect of DNA methylation on the transcriptional activity. Suppression of transcriptional activity, as measured by luciferase activity, was determined in comparison to the mock methylated control (Figure 3-2). As shown in Figure 3-2, when the *SORBS3* construct was methylated *in vitro* using the HhaI enzyme (G<u>C</u>GC, total of 8 CpG sites) transcriptional activity was not suppressed, but was significantly suppressed with the SssI enzyme methylation (CG, total of 59 CpG sites).

Predicted transcription factor binding analysis

To identify potential transcription factor binding that may be inhibited by *SORBS3* methylation, we analyzed sequences containing DMCs using PROMO (Messeguer et al., 2002). Transcription factor binding motifs were identified to overlap 13 of the 30 DMCs for SORBS3. The following are DMC positions with the disrupted binding of potential transcript factors: Chr.8:22,422,937: AP-2alphaA; Chr.8:22,422,953: Sp1; Chr.8:22,423,014 and 22,423,020: GCF; Chr.8:22,423,100: CREB; Chr.8:22,423,204, 22,423,205, 22,423,206, and 22,423,210: GCF; Chr.8:22,423,235: Sp1, Pax-5, and p53; Chr.8:22,423,689 and 22,423,690: GCF; and Chr.8:22,423,736: RXR-alpha.

Table 3-3. Association of *SORBS3* DMCs with characteristic data.

	BMI		Body Fat		TG		Chol.		HDL		LDL		FPG		FSI		M-value		HOMA-IR	
Chr. 8	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P
22411728	0.5	0.05	0.6	0.04											0.6	0.03			0.6	0.02
22422937															0.5	0.05				
22422940									-0.6	0.03										
22422953									-0.6	0.04										
22423014					0.6	0.03							0.7	0.002						
22423020					0.6	0.04														
22423091					0.6	0.04	0.7	0.02			0.6	0.03	0.6	0.02						
22423100					0.7	0.01							0.7	0.003						
22423111					0.7	0.004							0.9	0.0001						
22423186															-0.6	0.01	0.7	0.01	-0.6	0.04
22423204													0.7	0.01						
22423205													0.8	0.001						
22423206													0.5	0.05						
22423210													0.5	0.05						
22423519	0.8	0.002	0.6	0.02											0.7	0.01			0.7	0.01
22423529	0.6	0.02	0.5	0.05											0.5	0.04			0.6	0.02
22423568	0.5	0.05											0.5	0.04					0.6	0.03
22423702	0.6	0.03	0.5	0.04																
22423736			0.6	0.03																
22423852			0.5	0.05															0.6	0.04

Pearson's correlation coefficient, r and significance P≤0.05. Body mass index (BMI); Triglycerides (TG); Cholesterol (Chol.); High density lipoprotein (HDL); Low density lipoprotein (LDL); Fasting plasma glucose (FPG); Fasting serum insulin (FSI).

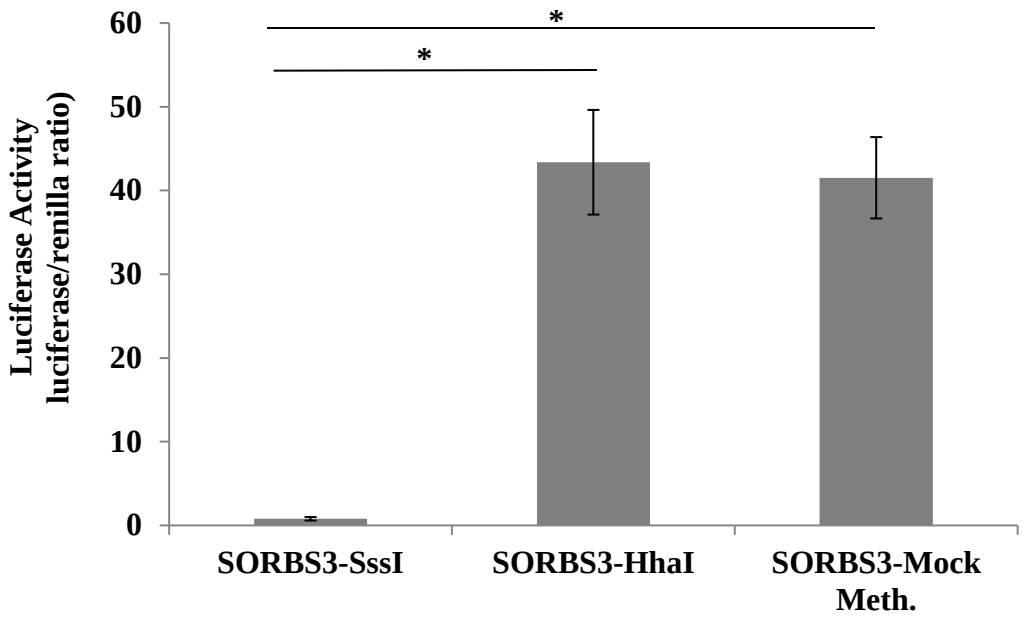

Figure 3-2. In vitro DNA methylation of the *SORBS3* human promoter is associated with decreased gene expression. The data is presented as mean ± SEM. The mean represents 4 independent experiments with 5 replicates per experiment. *Significance based on independent sample t-tests, $P < 0.0001$.

SORBS3 alterations with obesity and RYGB surgery

Increased DNA methylation in the promoter and 5'UTR of *SORBS3* with obesity were originally identified in our previous study (Day et al., 2016). In the RYGB cohort, methylation levels of *SORBS3* were found to decrease post-surgery. Upon comparing the 10 DMCs (9 increased and 1 decreased) from our previous, and the 30 DMCs (29 decreased and 1 increased) identified with RYGB surgery, we found sites to cluster in the same region, but no sites were identical between studies (Figure 3-3). We further assessed the average methylation levels of all DMCs (Figure 3-4a) and only DMCs consistent in the direction of methylation (3-4b). Both assessments presented similar average methylation levels between the lean and post-surgery, and the obese and pre-surgery (Figure 3-4).

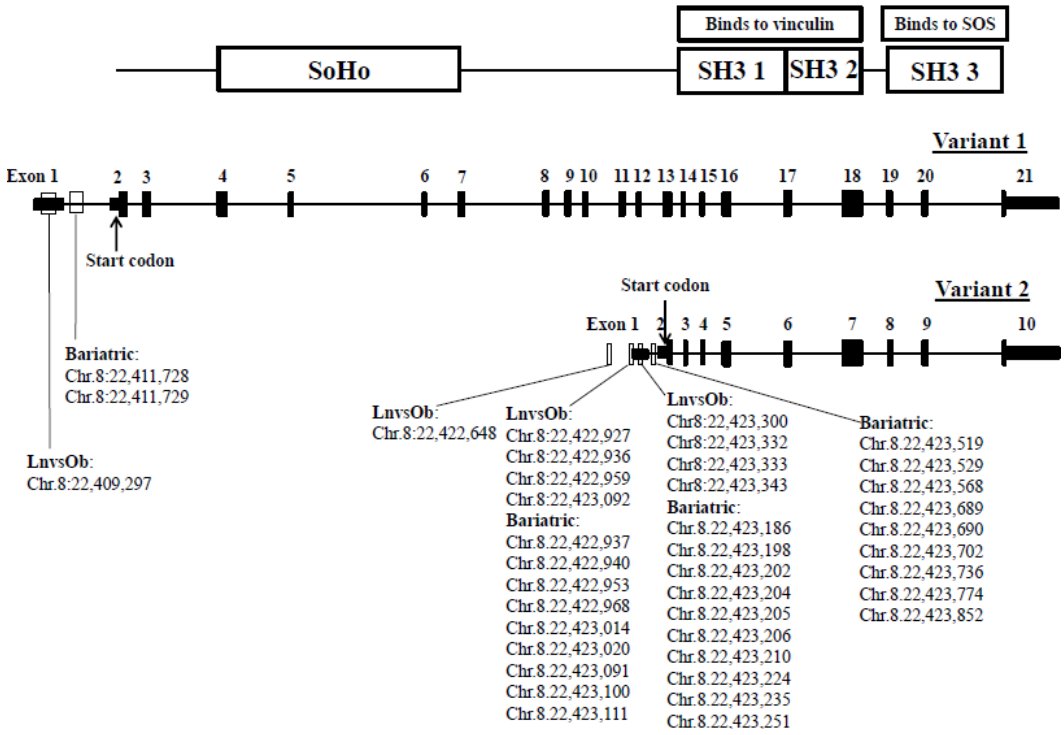

Figure 3-3. Differentially methylated cytosine (DMC) distribution among the promoter and 5'untranslated regions of sorbin and SH3 domain containing 3 (*SORBS3*) variant 1 and 2. The DMCs are derived from a previous study in obesity (LnvsOb) and the present study (Bariatric).

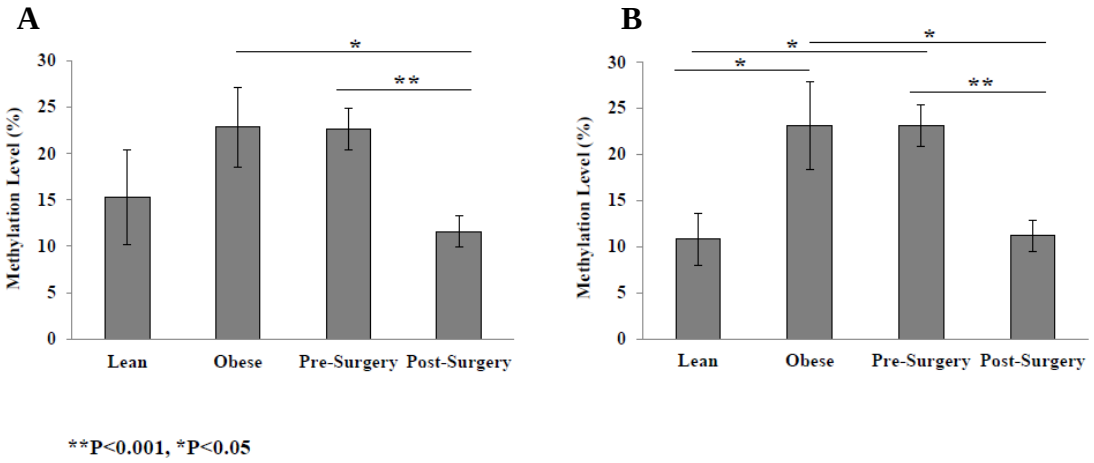

Figure 3-4. Average methylation levels of *SORBS3* DMCs from lean and obese participants in a previous study, and the present study pre- and post-surgery levels. The average methylation was assessed with all DMCs, regardless of methylation direction (A) and of only the DMCs that were consistent in the direction of methylation (B).

Discussion

Our previous study had identified *SORBS3* as an obesity-associated gene, whose expression may be epigenetically regulated (Day et al., 2016). We set out to further establish the relationship between *SORBS3* methylation and gene expression changes with obesity through a surgical weight-loss intervention. Three months following the RYGB surgery, there were significant reductions in weight and improvement of metabolic measures such as BMI, percent body fat and fasting plasma insulin levels. However, we did not observe an improvement in insulin-stimulated glucose disposal as determined by the euglycemic-hyperinsulinemic clamp. Our observations are consistent with another study, where insulin sensitivity was not markedly improved at 3 months, but was improved at 12 months with major weight loss (Albers et al., 2015). Therefore, we may have identified this improvement if measurements were assessed after the 3 months post-surgery. In addition, we did not identify a significant change in EGP at 3 months post-surgery. However, these results are also consistent with another study which had taken measures 1 week, 3 months, and 1 year after surgery (Bojsen-Moller et al., 2014). A significant decrease in EGP was identified 1 week after surgery, but then returned to pre-surgery measures at 3 months as fasting glucose and insulin concentrations were lowered (Bojsen-Moller et al., 2014). Moreover, HOMA-IR is primarily an indication of hepatic insulin resistance, in which our study was consistent with Bojsen-Møller *et al.* in showing hepatic sensitivity improvements at 3 months (Bojsen-Moller et al., 2014). Collectively, our data suggests improvements post-surgery are reflective of improved hepatic sensitivity.

Using both RRBS and pyrosequencing approaches, we identified significant decreases in DNA methylation located in promoter and untranslated regions of *SORBS3* post-surgery. Moreover, we found skeletal muscle expression of *SORBS3* significantly increased post-surgery compared to pre-surgery. The changes observed in DNA methylation and gene expression complement our previous findings (Day et al., 2016), where measurements post-surgery were relatively proportional to levels found in our lean individuals. The negative relationship between DNA methylation and gene expression were further established by the reduced transcriptional activity presented in the luciferase assay in response to DNA methylation in the *SORBS3* human promoter. The luciferase assay has been used in this study and in others (Barres et al., 2013; Ronn et al., 2013) as a reliable means of providing evidence that methylation in a particular promoter region can alter gene expression. We observed decreased gene expression for the methylation captured with SssI, but not with HhaI, suggesting the positioning of the sites in that promoter to be important. Another study has shown that the methylation of specific sites within a promoter region of a luciferase assay can result in varied outcomes, such as an increase or decrease in transcriptional activity (Petkova, Seigel, & Otteson, 2011). However, the exact mechanism in which our DNA methylation sites regulate the transcription of *SORBS3* has not been elucidated in this study. We have identified potential transcription factor binding motifs that may be affected by the presence of methylation, but require further investigation.

Environmental factors can influence transcriptional regulation through DNA methylation. Associations were found between *SORBS3* DMCs and a variety of participant characteristic measures, but most significantly with obesity measures,

triglycerides, and fasting glucose and insulin levels. Further assessment with gene expression only identified significant associations with BMI, percent body fat, and fasting insulin levels. Barres *et al.* had identified a significant positive correlation between PGC1α methylation levels and characteristics, such as BMI and triglycerides 6 months post-surgery (Barres et al., 2013). This study suggested that the correlation observed with multiple characteristics may suggest that methylation changes occur from our system as a whole (Barres et al., 2013). Collectively, our findings highlight the relationship between increased *SORBS3* DNA methylation in the presence of obesity and its underlying insulin resistance.

The findings post-surgery present an exciting new addition to further the understanding of DNA methylation associated with *SORBS3* expression. Not only have we detected differences associated with *SORBS3* in individuals with obesity and insulin resistance, but now have evidence of alterations in response to weight-loss by surgical intervention. However, we acknowledge the limitation of our sample size. Future studies will need to confirm our findings in a larger cohort. Moreover, we observed *in vitro* the suppression of *SORBS3* promoter DNA methylation on transcriptional activity. The specific placement of these sites can play an important role on the binding ability of transcription factors. We identified potential transcriptional regulators overlapping our methylation sites; however, follow-up studies will be necessary to refine the specific interaction.

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CHAPTER 4: POTENTIAL EPIGENETIC BIOMARKERS OF OBESITY RELATED INSULIN RESISTANCE IN HUMAN WHOLE BLOOD

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Abstract

Obesity can increase the risk of complex metabolic diseases including insulin resistance. Moreover, obesity can be caused by environmental and genetic factors. However the epigenetic mechanisms of obesity are not well defined. Therefore, identifying novel epigenetic biomarkers of obesity allows for a more complete understanding of the disease and its underlying insulin resistance. The aim of our study was to identify DNA methylation changes in whole blood that was strongly associated with obesity and insulin resistance. Whole blood was obtained from lean (n=10; BMI= 23.6±0.7 kg/m^2) and obese (n=10; BMI= 34.4±1.3 kg/m^2) participants in combination with euglycemic hyperinsulinemic clamps to assess insulin sensitivity. We performed reduced representation bisulfite sequencing (RRBS) on genomic DNA isolated from the blood. We identified 49 differentially methylated cytosines (DMCs; $q<0.05$) that were altered in the obese participants compared to the lean. We identified two sites (Chr.21:46,957,981 and Chr.21:46,957,915) in the 5' untranslated region of solute carrier family 19 member 1 (*SLC19A1*) that were both decreased in methylation in the obese participants (lean 0.73 ± 0.11 vs obese 0.09 ± 0.05; lean 0.68 ± 0.10 vs obese 0.09 ± 0.05

respectively). These two DMCs identified by obesity were also found to be significantly predicted by insulin sensitivity ($r = 0.68$, $P = 0.003$; $r = 0.66$; $P = 0.004$). In addition, we performed a differentially methylated region (DMR) analysis and demonstrated a decrease in methylation of Chr.21:46,957,915-46,958,001 in *SLC19A1* by -34.9% (70.4% lean vs 35.5% obese). The decrease in *SLC19A1* methylation in our obese participants in the whole blood was similar to the change observed in skeletal muscle (Chr.21:46,957,981; lean 0.70 ± 0.09 vs obese 0.31 ± 0.11 and Chr.21:46,957,915; lean 0.72 ± 0.11 vs obese 0.31 ± 0.13). Pyrosequencing analysis further demonstrated a decrease in methylation at Chr.21:46,957,915 in both the whole blood (lean 0.71 ± 0.10 vs obese 0.18 ± 0.06) and skeletal muscle (lean 0.71 ± 0.10 vs obese 0.30 ± 0.11). Our findings demonstrate a new potential epigenetic biomarker, *SLC19A1*, for obesity and its underlying insulin resistance.

Introduction

Obesity is an epidemic, and has become the fifth leading risk for global deaths (Kyrou, Randeva, & Weickert, 2000). Individuals with obesity have chronic low-grade inflammation (Coletta & Mandarino, 2011; Tateya, Kim, & Tamori, 2013). The expansion of white adipose tissue in obesity has been associated with increased proinflammatory cytokines, such as tumor necrosis factor-α (TNFα) and interleukin 6 (IL-6) (Tateya et al., 2013). These inflammatory cytokines circulate in the blood and can have negative effects on peripheral inulin responsive tissues, such as skeletal muscle and liver (Verdile et al., 2015). The activation of Toll-like and interleukin receptors have been proposed to reduce insulin signaling by promoting the signaling cascade of inflammatory kinases (Coletta & Mandarino, 2011). The reduction in insulin signaling, in part, is due to

phosphorylation of serine residues on the insulin receptor substrate 1 (IRS-1) in skeletal muscle, thus inhibiting its activity (Coletta & Mandarino, 2011; Olefsky & Glass, 2010). As such, the majority of individuals with obesity have an underlying insulin resistance (Tateya et al., 2013; Tilg & Moschen, 2006). This state of chronic inflammation associated with obesity can also exacerbate co-morbidities, including type 2 diabetes (T2D), hypertension, dyslipidemia, and cardiovascular disease (Kyrou et al., 2000; Shoelson, Herrero, & Naaz, 2007).

In order to better understand how to hinder disease progression, it has become important to find reliable biomarkers for early intervention (Dayeh et al., 2016). Biomarkers can be any biological characteristic that can be identified and/or monitored during the progression of a disease (Mikeska & Craig, 2014). This includes non-invasive measurements such as those currently used for identifying risk for the progression to type 2 diabetes and cardiovascular disease, such as high body mass index (BMI) and blood pressure (Singh et al., 2010). Other traditional biomarkers have included clinical measurements of glucose, hemoglobin A1c (HbA1c), and cholesterol levels from blood (Niswender, 2010). However, the progression of obesity and insulin resistance is a consequence of both environmental and genetic factors, and the above mentioned non-invasive traditional measurements do not provide insight into the molecular basis of the disease (O'Connell & Markunas, 2016).

Our previous work in whole blood assessed transcriptional changes in a Latino population from the Arizona Insulin Resistance (AIR) Registry (Kim, Campbell, Shaibi, & Coletta, 2015; Tangen et al., 2013). In one study, we demonstrated transcriptomic changes in genes involved in ribosome, oxidative phosphorylation and MAPK signaling

when analyzing the adults with and without metabolic syndrome (Tangen et al., 2013). In another study, we identified altered expression of genes involved in inflammatory pathways in adolescents with and without obesity (Kim et al., 2015). Our findings indicate potential biomarkers in whole blood for inflammation, insulin signaling, and mitochondrial function in obese and metabolic syndrome conditions. We believe that the transcriptomic changes observed in our cohorts are in part due to epigenetic regulation.

Epigenetics is a regulatory process that controls gene expression without altering the nucleotide sequence (Egger et al., 2004). DNA methylation is the epigenetic process of a methyl addition primarily to a cytosine residue preceding a guanine, termed CpG dinucleotide (Egger et al., 2004). DNA methylation marks residing in promoter and untranslated regions have been associated with gene silencing (Ling & Groop, 2009; Maussion et al., 2014; Yu et al., 2015). However, large-scale studies such as the Human Epigenome Project have found low correlations between gene expression and differential methylation (Eckhardt et al., 2006). Specifically, one-third of the differential methylation they identified in 5' untranslated regions were inversely correlated with transcription (Eckhardt et al., 2006). Epigenetic mechanisms have become important for determining the molecular basis of diseases, because they are due to both genetic and environmental factors (Ling & Groop, 2009). The influence of these factors on DNA methylation has also made it a promising biomarker for disease. The use of DNA methylation as an epigenetic biomarker has become attractive for clinical use due to its covalent bond, making it a robust mark for analysis (Mikeska & Craig, 2014).

A number of studies have focused on identifying epigenetic biomarkers in blood that were associated with obesity and insulin resistance (Aslibekyan et al., 2015; Dick et

al., 2014; Nilsson et al., 2015; Toperoff et al., 2012). In our study, we performed reduced representation bisulfite sequencing (RRBS) to assess DNA methylation at a whole genome level. Here we set out to identify changes in DNA methylation from obesity using the most readily available tissue, whole blood. Based on our previous transcriptomic findings in whole blood, we hypothesize that there will be alterations in DNA methylation of genes involved in inflammation, insulin signaling, and mitochondrial function. There is a potential we may have a low overall correspondence between our methylation data and our previous transcriptomic data based on the Human Epigenome Project findings (Eckhardt et al., 2006). Regardless, this study will allow us to identify novel epigenetic biomarkers that are associated with the obesity and insulin resistance in blood.

Material and methods

Participants

Ten participants with obesity (BMI $\geq$ 30 kg/m^2; 5M/5F; Age 23-52 years) and ten participants without obesity (BMI < 25 kg/m^2; 5M/5F; Age 21-43 years) took part in this study. The metabolic data for some of these participants were included in a previous publication (Day et al., 2016). Demographics, anthropometric measurements, and screening blood tests were obtained on all participants. Body impedance analysis (BIA) was used to assess percent body fat. A 75-g oral glucose tolerance test following a 10-12 hour overnight fast was used to assess normal glucose tolerance. No subject was taking any medication known to affect glucose metabolism. Written consent was obtained from all study participants. The study was approved by the Institutional Review Boards at Mayo Clinic in Arizona and Arizona State University.

Study Design

Fasted participants reported to the Clinical Studies Infusion Unit at the Mayo Clinic in Arizona. Blood was collected into PAXgene Blood DNA and RNA tubes (BD Diagnostics, Franklin Lakes, NJ) and stored at -80°C until processed. Following blood collection, a two hour euglycemic-hyperinsulinemic clamp (80 $mU.m^{-2}.min^{-1}$) to measure insulin sensitivity was performed as previously described (Day et al., 2016).

Substrate and Hormone Determinations

Fasted blood samples for comprehensive metabolic, lipid, and hemogram panels were performed by the Biospecimens Accessioning and Processing (BAP) Core at Mayo Clinic in Scottsdale. Plasma glucose concentration was determined by the glucose oxidase method on an YSI 2300 STAT plus (YSI INC., Yellow Springs, OH, USA). Plasma insulin was measured by a two-site immunoenzymatic assay performed on the DxI 800 automated immunoassay system (Beckman Instruments, Chaska, MN, USA).

Whole Blood Processing for DNA Isolation

Genomic DNA was isolated using the PAXgene Blood DNA Kit, as per the manufacturer's instructions (Qiagen, Valencia, CA). DNA quantity and quality was assessed using agarose gel electrophoresis and spectrophotometer A260/A280 values were determined using the NanoVue (GE Healthcare, United Kingdom).

Reduced Representation Bisulfite Sequencing (RRBS)

RRBS was performed on whole blood genomic DNA at the Mayo Clinic Genotyping Shared Resource facility, and library preparation was performed as previously described (Day et al., 2016). Sequencing data was analyzed using a streamlined analysis and annotation pipeline for reduced representation bisulfite sequencing, SAAP-RRBS (Day et

al., 2016; Sun et al., 2012). The methylation dataset supporting the conclusions of this article are available in the Gene Expression Omnibus repository, GSE85928 (http://www.ncbi.nlm.nih.gov/geo/). Furthermore, bigwig files were used to create a custom track on the UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgTracks?hgS_doOtherUser=submit&hgS_otherUserName=rlcolett&hgS_otherUserSessionName=blood%20Methylatio).

Whole Blood differentially methylated cytosines (DMC) analysis

To determine differences in methylation sites between groups, the aligned (Hg19) data was imported into the free open source R package, MethylSig (Park et al., 2014). A minimum of five reads and the recovery of the site in all ten participants from each group were required for the inclusion of a cytosine in subsequent analyses. The mean methylation values were adjusted by a beta binomial approach to account for biological variation among the groups being compared (Park et al., 2014). A comparison of the DNA methylation between groups with and without obesity at each site was based on a likelihood ratio test (nominal P value), and a Benjamini-Hochberg multiple testing correction was applied. Regional annotations for each DMC were imported from the University of California, Santa Cruz (UCSC) Genome Browser's RefSeq Genes and CpG Island tracks. Priority was given to annotating the site as a promoter or untranslated region if available in another transcript of the gene or in a different gene.

Whole blood differentially methylated region (DMR) analysis

Differences in methylated regions between groups were identified using the open source R package, dispersion shrinkage for sequencing data (DSS) (Wu et al., 2015). The

analysis included the BSmooth algorithm, which determined the level of methylation in a region for each sample and accounted for biological variation. The criteria for inclusion was: each region contained three CpGs supported with a read coverage of 5X, the recovery of the site in all ten participants from each group, and significance of $P<0.05$ (uncorrected) from the DMC analysis. DMRs were created by a sliding-window of 500 bp and a t-statistic cutoff of 2.5. The significance of a DMR was determined by the weight of the Area Stat, which is the sum of t-statistic values in each DMR. Regional annotations for the DMRs were imported from the UCSC Genome Browser's RefSeq Genes and CpG Island tracks. Priority was given to annotating the region as a promoter or untranslated region if available in another transcript of the gene or in a different gene.

Blood-based biomarkers of skeletal muscle DMC analysis

Skeletal muscle RRBS data from 11 lean (7F/4M; Age 21-43 years) and 9 obese (BMI > 30 kg/m^2; 4F/5M; Age 32-52 years) in our previous study (Day et al., 2016) was used for comparative analysis. Both the whole blood and skeletal muscle were analyzed using the program, MethylSig (Park et al., 2014). There were 9 lean and 6 obese that were the same as the individuals included in the whole-blood analyses. Promoter and untranslated region DMCs from both whole blood and skeletal muscle were merged based on matching chromosomal positions. The merged DMCs were then filtered for analogous direction and level of methylation in each tissue by grouping.

***SLC19A1* predictive transcription factor binding analysis**

Prediction of transcription factor binding was performed using the program PROMO version 3.0.2 (Messeguer et al., 2002). Analyses were performed with a 5 % maximum matrix dissimilarity rate using TRANSFAC version 8.3 database. The sequence from

Chr.21:46957905-46957991 was used to assess binding at the two *SLC19A1* DMCs. Furthermore, transcription factor binding was assessed for the *SLC19A1* DMR using the sequence from Chr.21:46957905-46958011.

Pyrosequencing

Confirmation of DNA methylation detected in both whole blood and skeletal muscle was performed using pyrosequencing, as previously described (Day et al., 2016). To assess the *SLC19A1* DMC (Chr.21:46,957,915), bisulfite-converted DNA was amplified by PCR using the following primers: forward 5'-GTTGGGTTGGAGGGTATTAT-3' and biotinylated reverse 5'-CCATCTTCCAAAATACCCTAACT-3'. Pyrosequencing was performed using the PyroMark Q96 MD system and the Gold Q96 kit with sequencing primers 5'-GGTTGGAGGGTATTATT-3' according to the manufacturer's instructions (Qiagen, Valencia, CA). Sequence analysis was performed using the PyroMark CpG SW 1.0 software (Qiagen, Valencia, CA).

Statistical analysis

Independent sample t-tests and chi-square were used to compare physical and metabolic characteristics between lean and obese groups. Non-normally distributed data were $\log_{10}$ or square root transformed. However, untransformed data are presented as a mean ± standard error of the mean (SEM) for ease of interpretation. Multiple regression analyses were performed with the purpose of adjustments for age, sex and/or BMI to estimate bivariate relationships between insulin sensitivity (i.e. M value) and significantly altered methylation. Pearson correlation analysis was performed to determine the relationship between whole blood and skeletal muscle methylation data. The SPSS 23.0

statistical software package was used. See above for the statistical analysis of the methylation data.

Results

Participants

Table 4-1 shows the phenotypic characteristics for participants with (n=10; BMI > 30 kg/m^2) and without (n=10) obesity. By design, the lean participants had a significantly lower body mass index (BMI). In addition, the lean participants had significantly lower measures of body fat percentage and waist circumference compared to the obese. As expected, the obese participants had higher fasting plasma insulin levels and lower M values compared to the lean.

Table 4-1. Characteristics of study participants (n=20) classified by body mass index.

Characteristics	Lean	Obese	Pvalue	Pvalue (age & sex)
Sex	5M/5F	5M/5F	1.0*	-
Age (years)	29.9 ± 2.2	35.9 ± 3.2	0.14	-
Body mass index (kg/m²)	23.6 ± 0.7	34.4 ± 1.3	<0.001	<0.001
Body fat (%)ǂ	25.0 ± 1.6	36.8 ± 2.2	<0.001	<0.001
Waist circumference (cm)	83.8 ± 2.9	103.6 ± 3.4	<0.001	0.0032
Systolic blood pressure (mmHg)	117.7 ± 2.2	119.4 ± 2.5	0.62	0.57
Diastolic blood pressure (mmHg)	72.0 ± 1.5	75.0 ± 2.0	0.24	0.52
Triglycerides (mg/dL)	101.1 ± 15.4	105.2 ± 15.0	0.85	0.77
Cholesterol (mg/dL)	174.3 ± 10.4	182.9 ± 10.7	0.57	0.61
High density lipoproteins (mg/dL)	52.5 ± 4.4	48.3 ± 3.3	0.45	0.45
Low density lipoproteins (mg/dL)	101.7 ± 8.7	113.5 ± 9.0	0.36	0.95
Hemoglobin A1c (%)	5.2 ± 0.04	5.3 ± 0.1	0.24	0.47
Fasting plasma glucose (mg/dL)	87.5 ± 2.0	90.2 ± 1.7	0.31	0.34
Fasting plasma insulin (µU/mL)	6.5 ± 1.3	13.4 ± 2.0	0.64	0.0054
M value (mg/kg.min)	7.7 ± 0.5	3.8 ± 0.5	<0.001	<0.001
M value (mg/kg.min·FFM)	10.2 ± 0.7	6.0 ± 0.8	<0.001	<0.001

Data presented as mean ± SEM, based on independent sample t-tests. Adjusted for age and sex by ANCOVA.*Calculated by Chi-Square Test. ǂBody fat determined by biometric impedance analysis (BIA). Fat-free mass (FFM).

Genome-wide methylation analysis in human whole blood

Using the next generation technique RRBS, there were 5,227,488 methylation sites captured in the blood methylation analysis from the lean and obese participants. The distribution of methylation sites were categorized by genic regions (Figure 4-1a) and CpG island features (Figure 4-1b). A large proportion of these sites fell within regulatory regions, with 22% in the promoter and 18% in the 3' and 5' untranslated regions (Figure 4-1a). When applying the proportion of CpG island features located within each genic region, we found CpG islands to be most concentrated in the promoter and 5'untranslated region (Figure 4-1b).

Whole blood differentially methylated cytosines (DMCs)

To identify potential blood biomarkers for obese insulin resistant states, sites within all genomic regions were considered for analysis. Of the 5,227,488 methylation sites captured, 52,995 sites were significantly altered (uncorrected $P<0.05$; Appendix D) between our groupings. The differentially methylated cytosines (DMC) were corrected by a false discovery rate (FDR; $q<0.05$), which identified 49 unique methylation sites (15 decreased; 34 increased; Appendix E).

Insulin sensitivity regression analysis of DMCs

We identified 49 DMCs that were altered with obesity. There is a strong association between obesity and insulin resistance (Shoelson et al., 2007). In this study, we observed that the M value, as measured by the euglycemic hyperinsulinemic clamp, and BMI measurements were significantly correlated ($r = -0.778$; $P = 0.00004$). Therefore, we further aimed to identify which DMCs were significantly associated with insulin sensitivity (i.e. M value). In multiple regression analyses with age, sex and M value as

the independent variables and methylation ratio of DMCs as the dependent variables, we found that M value independently explained a range of 25-54% of variance in 36 of the 49 DMCs (all P<0.05; Appendix F).

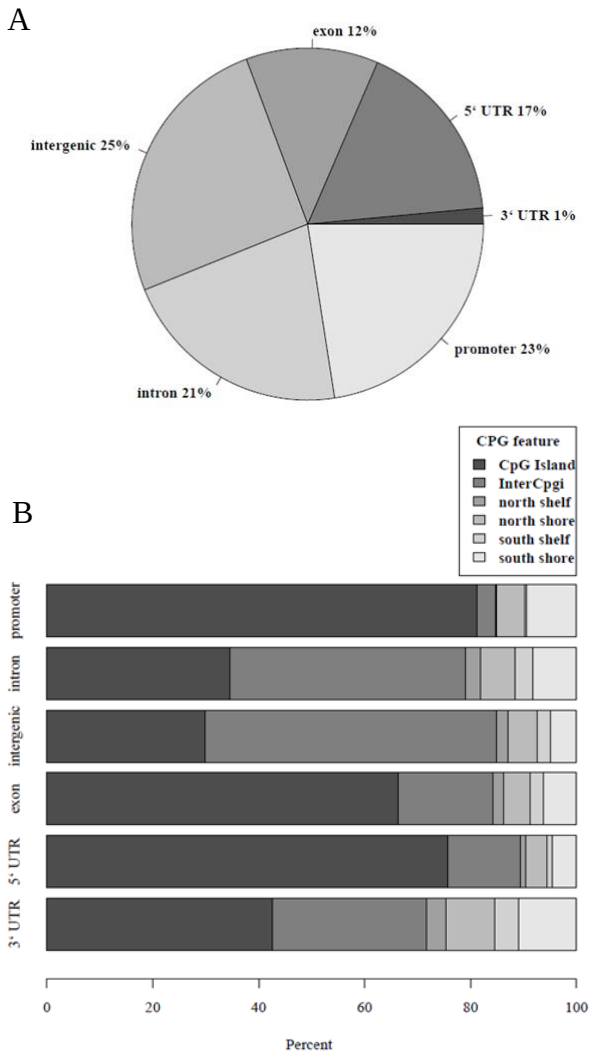

Figure 4-1. All methylation sites detected in our whole blood samples using reduced representation bisulfite sequencing technology were mapped (A) in the context of both gene regions and (B) CpG island features. Regions were defined using UCSC browser RefGene and CpG island tracks (see methods). The promoter region was defined as 1000 bp (basepairs) upstream of the transcription start site (TSS); untranslated region (UTR); CpG island is a 200-3000 bp stretch of DNA with a C+G content of 50% and observed CpG/expected CpG exceeding 0.6; North (N) and South (S) shores flank the CpG island by 0-2000 bp; the North (N) and South (S) shelf flank the shores by 2000 bp (2000-4000 bp from the island).

Whole blood differentially methylated regions (DMRs)

DNA methylation regulation can be mediated by a single CpG or by a group of CpGs in close proximity to each other. Therefore, a regional analysis was performed on the 52,995 blood DMCs that were significantly altered (uncorrected $P<0.05$). This analysis identified 74 DMRs (Appendix G). When the 74 blood DMRs were compared with the 49 blood DMCs ($q<0.05$), two genes (solute carrier family 19 member 1 (*SLC19A1*) and ephrin-A2 (*EFNA2*)) were in common between the analyses. The DMR (Chr.21:46,957,915-46,958,001) for *SLC19A1* was decreased in methylation by -34.9% (70.4% methylation in lean vs 35.5% methylation in obese) and the DMR for *EFNA2* (Chr.19:1,287,750-1,287,781) was increased by +14.3% (28.4% methylation in lean vs 42.7% methylation in obese) with obesity.

Potential blood-based biomarkers of skeletal muscle

Skeletal muscle is the major site for insulin-stimulated glucose disposal, making it an important target tissue for understanding insulin resistance (Abdul-Ghani & DeFronzo, 2010). However, accessibility of this tissue is more difficult compared with blood. Therefore, we set out to identify blood-based biomarkers of methylation sites in genes that were also identified in skeletal muscle using our previously published data (Day et al., 2016). The skeletal muscle results are based on 11 lean and 9 obese (BMI > 30 kg/m^2; Appendix H). Of these individuals, 9 lean and 6 obese were in common to the individuals included in the whole-blood analyses. When we compared the significantly changing whole blood DMCs (FDR; $q<0.05$) with our previously published skeletal muscle data (Day et al., 2016) we identified three sites that were in common. One site (Chr.21:46,927,138) was in collagen, type XVIII, alpha 1 (*COL18A1*) and the other two

sites (Chr.21:46,957,915 and Chr.21:46,957,981) were in the 5'UTR of *SLC19A1* (Figure 4-2).

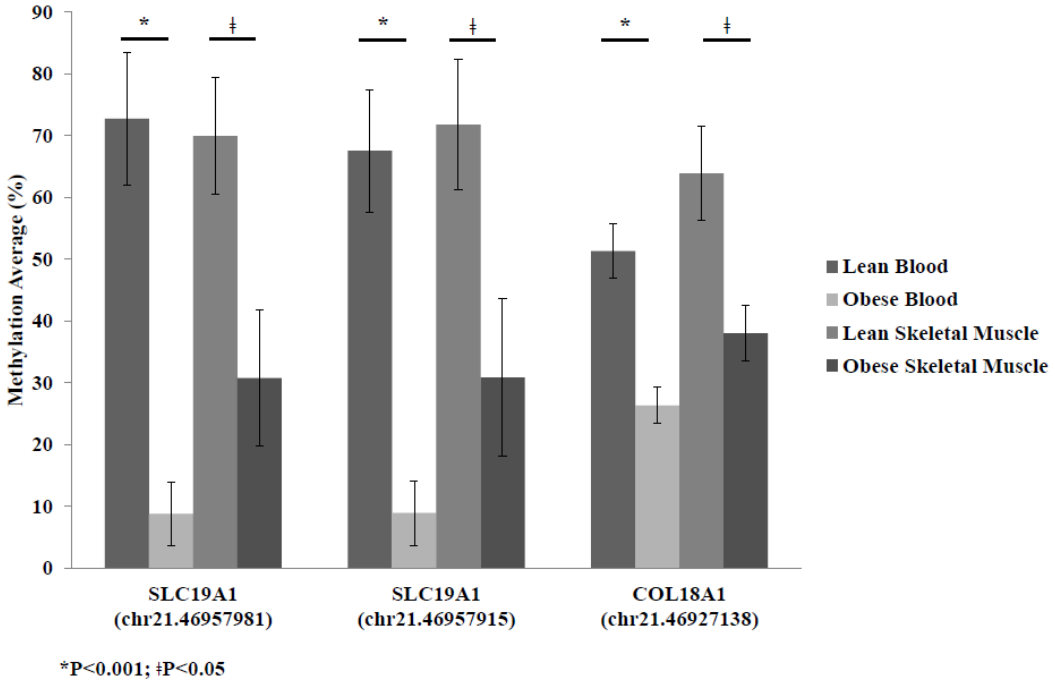

Figure 4-2. Average methylation detected by reduced representation bisulfite sequencing (RRBS) for *SLC19A1* sites Chr.21:46,957,981 and Chr.21:46,957,915 and *COL18A1* site Chr.21:46,927,138 for lean and obese in both blood and skeletal muscle. Significance based on independent sample t-tests.

SLC19A1 correlation analysis

The two significant (q<0.05) *SLC19A1* methylation sites, Chr.21:46,957,981 and Chr.21:46,957,915, are located downstream of two transcription start sites (TSSs) Chr.21:46,964,325 and Chr.21:46,962,385, based on the UCSC genome browser. The distances from the TSS are 6410bp and 4470bp for Chr.21:46957915, and 6344bp and 4404bp for Chr.21:46957981, respectively. These methylation sites were found in both whole blood and skeletal muscle methylation. In order to determine the relationship of methylation at those sites between tissues, Pearson correlation analysis was performed.

We found the methylation between the blood and skeletal muscle were significantly and positively correlated at both *SLC19A1* sites (Figure 4-3).

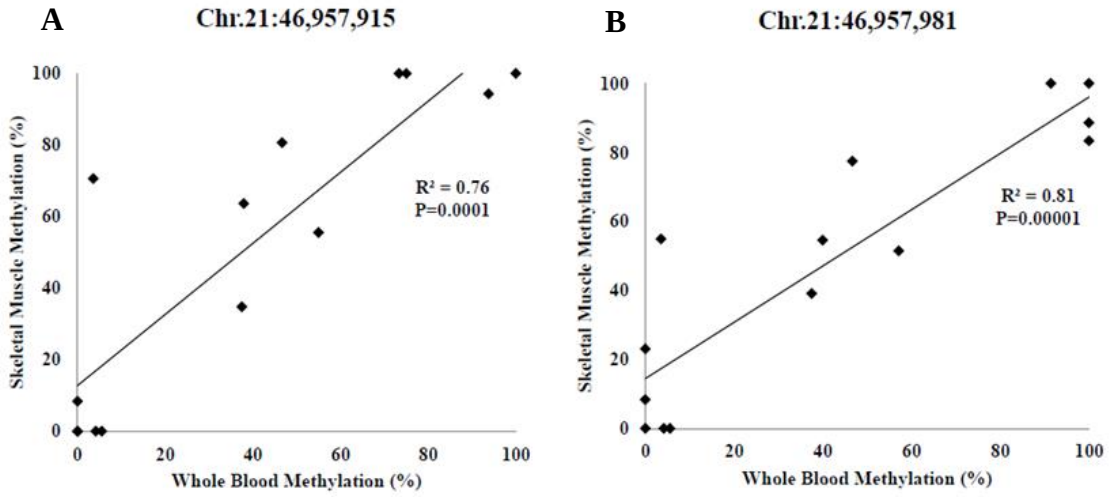

Figure 4-3. Pearson correlation analysis of the participants (8 lean; 6 obese) present in both whole blood and skeletal muscle methylation for *SLC19A1* (A) Chr.21:46,957,915 and (B) Chr.21:46,957,981.

SLC19A1 predicted transcription factor binding

We analyzed the sequences containing DMCs and the DMR associated with *SLC19A1* using the program PROMO (Messeguer et al., 2002). Transcription factor binding motifs were not identified to overlap our most significant DMCs, Chr.21:46,957,981 and Chr.21:46,957,915. However, using the DMR sequence (containing 4 CpGs: Chr.21:46957915, Chr.21:46957981, Chr.21:46957988, and Chr.21:46958001), we found two predicted transcription factors, forkhead box P3 (FOXP3) and glucocorticoid receptor (GR), to overlap a CpG site at position Chr.21:46957988.

SLC19A1 validation

The *SLC19A1* DMCs were demonstrated to be significantly altered in both whole blood and skeletal muscle. This may indicate that *SLC19A1* is tightly associated with

obesity. We confirmed the methylation changes between the lean and obese groups at Chr.21:46,957,915 using pyrosequencing. DNA methylation was significantly decreased in the obese participants compared to the lean in both whole blood and skeletal muscle (Figure 4-4). The changes observed in both tissues were comparable to the decreased methylation detected using RRBS.

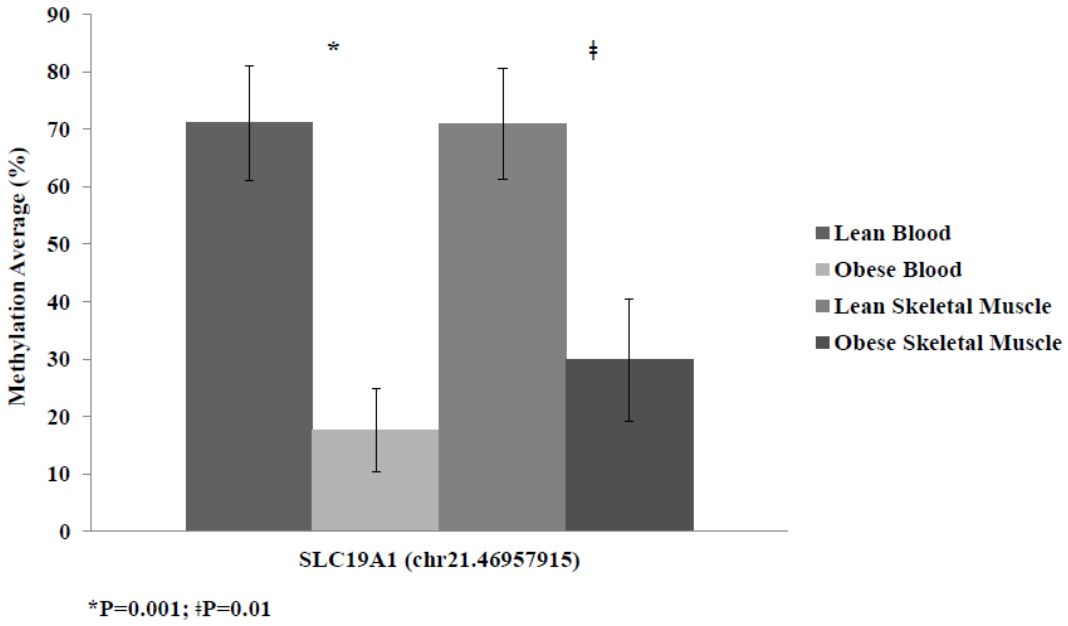

Figure 4-4. Average methylation of SLC19A1 site Chr.21: 46,957,915 detected by pyrosequencing validation for lean and obese samples in both blood and skeletal muscle. Significance based on independent sample t-tests.

Discussion

The present study was undertaken with the purpose of identifying whole blood biomarkers of DNA methylation that were altered in obesity and insulin resistance. Our genome-wide RRBS analysis demonstrated that the promoter region was most concentrated with CpG islands, which is well established in the field (Deaton & Bird, 2011). Interestingly, the distribution pattern of all detected CpG sites were similar to the patterns observed in our previous study in skeletal muscle (Day et al., 2016). The

consistent coverage of the genome regardless of tissue type presents RRBS as a viable technique for cross-tissue analysis.

Other recent studies have provided useful findings of DNA methylation differences in blood from obese compared to lean individuals (Ronn et al., 2015; X. Wang et al., 2010). Wang *et al.* identified methylation changes with obesity associated with *UBASH3A* and *TRIM3* from blood leukocytes in participants ranging 14-30 years of age (X. Wang et al., 2010). A study by Ronn *et al.* identified changes in methylation impacted by age, BMI and HbA1c levels in blood and adipose tissue using multiple cohorts whose ages collectively spanned 23-83 years (Ronn et al., 2015). We did not observe an overlap between our 49 corrected DMCs and the most significant sites identified from the aforementioned studies. The lack of overlap may be attributed to blood tissue type, age of cohorts and methylation detection technology used.

We determined that the most reliable biomarker of obesity and its underlying insulin resistance would be identified by using a number of analyses. Firstly, *SLC19A1* was identified as significantly decreased in obesity in both the whole blood DMC and DMR analyses. Furthermore, we set out to identify similarities between whole blood and the insulin responsive tissue, skeletal muscle. By using the skeletal muscle methylation changes assessed in our previous study (Day et al., 2016), we found *SLC19A1* to be a blood-based DNA methylation biomarker for that tissue. Not only were these DMCs decreased in methylation in both blood and skeletal muscle, but the level of change was to a similar extent. The confirmation of this finding through pyrosequencing leads us to believe that the altered *SLC19A1* methylation is strongly associated with obesity and its underlying insulin resistance, regardless of tissue. Others have also identified methylation

marks that are similar across different tissue types in association with a trait (Gillberg & Ling, 2015; Mikeska & Craig, 2014). Moreover, regression analyses of the *SLC19A1* sites demonstrated a significant relationship with insulin sensitivity. The utility of the euglycemic hyperinsulinemic clamp is considered a gold standard for measuring insulin sensitivity (DeFronzo et al., 1979). This was a key measurement to correlate with DNA methylation in order to identify epigenetic biomarkers for obese insulin resistance in whole blood.

*SLC19A1*codes for the protein reduced folate carrier (RFC), which contributes to methionine and *de novo* purine synthesis (Desmoulin, Hou, Gangjee, & Matherly, 2012). Folate is a methyl donor, and is suggested to have an important role in fetal programming by providing a substrate for DNA methylation (Yajnik & Deshmukh, 2008). Imbalances in folate levels, specifically high levels, have been predictive of adiposity and insulin resistance (Yajnik & Deshmukh, 2008). This observation has provided evidence for potential epigenetic influences on the risk of disease development. Rupasree *et al*. (Rupasree, Naushad, Rajasekhar, & Kutala, 2014), conducted a study in systemic lupus erythematosus (SLE) cases and identified differential methylation in blood lymphocytes of genes involved in one-carbon metabolism. They found a decrease in *SLC19A1* promoter methylation in SLE cases that were positive for anti-ribonucleoprotein (RNP) antibodies. The detection of anti-RNP in SLE patients is used for further classification of connective tissue disease such as Raynaud's phenomenon (Migliorini, Baldini, Rocchi, & Bombardieri, 2005). The chronic inflammation in SLE may contribute to the similarities in decreased *SLC19A1* methylation found in both obese, insulin resistant states from our study and the anti-RNP positive SLE cases (Coletta & Mandarino, 2011; Podolska,

Biermann, Maueroder, Hahn, & Herrmann, 2015). Increased levels of TNFα and IL-6 have been associated with both obesity and SLE (Agha-Hosseini, Moosavi, & Hajifaraj Tabrizi, 2015; Tateya et al., 2013). However, these measurements were not taken in this study, so inflammation cannot be confirmed in our participants. He *et al*. (He et al., 2016), found a significant decrease in methylation in the promoter of *SLC19A1* in the placenta of intrauterine growth restricted (IUGR) samples. This study speculated that the change in methylation may play a role in *in utero* development. IUGR has been associated with increased risk for type 2 diabetes, metabolic syndrome, cardiovascular and heart disease (Chernausek, 2012). It is interesting to speculate that the decreased *SLC19A1* methylation with obesity from our study may stem from a developmental origin. Although our study identified different chromosomal positions from the above mentioned studies, we believe the methylation status of both Chr.21:46,957,915 and Chr.21:46,957,981 provide new potential epigenetic biomarkers for better understanding obesity related insulin resistance.

Our study focused on the identification of novel epigenetic biomarkers. Based on previous transcriptomic findings from our lab, we hypothesized that we would identify altered methylation of genes involved in inflammation, insulin signaling, and mitochondrial function (Kim et al., 2015; Tangen et al., 2013). We identified three genes, integrin alpha E (*ITGAE*), RNA binding motif protein 5 (*RBM5*), and *SLC19A1*, that were in common with our previous findings (Tangen et al., 2013). We expected to find more genes that were in common between the transcriptomic and epigenomic datasets. The lack of concordance across the datasets could be explained by differences in ethnicity and lower number of subjects study (Cossrow & Falkner, 2004). However, the occurrence of

SLC19A1 in the transcriptomic dataset (Tangen et al., 2013) with the present study solidifies its connection to obesity.

In this study we have focused primarily on *SLC19A1*, however there are other genes from the list of 49 DMCs ($q<0.05$) that could potentially be relevant to obesity. One such gene is *EFNA2*, which codes for the glycosylphosphatidylinositol (GPI)-linked ephrin-A ligand. *EFNA2* interacts with Eph receptor tyrosine kinases thereby affecting the activities of actin cytoskeleton, cell motility, proliferation, and secretion (Pasquale, 2008). We (Coletta & Mandarino, 2011; Hwang et al., 2010) have previously found a reduction in actin cytoskeleton proteins with insulin resistance. Another gene was *COL18A1*, which was found to have similar methylation changes in both whole blood and skeletal muscle. *COL18A1* codes for a multiplexin localized at the basal lamina (Halfter, Dong, Schurer, & Cole, 1998). We (Berria et al., 2006; Coletta & Mandarino, 2011; Richardson et al., 2005) and others (Kang et al., 2011) have shown increased collagen content in insulin resistant skeletal muscle. Taken together, the changes in methylation for *EFNA2* and *COL18A1* suggest that these genes may be relevant epigenetic biomarkers of blood in obesity.

Although we have described novel epigenetic biomarkers of blood with obesity and its underlying insulin resistance, we acknowledge the shortcomings of our study. Whole blood has a heterogeneous cell composition, and potential differences in inflammation between our groups could confound the DNA methylation results (Houseman, Kim, Kelsey, & Wiencke, 2015). Furthermore, we identified new potential biomarkers for obesity related insulin resistance, but within a limited sample size. Future studies could fractionate blood cell types to avoid confounding composition effects, and will need to

replicate our findings in larger cohorts in order to be considered candidate biomarkers. Our study was novel in that we identified epigenetic changes in whole blood using RRBS. Specifically, we identified *SLC19A1* from participants with obesity as a potential epigenetic biomarker that is significantly predicted by insulin sensitivity (i.e. M value). Moreover, the blood methylation for *SLC19A1* was positively correlated with skeletal muscle methylation. Our transcription factor binding analysis found potential binding within the *SLC19A1* DMR, but not at the most significant DMC sites. However, we speculate that methylation at those sites may have a regulatory affect through the recruitment of methylcytosine-binding proteins (Attwood et al., 2002). These proteins can associate with protein complexes that contain corepressors and histone deacetylases, and could influence the chromatin structure (Attwood et al., 2002). Our findings demonstrate the DNA methylation status associated with *SLC19A1* as a promising biomarker for obesity and its underlying insulin resistance, as it is present in both skeletal muscle and blood.

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CHAPTER 5: CONCLUSION

In this dissertation, we set out to decipher the epigenetic basis of obesity and its underlying insulin resistance. Using next generation sequencing and transcriptomic approaches, we were able to test our specific hypotheses, as well as generate novel findings in obesity. In addition, we were able to further investigate our novel obesity epigenetic finding in a cohort that underwent weight-loss induced by RYGB surgery. Lastly, we were able to evaluate our findings in human skeletal muscle from obese participants with whole blood to identify if this tissue could be a surrogate for muscle.

The aim of our first study was to investigate the role of skeletal muscle DNA methylation in combination with transcriptomic changes with obesity and insulin resistance. We identified an increased methylation mainly concentrated in promoter and 5'UTR of *SORBS3* variant 2, and decreased gene expression with obese, insulin resistant individuals. To date, the only other studies to have identified differential methylation for *SORBS3* have focused on Alzheimer's disease (Sanchez-Mut et al., 2013; Siegmund et al., 2007). In human brain tissue with Alzheimer's disease, there was increased *SORBS3* methylation coordinated with decreased expression (Sanchez-Mut et al., 2013). Beyond epigenetic studies, *SORBS3* gene expression has been demonstrated to be diminished with liver cancer (Roessler et al., 2012). Coincidentally, among all disease states, *SORBS3* expression has been reduced. As described in Chapter 2, there were multiple characteristics that were significantly different between our lean and obese groupings. These characteristics included BMI, body fat, waist circumference, fasting plasma insulin, *M* value, and age. Although we provided statistical evidence that age was not

driving the significant changes in methylation for *SORBS3*, we decided to further evaluate our *SORBS3* methylation findings in a RYGB surgery cohort.

In order to examine the influential characteristics on DNA methylation and gene expression changes in *SORBS3*, we investigated the skeletal muscle from morbidly obese (BMI >40 kg/m^2) women before and 3 months after RYGB surgery. Post-surgery, we identified decreased methylation in promoter and 5'UTR, and an increase in the expression of *SORBS3*. Again, the methylation was concentrated in the *SORBS3* variant 2 as observed in our previous study (Day et al., 2016), described in Chapter 2. In Chapter 3, we had further demonstrated a negative relationship between DNA methylation in the human promoter of *SORBS3* variant 2 with decreased gene expression measured by luciferase activity. Furthermore, we identified obesity measures and insulin levels as environmental factors potentially influencing epigenetic and transcript changes post-surgery. Altogether, the findings from Chapter 2 and 3 provide evidence for obesity and insulin resistant associated transcriptional regulation of *SORBS3* through increased DNA methylation, which can be restored to normal levels through weight-loss induced by RYGB surgery. Identifying alterations in skeletal muscle, such as this novel epigenetic change associated with SORBS3 is important, as muscle is a primary tissue for studying obesity induced insulin resistance. However, this tissue is not easily accessible. Therefore, set out to identify if whole blood presented similar changes in DNA methylation as skeletal muscle.

Whole blood is an easily accessible tissue for studying a disease state. In Chapter 4, we assessed if whole blood could be used as a surrogate for our epigenetic finding in skeletal muscle. We observed no methylation change with *SORBS3* in whole blood,

which suggests that *SORBS3* is a marker for obesity in skeletal muscle. This discordance between epigenetic findings in whole blood and skeletal muscle were not completely unexpected, based on transcriptomic results from our previous whole blood study (Tangen et al., 2013). Genes from pathways such as oxidative phosphorylation and MAPK signaling were shown to have reversed expression outcomes in whole blood than seen in skeletal muscle (Tangen et al., 2013). In another study, blood and brain gene expression were not observed to be highly correlated overall, but some correlations were identified with genes involved in basic processes, such as infection mechanisms and post-translational modification (Cai et al., 2010). Therefore, blood may be useful for identifying biomarkers, but not as a complete surrogate for tissue-specific processes. Our study identified a potential epigenetic biomarker of blood in our obese, insulin resistant participants. We found significantly decreased methylation in the 5'UTR of *SLC19A1* in whole blood. Interestingly, *SLC19A1* was also identified to be decreased in methylation in skeletal muscle of obese, insulin resistant participants. *SLC19A1* codes for the protein reduced folate carrier (RFC), which can contribute to methionine synthesis and ultimately S-adenosylmethionine (SAM) required for DNA methylation (Desmoulin et al., 2012). Upon initial speculation, a potential increased availability of SAM in obese, insulin resistant participants could have contributed to the increased methylation found in skeletal muscle for *SORBS3*. However, gene expression measurements of *SLC19A1* in both blood (FC: -3.0, P=0.06) and skeletal muscle (FC: -1.4, P=0.08) were not significantly altered, and trended towards decreased expression with obesity and insulin resistance (unpublished data). Therefore, the *SLC19A1* methylation identified does not seem to contribute to transcriptional regulation and may not alter biological function.

Findings from the Human Epigenome Project had shown that differential 5'UTR methylation may not always lead to inhibition of gene expression, where one-third of their 5'UTR methylation sites were inversely correlated with transcription (Eckhardt et al., 2006). Differential DNA methylation sites alone are useful biomarkers, as they are stable marks that can be influenced by environmental factors (Mikeska & Craig, 2014). Therefore, the reduction in methylation for *SLC19A1* seems to be tightly associated with obesity and insulin sensitivity measures and can be used as a potential biomarker. Further studies are warranted to assess these methylation sites in a larger cohort.

In this dissertation, we highlight the collective data for *SORBS3,* as it may play a role in our proposed model for obesity and its underlying insulin resistance in skeletal muscle. In previous work, we had identified increased collagen (extracellular matrix) (Berria et al., 2006; Richardson et al., 2005) and decreased cytoskeletal proteins (Hwang et al., 2010) in response to obese, insulin resistant states. We have proposed that changes in the extracellular matrix can affect the cytoskeletal sensing of contractile activity, which alters the mechanosignaling for gene expression changes in mitochondrial biogenesis (Coletta & Mandarino, 2011). This can potentially lead to a reduction and abnormal function of mitochondria, and ultimately lead to the cellular abnormalities (lipid accumulation, reduced fat oxidation and insulin signaling) related to insulin resistance (Coletta & Mandarino, 2011). We had set out to determine if these changes were a result of epigenetic regulation by differential DNA methylation.

As described in Chapter 2 and 3, we identified alterations in promoter and 5'UTR methylation concentrated near *SORBS3* variant 2. *SORBS3* codes for two isoforms of the adapter protein vinexin α and β, by use of two distinct alternative promoters (Kioka et al.,

1999). Vinexin α contains a sorbin homology (SoHo) domain at the N-terminus and three SRC homology 3 (SH3) domains at the C-terminus, whereas vinexin β is a shorter isoform only containing the same C-terminal end (Kioka et al., 1999). Our data indicates that obese, insulin resistant states may be influencing the epigenetic down-regulation of variant 2, coding for vinexin β. An important binding partner for vinexin β is vinculin through the first two SH3 domains, localized at actin-binding cytoskeletal protein cell-extracellular matrix (ECM) and cell-cell adhesion sites (Kioka et al., 1999). The cooperative function between vinculin and vinexin β enhances cell spreading (Kioka et al., 1999). Moreover, cell spreading can be regulated by the interaction of vinexin β with extracellular signal-regulated kinase 1/2 (ERK1/2) (Mitsushima, Suwa, Amachi, Ueda, & Kioka, 2004; Mizutani et al., 2007). Upon growth-factor stimulation, ERK1/2 can phosphorylate vinexin β and attenuate cell spreading and migration (Mitsushima et al., 2004; Mizutani et al., 2007). It is tempting to speculate that a reduction in vinexin β abundance may play a role in the altered cytoskeletal organization for mechanosignal transduction proposed with insulin resistance. However, we currently do not have evidence of vinexin β protein changes in skeletal muscle from our cohorts. Initial attempts of western blotting for vinexin β are inconclusive (unpublished data, Appendix I), and require further investigation.

Our work is summarized in Figure 5-1, where epigenetic changes associated with *SORBS3* can be identified in skeletal muscle of individuals with obesity and insulin resistance, and after weight-loss induced by surgery. This may correspond to protein changes in vinexin β, and provide an additional piece to our working model of obesity and its underlying insulin resistance.

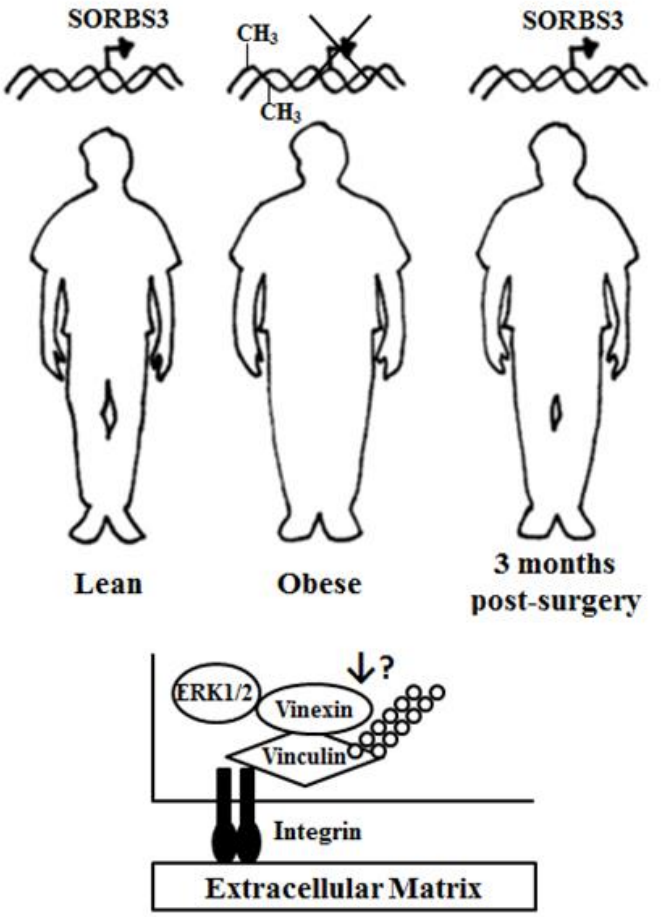

Figure 5-1. Working model of *SORBS3*. Increased DNA methylation in the promoter and 5'untranslated region was observed in obese, insulin resistant participants. This increased methylation corresponded to a decrease in *SORBS3* gene expression. Weight-loss induced 3 months after surgery had shown decreased *SORBS3* methylation and increased gene expression. The future direction for this model is to identify whether there are protein abundance changes in the *SORBS3* gene coding for vinexin. If protein changes follow our working model, vinexin would be decreased (indicated by ↓) in abundance with obesity and insulin resistance. However, the outcome is currently unknown (indicated by ?), and requires further investigation.

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APPENDIX A

THE 13,130 DIFFERENTIALLY METHYLATED CYTOSINES (DMCS; $P<0.05$) THAT WERE LOCATED WITHIN A PROMOTER OR UNTRANSLATED REGIONS (3' AND 5')

[CONSULT ATTACHED FILES]

APPENDIX B

THE PROBES (N=99) REMAINING FROM THE MICROARRAY DATA THAT MET THE FALSE DISCOVERY RATE (FDR) CORRECTION CRITERIA OF P<0.05

[CONSULT ATTACHED FILES]

APPENDIX C

THE 170 DIFFERENTIALLY METHYLATED REGIONS (DMRS; P<0.05) THAT WERE LOCATED WITHIN A PROMOTER OR UNTRANSLATED REGIONS (3' AND 5')

[CONSULT ATTACHED FILES]

APPENDIX D

THE 52,995 DIFFERENTIALLY METHYLATED CYTOSINES (DMCS; $P<0.05$)

[CONSULT ATTACHED FILES]

APPENDIX E

WHOLE BLOOD DIFFERENTIALLY METHYLATED CYTOSINES (DMCS; Q<0.05) BETWEEN LEAN AND OBESE GROUPINGS

		DNA Methylation (%)					
Chr. Position	Gene	Lean	Obese	P value	q value	Genic Region	CpG Island Region
chr21.46957981	*SLC19A1*	0.73 ± 0.11	0.09 ± 0.05	<0.01	0.01	5'UTR	South Shore
chr21.46957915	*SLC19A1*	0.68 ± 0.10	0.09 ± 0.05	<0.01	0.01	5'UTR	CpG Island
chr6.3771940	-	0.76 ± 0.03	0.38 ± 0.07	<0.01	0.05	Intergenic	InterCpG
chr5.80690459	*ACOT12*	0.43 ± 0.04	0.15 ± 0.02	<0.01	<0.001	Promoter	South Shore
chr11.2883716	-	0.52 ± 0.06	0.22 ± 0.04	<0.01	0.05	Promoter	CpG Island
chr21.46927138	*COL18A1*	0.51 ± 0.04	0.26 ± 0.03	<0.01	0.04	Intron	North Shelf
chr8.145702503	*FOXH1*	0.93 ± 0.02	0.71 ± 0.02	<0.01	<0.001	Promoter	South Shore
chr2.121283801	-	0.93 ± 0.02	0.79 ± 0.03	<0.01	0.05	Intergenic	South Shelf
chr19.46456403	*NOVA2*	0.12 ± 0.03	0.02 ± 0.01	<0.01	0.02	Intron	CpG Island
chr6.146348971	*GRM1*	0.16 ± 0.02	0.06 ± 0.01	<0.01	0.04	Promoter	North Shore
chr4.2648590	*FAM193A*	0.99 ± 0.01	0.90 ± 0.02	<0.01	0.04	Intron	InterCpG
chr1.214504377	*SMYD2*	0.92 ± 0.01	0.85 ± 0.01	<0.01	0.02	Exon	InterCpG
chr20.32856825	*ASIP*	0.06 ± 0.02	0.0 ± 0.0	<0.01	0.05	Exon	CpG Island
chr9.139085246	-	0.03 ± 0.01	0.0 ± 0.0	<0.01	<0.001	Intergenic	CpG Island
chr19.55898053	*RPL28*	0.05 ± 0.02	0.0 ± 0.0	<0.01	0.01	Promoter	CpG Island
chr5.72742630	*FOXD1*	0.01 ± 0.0	0.05 ± 0.01	<0.01	0.05	3'UTR	South Shore
chr8.132917158	*EFR3A*	0.0 ± 0.0	0.06 ± 0.02	<0.01	0.04	Intron	South Shore
chr16.28993311	*LAT*	0.94 ± 0.02	1.0 ± 0.0	<0.01	0.05	Exon	InterCpG
chr17.1184167	*TUSC5*	0.92 ± 0.02	1.0 ± 0.0	<0.01	0.01	Intron	InterCpG
chr7.157655513	*PTPRN2*	0.94 ± 0.02	1.0 ± 0.0	<0.01	<0.001	Exon	North Shelf
chr4.843782	*GAK*	0.92 ± 0.01	0.99 ± 0.01	<0.01	0.04	Exon	CpG Island
chr11.31827022	*PAX6*	0.01 ± 0.0	0.07 ± 0.01	<0.01	<0.001	Intron	South Shore
chr6.158072851	*ZDHHC14*	0.95 ± 0.02	1.0 ± 0.0	<0.01	0.02	Intron	InterCpG
chr12.81444314	-	0.93 ± 0.02	1.0 ± 0.0	<0.01	<0.001	Intergenic	InterCpG
chr7.23646672	*CCDC126*	0.92 ± 0.01	0.99 ± 0.01	<0.01	0.03	5'UTR	InterCpG

chr6.74371204	-	0.93 ± 0.02	1.0 ± 0.0	<0.01	0.05	Intergenic	InterCpG
chr2.158272554	*CYTIP*	0.92 ± 0.02	1.0 ± 0.0	<0.01	0.01	Exon	InterCpG
chr17.21219144	-	0.87 ± 0.01	0.96 ± 0.01	<0.01	0.05	Intergenic	North Shore
chr1.87429560	*HS2ST1*	0.90 ± 0.02	0.99 ± 0.01	<0.01	0.03	Intron	InterCpG
chr8.111697045	-	0.91 ± 0.02	1.0 ± 0.0	<0.01	<0.001	Intergenic	InterCpG
chr17.19743530	*ULK2*	0.90 ± 0.03	1.0 ± 0.0	<0.01	<0.01	Intron	InterCpG
chr5.61058332	-	0.91 ± 0.02	1.0 ± 0.0	<0.01	0.04	Intergenic	InterCpG
chr3.50131816	*RBM5*	0.90 ± 0.02	1.0 ± 0.0	<0.01	0.04	Intron	InterCpG
chr9.139093743	*LHX3*	0.0 ± 0.0	0.09 ± 0.02	<0.01	<0.01	Intron	CpG Island
chr7.87256217	*ABCB1*	0.88 ± 0.02	0.99 ± 0.01	<0.01	<0.01	Promoter	North Shore
chr17.3701518	*ITGAE*	0.92 ± 0.02	1.0 ± 0.0	<0.01	0.04	Intron	InterCpG
chr9.99791494	-	0.90 ± 0.03	1.0 ± 0.0	<0.01	<0.01	Intergenic	InterCpG
chrX.933751	-	0.86 ± 0.02	0.97 ± 0.02	<0.01	0.02	Intergenic	InterCpG
chr15.62511245	-	0.89 ± 0.03	1.0 ± 0.0	<0.01	<0.01	Intergenic	InterCpG
chr20.43948457	-	0.02 ± 0.01	0.13 ± 0.02	<0.01	<0.01	Intergenic	South Shelf
chr5.172090073	*NEURL1B*	0.89 ± 0.02	1.0 ± 0.0	<0.01	0.04	Intron	InterCpG
chr20.1276940	*SNPH*	0.86 ± 0.02	0.99 ± 0.01	<0.01	<0.01	5'UTR	CpG Island
chr12.132871826	*GALNT9*	0.86 ± 0.02	0.98 ± 0.01	<0.01	<0.001	Intron	North Shore
chr7.140180051	*MKRN1*	0.81 ± 0.03	0.98 ± 0.01	<0.01	0.05	Promoter	South Shore
chr19.1289934	*EFNA2*	0.73 ± 0.03	0.90 ± 0.02	<0.01	0.03	Intron	North Shore
chr9.129088683	*FAM125B*	0.19 ± 0.02	0.36 ± 0.03	<0.01	<0.01	Promoter	CpG Island
chr1.103319604	-	0.62 ± 0.07	0.86 ± 0.03	<0.01	0.04	Intergenic	InterCpG
chr8.22560981	-	0.18 ± 0.02	0.39 ± 0.05	<0.01	0.02	Intergenic	CpG Island
chr7.1659260	-	0.74 ± 0.06	0.98 ± 0.01	<0.01	0.02	Intergenic	CpG Island

Methylation data presented as mean ± SEM. q value generated by Benjamini-Hochberg multiple testing correction. CpG island is a 200-3000 bp stretch of DNA with a C+G content of 50% and observed CpG/expected CpG exceeding 0.6; North (N) and South (S) shores flank the CpG island by 0-2000 bp; the North (N) and South (S) shelf flank the shores by 2000 bp (2000-4000 bp from the island). InterCpG are locations between CpG islands.

APPENDIX F

REGRESSION ANALYSIS OF THE DIFFERENTIALLY METHYLATED CYTOSINES (DMCS; Q<0.05) PREDICTED BY M VALUE AFTER ADJUSTING FOR AGE AND SEX

Chromosome Position	Gene	Genic Region	CpG Island Region	partial *r*	P value
chr21.46957981	SLC19A1	5'UTR	South Shore	0.68	0.003
chr21.46957915	SLC19A1	5'UTR	CpG Island	0.66	0.004
chr6.3771940	-	Intergenic	InterCpG	0.615	0.009
chr5.80690459	ACOT12	Promoter	South Shore	0.563	0.019
chr11.2883716	-	Promoter	CpG Island	0.657	0.004
chr21.46927138	COL18A1	Intron	North Shelf	0.705	0.002
chr8.145702503	FOXH1	Promoter	South Shore	0.683	0.002
chr2.121283801	-	Intergenic	South Shelf	0.711	0.001
chr19.46456403	NOVA2	Intron	CpG Island	0.51	0.037
chr6.146348971	GRM1	Promoter	North Shore	0.616	0.009
chr4.2648590	FAM193A	Intron	InterCpG	0.59	0.013
chr1.214504377	SMYD2	Exon	InterCpG	0.558	0.02
chr20.32856825	ASIP	Exon	CpG Island	0.569	0.017
chr5.72742630	FOXD1	3'UTR	South Shore	-0.565	0.018
chr16.28993311	LAT	Exon	InterCpG	-0.497	0.043
chr17.1184167	TUSC5	Intron	InterCpG	-0.577	0.015
chr4.843782	GAK	Exon	CpG Island	-0.556	0.021
chr11.31827022	PAX6	Intron	South Shore	-0.581	0.014
chr12.81444314	-	Intergenic	InterCpG	-0.498	0.042
chr7.23646672	CCDC126	5'UTR	InterCpG	-0.546	0.023
chr17.21219144	-	Intergenic	North Shore	-0.589	0.013
chr1.87429560	HS2ST1	Intron	InterCpG	-0.674	0.003
chr5.61058332	-	Intergenic	InterCpG	-0.644	0.005
chr3.50131816	RBM5	Intron	InterCpG	-0.689	0.002
chr9.139093743	LHX3	Intron	CpG Island	-0.589	0.013
chr7.87256217	ABCB1	Promoter	North Shore	-0.67	0.003
chr9.99791494	-	Intergenic	InterCpG	-0.506	0.038
chrX.933751	-	Intergenic	InterCpG	-0.692	0.002
chr15.62511245	-	Intergenic	InterCpG	-0.568	0.017
chr5.172090073	NEURL1B	Intron	InterCpG	-0.637	0.006
chr7.140180051	MKRN1	Promoter	South Shore	-0.498	0.042
chr19.1289934	EFNA2	Intron	North Shore	-0.737	0.001
chr9.129088683	FAM125B	Promoter	CpG Island	-0.721	0.001
chr1.103319604	-	Intergenic	InterCpG	-0.568	0.017
chr8.22560981	-	Intergenic	CpG Island	-0.58	0.015
chr7.1659260	-	Intergenic	CpG Island	-0.637	0.006

APPENDIX G

THE 74 DIFFERENTIALLY METHYLATED REGIONS (DMRS; P<0.05)

[CONSULT ATTACHED FILES]

APPENDIX H

CHARACTERISTICS OF SKELETAL MUSCLE STUDY PARTICIPANTS (N=20)

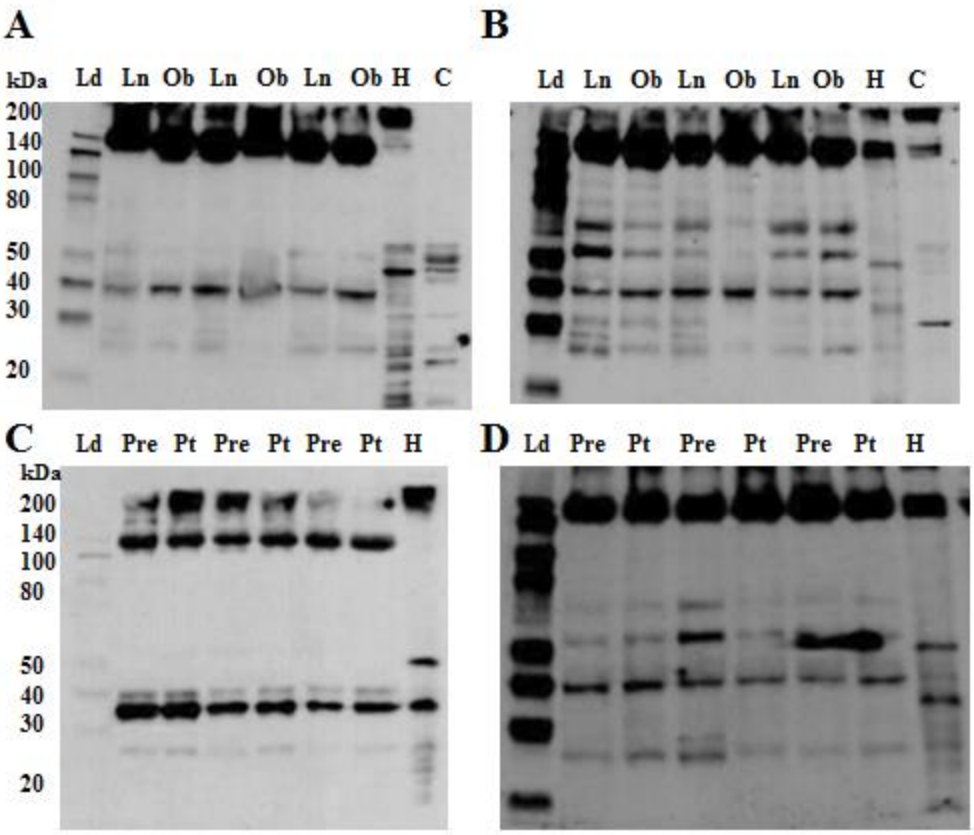
CLASSIFIED BY BODY MASS INDEX

Characteristics	Lean	Obese	P value	P value (age and sex)
Sex	7F/4M	4F/5M	0.4	-
Age (yrs)	29.3 ± 2.2	41.8 ± 2.2	0.0008	-
Body mass index (kg/m²)	23.1 ± 0.7	32.7 ± 0.8	0.00000004	0.00003
Body fat (%)	25.8 ± 1.4	34.1 ± 2.1	0.003	0.001
Waist circumference (cm)	81.1 ± 3.2	102.6 ± 2.1	0.0003	0.2
Systolic blood pressure (mmHg)	119.2 ± 2.6	124.7 ± 3.4	0.2	0.06
Diastolic blood pressure (mmHg)	72.2 ± 1.6	78.4 ± 1.4	0.010	0.05
Triglycerides (mg/dL)	92.5 ± 13.9	121.9 ± 14.9	0.2	0.8
Cholesterol (mg/dL)	178.5 ± 9.7	192.8 ± 10.3	0.3	0.9
High density lipoproteins (mg/dL)	59.1 ± 5.2	50.4 ± 3.8	0.2	0.8
Low density lipoproteins (mg/dL)	101.0 ± 7.9	118.0 ± 10.0	0.2	0.7
Hemoglobin A1c (%)	5.2 ± 0.04	5.4 ± 0.1	0.1	0.4
Fasting plasma glucose (mg/dL)	85.7 ± 1.7	88.3 ± 1.4	0.3	0.7
Fasting plasma insulin (μU/mL)	6.3 ± 1.2	11.2 ± 1.1	0.007	0.1
Clamp Rd (mg/kg.min)	7.2 ± 0.6	4.8 ± 0.7	0.02	0.03

Data presented as mean ± SEM, based on independent sample t-tests. Adjusted for age and sex by ANCOVA.*Calculated by Chi-Square Test. ǂBody fat determined by biometric impedance analysis (BIA).

APPENDIX I

WESTERN BLOT IMAGES



(A) Beta actin antibody (1/1000 dilution) expected detection around 45kDa was exposed for 30 sec, (B) Vinexin antibody (1/100 dilution) expected detection around 41kDa was exposed for 30 sec, (C) Glyceraldehyde 3-phosphate dehydrogenase antibody (0.47µg/mL dilution) expected detection around 37kDa was exposed for 5 secs, and (D) Vinexin antibody (1/100 dilution) expected detection around 41kDa was exposed for 1 min. Abbreviations were used for ladder (Ld), lean (Ln), obese (Ob), pre-surgery (Pre), post-surgery (Pt), HeLa whole cell lysate (H), and C2C12 whole cell lysate (C). The lean, obese, pre- and post-surgery samples were all human skeletal muscle lysate.

APPENDIX J

PERMISSIONS STATEMENT

All co-authors have granted permission for the use of the articles presented in this dissertation.