Genetic regulation of enoyl-CoA hydratase domain-containing 3 in adipose tissue
determines insulin sensitivity in African Americans and Europeans

Short title: Adipose tissue cis-eGene and insulin resistance

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**Abstract:**

Insulin resistance (IR) is a harbinger of type 2 diabetes (T2D) and partly determined by genetic factors. However, genetically-regulated mechanisms of IR remain poorly understood. Using gene expression, genotype, and insulin sensitivity data from an African American cohort (AAGMEx), we performed transcript-wide correlation and expression quantitative trait loci (eQTL) analyses to identify IR-correlated *cis*-regulated transcripts (*cis*-eGenes) in adipose tissue. These IR-correlated *cis*-eGenes were tested in a European ancestry cohort (METSIM) for trans-ethnic replication. Comparison of Matsuda index-correlated transcripts in AAGMEx with the METSIM study identified significant correlation of 3,849 transcripts, with concordant direction of effect for 97.5% of the transcripts. *Cis*-eQTLs for 587 Matsuda index-correlated genes were identified in both cohorts. The enoyl-CoA hydratase domain-containing 3 (*ECHDC3*) was the top ranked Matsuda index-correlated *cis*-eGene. Expression levels of *ECHDC3* were positively correlated with Matsuda index, and regulated by *cis*-eQTL, rs34844369 being the top *cis*-eSNP in AAGMEx. Silencing of *ECHDC3* in adipocytes significantly reduced insulin-stimulated glucose uptake and Akt-Ser\(^{473}\) phosphorylation. RNA-seq analysis identified 691 differentially-expressed genes in *ECHDC3*-knockdown adipocytes, which were enriched in \(\gamma\)-linolenate biosynthesis, and known IR genes. Thus, our studies elucidated genetic regulatory mechanisms of IR, and identified genes and pathways in adipose tissue that are mechanistically involved in IR.
**Introduction:**

The deterioration of systemic insulin responses related to glucose (and other metabolite) handling is referred to as insulin resistance (IR) (1). Reduced insulin sensitivity, or IR, promotes glucose intolerance and is a forerunner of type 2 diabetes (T2D) (1). Longitudinal studies in high-risk individuals suggest that IR occurs years before glucose intolerance or β-cell failure (2). Thus, reduced insulin sensitivity is an intermediate phenotype and early marker of T2D risk. Diet, lifestyle and other environmental factors play important roles in the etiology of IR. However, several lines of evidence, including differences in the prevalence of IR among ethnic groups, suggest that genetic factors are important influences on IR (3). Deciphering the underlying molecular defects and genetic regulatory mechanisms of insulin sensitivity is an unmet need to develop novel and safe therapeutic options to prevent IR and slow or halt progression to T2D and its devastating complications.

Previous efforts to understand the role of genetic factors in determining IR were primarily focused on identifying susceptibility loci (e.g. single nucleotide polymorphisms (SNPs)) by genetic association studies in human populations. Large-scale genetic association studies provide valuable insights on the genetic architecture of phenotypes such as fasting glucose and insulin (4-7), IR, whether derived from oral glucose tolerance test (OGTT), frequently sampled intravenous- glucose tolerance tests (FSIGT), or euglycemic clamp studies (8-10), and of T2D. Functional interpretation of those association signals remains challenging, and recent efforts were only nominally successful in defining genes specifically modulated by these SNPs (11;12). Alternative strategies are required for a more precise understanding of genetic regulatory mechanisms of IR.

Adipose tissue integrates various physiologic pathways, including glucose and fatty acid homeostasis (13). The failure of adipocytes to sequester excess fuel during nutritional abundance results in ectopic fat accumulation and is proposed as a trigger for systemic IR (1;14;15). Transcriptomic analyses by us and others suggest that IR results from a derangement in gene expression in tissues involved in glucose homeostasis, including adipose tissue (16-18). Interactions among genetic factors and dietary components likely determine variability in adipose tissue remodeling and expansion by modulating gene expression, leading to adipose tissue dysfunction.
and onset of IR. Genetic variants such as SNPs may determine tissue gene transcript levels. Thus, to identify genetic IR regulatory mechanisms, we focused on adipose tissue IR-correlated transcripts modulated by expression regulatory loci (eQTLs). We hypothesized that expression of a substantial subset of IR-correlated genes, in part, are genetically-driven and causally linked to altered insulin sensitivity. Further, we hypothesized that focused interrogation of these genes would help to identify novel susceptibility loci within a functional context.

Similar to most complex diseases, IR is a heterogeneous mix of molecular phenotypes caused by perturbations of multiple genes contributing to altered insulin sensitivity. To identify genetic IR regulatory mechanisms that are common in both African and European ancestry populations, we implemented a systems biological approach. Using gene expression, genotype, and insulin sensitivity data from an African American cohort, we identified insulin sensitivity-correlated cis-regulated transcripts (cis-eGenes) in adipose tissue. Data from a European ancestry cohort replicated 587 insulin sensitivity-correlated cis-eGenes. These analyses suggested that genetically-regulated expression of adipose tissue enoyl-CoA hydratase domain-containing 3 (ECHDC3) may play an important role in determining insulin sensitivity. In vitro genetic silencing studies in a human adipocyte model were successful in elucidating cellular and molecular mechanisms for the role of ECHDC3 in IR.

**Research Design and Methods:**

**Human Subjects:** Gluco-metabolic phenotype, gene expression, and genotype data available from 256 unrelated and non-diabetic individuals in the “African American Genetics of Metabolism and Expression” (AAGMEx) cohort (18;19) were used to identify genetically-regulated adipose tissue insulin sensitivity-correlated transcripts. Cohort participants were healthy, self-reported African American men and women residing in North Carolina, aged 18-60 years, with a body mass index (BMI) between 18 and 42 kg/m². All participants provided written informed consent under protocols approved by the Institutional Review Boards at Wake Forest School of Medicine. A standard 75-g OGTT was used to evaluate insulin sensitivity and exclude individuals with diabetes. Fasting blood samples were drawn for DNA isolation and biochemical analyses. Subcutaneous adipose tissue biopsies were collected by Bergstrom needle from participants after an overnight
fast. Clinical, anthropometric, and physiological characteristics of the AAGMEx cohort have been described (18).

We sought to replicate our findings in AAGMEx cohort using adipose tissue gene expression and genotype data from the European Ancestry individuals in Metabolic Syndrome in Men cohort (METSIM, Finland; N=770) (20). In AAGMEx, insulin sensitivity was measured by both OGTT and FSIGT. However, the Matsuda index, a measure of insulin sensitivity derived from OGTT was available for both the AAGMEx and METSIM cohorts and was used in this study as the primary phenotype.

Laboratory, statistical and bioinformatic methods are described briefly below.

**Laboratory measures and physiologic phenotypes:** Details of clinical laboratory measures have been described (18;20). In brief, for the AAGMEx cohort, plasma glucose levels were analyzed at a CLIA-certified commercial laboratory (LabCorp). Plasma insulin was measured using an immuno-chemiluminometric assay (Invitron Limited, Monmouth, UK). Plasma glucose and insulin data from five OGTT time points (0, 30, 60, 90, and 120 min) were used to calculate the Matsuda insulin sensitivity index (http://mmatsuda.diabetes-smc.jp/MIndex.html). Insulin sensitivity in the METSIM cohort was evaluated by calculating Matsuda index from three OGTT data points (0, 30, and 120 min).

**Gene expression analyses and genotyping methods:** Details of adipose tissue gene expression analyses, genotyping, and data quality control methods for both cohorts have been published (18-20). Genome-wide expression data in AAGMEx were generated using HumanHT-12 v4 Expression BeadChip (Illumina, San Diego, CA) whole-genome gene expression arrays. Data were submitted to the Gene Expression Omnibus (GEO; id #GSE95674). In the METSIM cohort, subcutaneous adipose tissue expression data were generated by Affymetrix U219 arrays and submitted to GEO (id #. GSE70353). Infinium HumanOmni5Exome-4 v1.1 DNA Analysis BeadChips (Illumina) were used to genotype DNA samples from the AAGMEx cohort based on the manufacturer’s instructions. METSIM samples were genotyped using the Illumina Human OmniExpress BeadChip array and the Illumina Human CoreExome array.
Bioinformatics and statistical analyses: As described previously (18), in the AAGMEx data set, genome-wide gene expression data (probe level) were extracted using Illumina GenomeStudio V2011.1. Expression levels were log$_2$ transformed, robust multi-array average normalized (RMA, includes quantile normalization), and batch-corrected using ComBat (https://www.bu.edu/jlab/wp-assets/ComBat/). Details of the gene expression data processing for METSIM are presented elsewhere (20).

In AAGMEx, genotype data were examined to verify sample and SNP quality. Genotype assays of 4,210,443 SNPs passed technical quality filters. Genotypes from 2,296,925 autosomal SNP assays (representing 2,210,735 unique high-quality genotyped SNPs with minor allele frequency (MAF) >0.01 and Hardy-Weinberg equilibrium-p-value >1x10$^{-6}$) were used in our initial eQTL analyses (19). We imputed these genotyped SNPs to the 1000 Genomes dataset (1KGP, phase 3 cosmopolitan reference panel) using the genotype imputation program Minimac3, implemented on the Michigan Imputation Server (https://imputationserver.sph.umich.edu/). The combined set of 14,502,313 autosomal genotyped and imputed SNPs was used for expanded eQTL analyses for selected Matsuda index-correlated transcripts. After quality control and genotype imputation of the 681,789 directly genotyped variants, the METSIM study used 7,677,146 variants (MAF ≥ 0.01) for eQTL analysis (20).

To test for associations between insulin sensitivity and expression level in the AAGMEx cohort, we computed a linear regression model using R(glm) software with the Matsuda index (natural log transformed) as the outcome and expression level (log$_2$) as the predictor. Models included age, gender, and African ancestry proportion (admixture estimates were computed using the ADMIXTURE program; http://software.genetics.ucla.edu/admixture/index.html) as covariates (Supplementary Figure-1). A secondary analysis included BMI as an additional covariate. We computed p-values adjusted for Benjamini-Hochberg false discovery rate (q-value). Expression of a transcript probe correlated with Matsuda index at q<0.01 was considered significant, excluding probes with a SNP within the probe sequence. We also correlated the Matsuda index with each transcript using Spearman semi-partial correlation coefficients adjusted for age, gender and ancestry proportion (admixture). We used the DAVID functional annotation tool (https://david.ncifcrf.gov/;
v6.9) and Ingenuity Pathway Analysis (IPA; https://apps.ingenuity.com/, Build version: 470319M, Content version: 43605602) to identify enrichment of Matsuda index-correlated genes in biological pathways. In the METSIM dataset, regression analyses were computed to evaluate correlations between adipose tissue gene expression and cardio-metabolic-related traits, including the Matsuda index (20). We compared results from the METSIM and AAGMEx cohorts. Expression of a transcript correlated with Matsuda index at q<0.05 in both cohorts and showing the same direction of effect was considered statistically replicated.

We also conducted cis-eQTL analyses (i.e., within ±500kb 5’ and 3’ of respective transcript). For each transcript probe, we computed linear regression with the log$_2$ transformed expression value as the outcome and an additive genetic model for the SNP as implemented in the R-package MatrixEQTL, with age, gender, and African ancestry proportion as covariates in AAGMEx. Cis-eQTLs with p< 2.96 X 10$^{-5}$ corresponding to a q-value <0.01 (or <1.0%) were considered significant (19). A secondary eQTL analysis included BMI as an additional covariate. Results from factored spectrally transformed linear mixed models (FaST-LMM) eQTL analysis in the METSIM cohort (https://systems.genetics.ucla.edu/) were used for comparison (20). Considering the difference in sample sizes for eQTL analyses, transcripts with cis-eQTL p-value < 2.46 X 10$^{-4}$ in METSIM (corresponding to 1% FDR) (20) and p≤ 1.75 X 10$^{-4}$ (corresponding to q<0.04) in AAGMEx were considered statistically replicated cis-eGenes.

To test for an association between a SNP and Matsuda index (natural log transformed) in AAGMEx, a linear regression model (SNPLASH; https://www.phs.wakehealth.edu/public/home.cfm) was computed that included age, gender, and African ancestry proportion as covariates. The primary inference was based on the additive genetic model. Similar linear regression model analysis adjusting for age was implemented in R to test association between cis-eSNPs in selected loci and Matsuda index in METSIM.

**Studies in cultured adipocyte models:** Gene expression analysis in adipose tissue from human cohorts identified enoyl-CoA hydratase domain-containing 3 (ECHDC3) as the top-ranked Matsuda index-correlated cis-eGene (see Results). Thus, we studied the expression of ECHDC3 at different stages of differentiation in human adipose stroma-derived stem cells (hADSCs). The hADSCs (AG17928 and AG172929) derived from
abdominal subcutaneous tissue samples donated by two different women were obtained from Coriell Cell Repositories (Camden, NJ). To identify the role of \textit{ECHDC3} in adipocytes by knocking down its expression, we used Simpson-Golabi-Behmel syndrome (SGBS) pre-adipocytes, a well-characterized human adipocyte cell model (21) that is more amenable to genetic manipulation than hADSC’s and has an expression profile after differentiation that closely mimics mature adipocytes. We grew hADSC and SGBS cells under standard culture condition in DMEM/Ham’s F-12 (1:1 v/v) adipocyte basal medium (BM-1, Zenbio, Inc; Research Triangle Park, NC) supplemented with 10% FBS and antibiotics. Cells were differentiated to adipocytes using adipocyte differentiation medium (DM2, Zen Bio) for seven days and maintained for an additional seven days in adipocyte maintenance medium (AM-1, ZenBio) for complete maturation of adipocytes following the vendor-recommended protocol.

For stable RNA interference, the \textit{ECHDC3} gene was silenced by infecting the SGBS preadipocytes with lentiviral particles (lv) to deliver gene-specific shRNA expression vectors (sc-90758-V, Santa Cruz Biotechnology Inc., Santa Cruz, CA; a pool of transduction-ready viral particles containing three target-specific constructs that encode shRNA) in the presence of polybrene (sc-134220, Santa Cruz; 8 µg/ml) according to the manufacturer’s protocol. A control shRNA lentiviral Particles-A (sc-108080, Santa Cruz; encodes a scrambled shRNA sequence that will not lead to the specific degradation of any known cellular mRNA) was used as a negative control. Cells successfully transduced and stably expressing shRNA were selected using 2 µg/ml of puromycin (A1113803, Gibco, Thermo Fisher Scientific, USA).

Total RNA was isolated using RNAeasy kit (Qiagen) from three sets of cells: 1) undifferentiated hADSC and SGBS cells; 2) hADSC and SGBS cells at different stages of differentiation; and 3) \textit{ECHDC3}-shRNA and control-shRNA-expressing SGBS cells at 14th-day of differentiation. RNA samples were reverse-transcribed using QuantiTect reverse transcription kit (Qiagen) based on the manufacturer’s protocol. To determine expression levels at different stages of adipocyte differentiation and to confirm shRNA-mediated downregulation, \textit{ECHDC3} expression was measured in cDNA by quantitative real-time PCR (qRT-PCR) using Power SYBR green chemistry (Applied Biosystems, Inc., Foster City, CA). Oligonucleotide primers used to
amplify ECHDC3 were 5’-ACGGCATAAGGAACATCGTC-3’ (forward) and 5’-AAAACACAGGCCCTCAG-3’ (reverse). The expression of target genes was normalized to the expression of an endogenous control gene, 36B4/RPLP0. Two independent experiments with three biological replicates for each treatment condition were performed for confirmation of successful ECHDC3 knock-down by gene-specific shRNA.

Glucose uptake assay: Similar to a published study (22), on day 14 of differentiation, the control-shRNA and ECHDC3-shRNA (ECHDC3-KD) SGBS cells grown in biological triplicates in 6-well plates were washed with DPBS and pre-incubated with HEPES-buffered Krebs-Ringer solution (Alfa Aesar J67795) for 6 h at 37°C. After pre-incubation, the cells were incubated with or without 1 µM insulin in Krebs-Ringer solution for 20 min. Next, 0.5 µCi/mL labeled 2-deoxy-D-[3H] glucose and 0.25 mmol/L d-glucose were added for an additional 20 min at 37°C. Reactions were terminated by placing the cells onto the ice and washing 3 times with ice-cold DPBS. The cells were solubilized with 1 mL of 0.2 N NaOH per well and incubated overnight at room temperature with constant shaking. Radioactivity and protein content in cell lysates were measured using liquid scintillation spectroscopy and bicinchoninic acid assays, respectively. Cellular radiolabeled glucose uptake was normalized to cell protein content.

Western blots to determine insulin-stimulated Akt phosphorylation: On the 14th day of differentiation, control-shRNA and ECHDC3-shRNA (ECHDC3-KD) SGBS cells (in 6-well plates) were washed once with 0.1 % bovine serum albumin -PBS and serum-starved in 0.1 %BSA-PBS for 30 min. Cells were then stimulated with or without 100 nM insulin for 15 min (23). Cells were harvested using 200 µl of RIPA buffer (no SDS) per well of a 6-well plate. Cell lysates were sonicated (3 pulses for 3 seconds) on ice, centrifuged (12000 rcf for 15 minutes) and protein concentrations in clear lysates were measured by bicinchoninic acid assays. Molecular weight markers and heat-denatured cell lysates containing 10 µg protein were loaded in each well for electrophoretic separation on a 10% SDS-PAGE gel (30 minutes at 80 V and 90 minutes at 110 V). Protein bands were transferred to polyvinylidene difluoride membranes (3.5-4 hours at 65 V or 400 mA). Membranes were blocked with 5% non-fat milk and 0.1% Tween 20 in Tris-buffered saline (0.1% TBST) for 1 hour at room
temperature. Membranes were washed three times (5 minutes each) in 0.1% TBST. Blocked and washed membranes were then incubated overnight at 4°C in 0.1% TBST buffer containing 5% fatty acid free-bovine serum albumin, and primary phosphorylated Akt at Ser^{473} (1:1000; Cell Signaling Technologies, cat no:4060 ), total Akt (1:1000; Cell Signaling Technologies, cat no: 4691), or GAPDH (1:2000; Santa Cruz Biotechnology, cat no: sc-32233) antibody. Membranes were then washed three times in 0.1% TBST (5 minutes each), and incubated with conjugated secondary antibody (1:5000; Goat anti-rabbit IgG-HRP , Sigma-Aldrich, cat no: A9169; Goat anti-mouse IgG-HRP Santa Cruz Cat no: sc-2031 ) in 0.1% TBST buffer containing 5% non-fat milk at room temperature for 1-2 hours. Finally, membranes were washed (three washes of 0.1% TBST, 5 minutes each) and images were acquired using darkroom development techniques or cold camera for chemiluminescence.

**RNA-seq and bioinformatic analyses:** We performed genome-wide expression profiling of control- and ECHDC3-shRNA SGBS cells in triplicate at day 14 of differentiation by RNA sequencing. RNA sequencing experiments were performed at Beijing Genome Institute (BGI Co., Ltd, China). In brief, high-quality total RNA samples (RIN>9.0) isolated from SGBS cells were treated with DNase I to remove DNA contamination, and then the mRNA was enriched by using oligo (dT) magnetic beads. Sequencing adaptor-ligated cDNA libraries were prepared from mRNA templates and were used with BGISEQ-500 RNA-sequencing technology to obtain >20 million single-end 50 bp reads per sample. On average 24,135,731 raw sequencing reads were generated per sample and after filtering low-quality reads, on average 23,841,269 clean reads were obtained. Clean reads were mapped to the human reference genome using the HISAT/Bowtie2 tool (24;25). The average mapping ratio with the reference gene was 84.41%, and average genome mapping ratio was 96.51%. Gene expression levels were quantified as fragments per kilobase million (FPKM) values using RSEM software (26). Differentially expressed genes (DEGs) between groups were screened using NOIseq (27). DEGs with Bayes posterior probability >70% in NOISeq and average log$_2$ fold change (FC) +/-0.58 were considered significant. Ingenuity Pathway Analysis (IPA; https://apps.ingenuity.com/, Build version: 481437M, Content version: 39480507) was used to identify enrichment of differentially expressed genes in biological pathways.
Results:

Expression of subcutaneous adipose tissue transcripts enriched for salient biological pathways is correlated with Matsuda index of insulin sensitivity in African Americans. Individuals in the AAGMEx cohort had a broad range of insulin sensitivity as measured by OGTT-derived Matsuda index (6.2±6.7). High-quality adipose tissue gene expression and Matsuda index data (N= 246 subjects) was used to determine correlations between insulin sensitivity and adipose tissue transcript expression levels. Expression of 5,102 transcripts (probes representing 4,273 Entrez Gene ids) in adipose tissue was significantly correlated (q-value <0.01) with Matsuda index (Supplementary Table 1). Matsuda index was positively and negatively correlated, respectively, with the expression of 2,645 and 2,457 adipose transcripts. Among these transcripts 83% (4239 probes) remain significantly associated (p<0.05) with Matsuda index even after additional adjustment for BMI. Membrane-spanning 4-domains subfamily-A member 6A (MS4A6A) was the most significant negatively (inversely) correlated transcript (ILMN_1721035, β= -1.06, p= 4.27 X 10⁻²⁴), whereas zinc-binding alpha-2-glycoprotein 1 (AZGP1) was the most significant positively correlated transcript (ILMN_1797154, β= 1.33, p= 2.96 X 10⁻²³) with the Matsuda index (Table 1). Multiple probes putatively representing isoforms of 707 Entrez id genes were correlated with Matsuda index-including probes for 691 Entrez id genes (e.g. three probes for MS4A6A) showing the same effect direction. However, probes for 16 Entrez id genes (e.g. two probes for clusterin (CLU)) showed discordant directions of effect, suggesting isoform-specific roles of these genes in insulin sensitivity.

We found significant enrichment (B-H corrected p< 0.05) of 205 canonical pathways (in IPA knowledgebase) among these Matsuda index-correlated transcripts in adipose tissue (Supplementary Table 2A). Gene expression profiles also suggested significant activation (activation z-score >2) or repression (z< -2) of 41 pathways enriched in adipose tissue of insulin sensitive individuals. Among the enriched pathways, expression profile of genes in oxidative phosphorylation (B-H corrected p = 1.58 X10⁻¹², z= 7.216), EIF2 signaling (p= 6.17 X 10⁻⁸, z= 3.111), and valine degradation (p= 7.24 X 10⁻⁷, z= 3.357) pathways suggested significant activation, whereas genes in integrin signaling (p= 4.47 X 10⁻⁹, z= -3.801), IL-8 signaling (p= 1.70 X10⁻⁶, z= -
3.204) and Fcγ receptor-mediated phagocytosis in macrophages and monocytes (p= 2.88 X10^-6, z= -3.904) pathways suggested significant repression in insulin-sensitive individuals. Expression levels of 55 genes in oxidative phosphorylation pathways were positively, and 33 genes in Fcγ receptor-mediated phagocytosis in macrophages and monocytes pathway were inversely correlated with Matsuda index. DAVID functional annotation analysis using KEGG and GO annotation also validated the enrichment of multiple pathways (Supplementary Table 2B).

Correlation of adipose tissue transcript levels with Matsuda index in African Americans replicates in an independent European ancestry cohort. Using adipose tissue expression data from METSIM and European ancestry cohorts, we sought replication of Matsuda index-correlated transcripts identified in African Africans. Individuals in the METSIM cohort also had a broad range of Matsuda index values (7.3±4.3). We compared the most significant Matsuda index-correlated probe for each gene in METSIM study with transcripts significantly correlated with Matsuda index and matched for gene symbol annotation in AAGMEx. A total of 3,849 transcripts were significantly correlated (q<0.05) with Matsuda index in both cohorts (Supplementary Table 3). Matsuda index was positively and inversely correlated with the expression of 2,047 and 1,708 adipose transcripts in both cohorts, respectively. (Supplementary Figure 2A). Thus, our results suggest a strong replication of correlation and direction of effect for 97.5% of Matsuda index-correlated adipose tissue transcripts in independent cohorts of African and European ancestry individuals.

Matsuda index-correlated transcripts are regulated by eQTLs in African and European ancestry individuals. Our analyses in these cohorts suggested deranged expression of thousands of adipose tissue genes in those with IR. However, it is difficult to differentiate causal effects from reactive effects based on transcriptomic data. Genotype variations, including SNPs in regulatory regions of the genome or eQTL, may determine tissue transcript levels. The eQTL analyses integrate SNP genotypes with transcript expression profiles, and provide evidence for genotype-dependent variations in transcript abundance. eQTL analyses identified significant cis-eSNPs for 587 Matsuda index-correlated transcripts in adipose tissue of AAGMEx and METSIM participants (FDR 4% and FDR 1%, respectively) (Supplementary Table 4). Top cis-eSNPs in
one cohort show high concordance in effect direction, and strong correlation of effect sizes in the other cohort (Supplementary Table 4A and 4B, Supplementary Figure 3). In AAGMEx cohort, we also compared the eQTL analysis with and without adjustment for BMI. Of the 587 Matsuda index-correlated cis-eGenes we report, 474 are significant in BMI corrected eQTL analysis (FDR<4%; in Supplementary Table 4C), and results from both analysis (β and –log10 p-value) were highly correlated (r²>0.9). Thus, adipose tissue expression levels of these 587 transcripts are correlated with insulin sensitivity and are genetically regulated in both ethnicities. These transcripts were ranked based on their statistical significance of correlation with Matsuda index and association with genotype in eQTL analysis in AAGMEx and METSIM cohorts. The top ten Matsuda index-correlated cis-eGenes are shown in Table 2. Based on average ranking, the enoyl Coenzyme-A hydratase domain containing-3 (ECHDC3) gene was the top-ranked Matsuda index-correlated cis-eGene. Its expression was positively correlated with Matsuda index (Figure 1A; ILMN_2072178, β= 1.009, p= 6.29 X10⁻¹⁵ in AAGMEx; and Supplementary Figure 2B; 11728810_a_at, β= 0.34, p=2.35 X 10⁻²² in METSIM). Adipose tissue ECHDC3 expression was strongly regulated by cis-eQTL (p= 1.94 X 10⁻⁹ in AAGMEx and p= 8.26 X10⁻⁵² in METSIM; Figure 1B and Supplementary Figure 2C, D).

Ten genotyped SNPs (within ±500kb of transcript and MAF>0.01) showed significant association (q<0.04) with ECHDC3 transcript levels in adipose tissue of African Americans in the AAGMEx cohort, with rs200943982_T being the strongest genotyped cis-eSNP (MAF= 0.393, β= -0.281, p= 1.94 X10⁻⁹, Figure 1B). Expanded eQTL analysis using imputed SNPs identified 53 cis-eSNPs (p< 1X10⁻⁴) in AAGMEx; rs34844369_A, an imputed SNP, was the strongest cis-eSNP (MAF= 0.438, β= -0.283, p= 9.37 X10⁻¹⁰; Figure 1C) for ECHDC3 (Supplementary Table 5). ECHDC3 expression was positively correlated with Matsuda index in all genotype groups (Supplementary Figure-4). The rs34844369 SNP is located in the intron of ECHDC3 and rs200943982 is located downstream (~6.2Kb 3’ of ECHDC3). These two cis-SNPs are 7,717 bp apart on chromosome 10, but showed moderate linkage disequilibrium (D’= 0.89, r²= 0.67). Neither SNP was significantly associated with ECHDC3 expression when adjusting for the other. Stepwise regression analysis identified no other significant eSNPs after adjusting for rs200943982 and rs34844369. Haplotype analyses of rs34844369 and
rs200943982 suggested significant associations with \textit{ECHDC3} expression level (global haplotype test $p=1.32 \times 10^{-13}$), with the A-T haplotype (rs34844369\_A-rs200943982\_T) associated with reduced \textit{ECHDC3} expression ($\beta= -0.2976$, $p = 2.75 \times 10^{-15}$, adjusted for age, sex, admixture). In the METSIM cohort, rs34844369 was a significant \textit{ECHDC3} \textit{cis}-eSNP ($p=1.41 \times 10^{-32}$, \textbf{Supplementary Table 6}). However, in METSIM, the strongest \textit{ECHDC3} \textit{cis}-eQTL signal was identified at SNP rs3814627\_G (MAF=0.312, $\beta= -0.755$, $p= 8.26 \times 10^{-52}$, \textbf{Supplementary Figure 1D}), located in the putative \textit{ECHDC3} promoter region (-482 bp upstream of the TSS). The SNP rs3814627 was not a significant \textit{cis}-eSNP in AAGMEx (MAF =0.122, $p= 0.68$); however, another SNP near this putative promoter region (rs10906007\_G, p= 8.44X10-7, -1157 bp upstream of TSS) was significantly associated, suggesting the presence of common and ethnic-specific genetic regulation for \textit{ECHDC3} expression in adipose tissue. Epigenetic regulatory annotation of SNPs in \textit{ECHDC3} \textit{cis}-eQTL identified in the AAGMEx and METSIM cohorts, and the GTEx study by HaploReg v4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php) suggested the presence of promoter or enhancer histone marks, and altered transcription factor binding motifs for many of these \textit{cis}-eSNPs (\textbf{Supplementary Table 7A}). Bioinformatic analysis using RegulomeDb-v1.1 (http://regulomedb.org/) and SNP2TFBS (https://ccg.vital-it.ch/snp2tfbs/) also supported the putative regulatory role and ability to alter transcription factor binding of these SNPs in \textit{ECHDC3} \textit{cis}-eQTL (\textbf{Supplementary Table 7B, 7C}). Despite strong effect of regulatory genetic variants on proximal molecular phenotypes, i.e., transcript expression, these \textit{cis}-eSNPs individually showed no statistically significant association with Matsuda index in either AAGMEx or METSIM cohort (\textbf{Supplementary Table 5 and 6}). However, large GWAS data sets curated in AMP-T2D knowledge portal (http://www.type2diabetesgenetics.org/) for glycemic traits showed significant association (below genome wide threshold) of top \textit{ECHDC3} \textit{cis}-eSNPs, and allele effect direction on traits were consistent with their effect on gene expression in our eQTL analysis. For example, rs3814627\_G allele that reduces \textit{ECHDC3} expression, was associated with reduced Stumvoll insulin sensitivity index ($\beta= -0.023$, $p= 0.0448$) and increased HbA1c ($\beta= 0.027$, $p= 0.0309$) in MAGIC GWAS data sets.
**ECHDC3 is primarily expressed in adipocytes.** Human cohort analyses suggested genetically-regulated expression of ECHDC3 in adipose tissue may be important in determining insulin sensitivity. Defining the contribution of adipose tissue cell types in ECHDC3 expression levels may help identify cell-specific roles in correlations between ECHDC3 expression and the Matsuda index. Thus, we fractionated fresh subcutaneous adipose tissue into adipocytes and adipose stromal vascular cells as described previously (28). Total RNA extracted from 5-6 African American individuals in the AAGMEx cohort was used to prepare separate pools of adipocyte fraction and stromal vascular fraction cDNA. In a qRT-PCR assay, ECHDC3 expression was 13.6-fold higher in the adipocyte fraction (relative expression 11.53±0.04) than the stromal vascular fraction (relative expression 0.85±0.22; Figure 2A), suggesting that ECHDC3 expression is primarily attributable to adipocytes.

**Adipocyte differentiation induces ECHDC3 expression.** We analyzed the expression of ECHDC3 transcripts at different stages of in vitro differentiation of hADSCs and SGBS cells. ECHDC3 mRNA expression was low in undifferentiated hADSCs (relative expression 0.05±0.01), began to rise in cells after 7 days in differentiation media, and was strongly induced at 14 days (relative expression 1.01±0.02, Figure 2B). Similarly, ECHDC3 mRNA expression at 14 days was 23-fold higher in SGBS cells compared to undifferentiated cells (Figure 2C).

**ECHDC3 silencing in SGBS adipocytes decreases insulin sensitivity.** Individuals with low mRNA abundance of ECHDC3 in adipose tissue had low insulin sensitivity. Thus, we next determined whether decreasing ECHDC3 expression in cultured human adipocytes reduced insulin sensitivity. SGBS cells stably expressing ECHDC3-specific shRNA (ECHDC3-KD) showed 70% downregulation (p= 1.20X10^-5) of ECHDC3 mRNA (Figure 3A) compared with cells stably expressing control shRNA at 14 days after the induction of differentiation. Insulin (1 µM) stimulated glucose uptake by 84% in control cells (p=0.009) relative to PBS controls, but only 32% in ECHDC3-KD cells, demonstrating that insulin-stimulated glucose uptake was significantly (p= 0.03) decreased in ECHDC3-shRNA SGBS cells (ECHDC3-KD) compared to controls (Figure 3B). As shown in a representative western blot in Figure 3C, Akt phosphorylation (pAkt-Ser\(^{473}\)) after insulin stimulation was significantly decreased (p=0.0004, Figure 3D) in ECHDC3-KD cells compared to controls.
**ECHDC3 silencing in adipocytes modulates many pathways and genes correlated with IR and metabolism of fatty acid and triacylglycerol.** The functional role of ECHDC3 in adipocytes or other cells is unknown. Our qRT-PCR analyses suggested significant downregulation of three genes important in insulin resistance - adiponectin (*ADIPOQ*; \( p = 1.15 \times 10^{-3} \)), facilitated glucose transporter-4 (GLUT4/SLC2A4; \( p = 3.36 \times 10^{-6} \)), and peroxisome proliferator-activated receptor gamma (*PPARG*; \( p = 2.51 \times 10^{-6} \)) in ECHDC3-shRNA SGBS cells (ECHDC3-KD) compared to controls (Figure 4A). To identify genes modulated by ECHDC3 and to define its biological role, we compared triplicate cultures of ECHDC3-shRNA SGBS cells (ECHDC3-KD) with control-shRNA SGBS cells at day 14 of differentiation by RNA-seq analysis. This genome-wide analysis identified 691 DEGs (at Bayes posterior probability \( >70\% \) and average \( \log_2 FC +/-0.58 \)) in ECHDC3-KD cells compared to control cells (Figure 4B; Supplementary Table 8), including 479 downregulated and 212 upregulated genes. IPA result suggested an enrichment of 63 canonical pathways (\( p<0.05 \)) among DEGs, with the \( \gamma \)-linolenate Biosynthesis pathway as the most enriched (\( p = 3.8 \times 10^{-8} \); Supplementary Table 9). Eight genes in this pathway (e.g. *FADS1, FADS2, ACSL1, ACSL5*) were downregulated in ECHDC3-KD cells. Downregulation of *FADS1* (\( p = 9.44 \times 10^{-5} \)) in ECHDC3-KD cells was validated by qRT-PCR in independent experiments (Figure 4C). Canonical pathways for fatty acid, triglycerides, and cholesterol biosynthesis were also enriched among DEGs. Among the enriched pathways, expression profile of genes suggested significant inactivation of LXR/RXR activation pathway (\( p= 2.04 \times 10^{-7} \), \( z= -2.53 \)). The IPA results also suggested enrichment of disease or biological function, including metabolism of triacylglycerol (17 genes, \( p= 4.54 \times 10^{-13} \)), fatty acid metabolism (51 genes, \( p= 1.54 \times 10^{-10} \)), and insulin resistance (25 genes, \( p= 1.63 \times 10^{-8} \); Figure 4D, Supplementary Table 10). Regulator Effects analysis considered DEGs in ECHDC3-KD cells and suggested an effect of upstream regulators on the expression of downstream target molecules and impact on biological function and diseases. The top two regulatory networks with a consistency score \( >20 \) suggested derangement in the accumulation of lipid, adipogenesis, and fatty acid metabolism in ECHDC3-KD cells (Figure 4E, Supplementary Table 11). Thus ECHDC3 knockdown in adipocytes may cause insulin resistance by deranging multiple key biological pathways.
Discussion:

Our results suggest that gene expression levels in adipose tissue are key trans-ethnic (trans-ancestral) biomarkers strongly correlated with modulation of insulin sensitivity. Expression levels of transcripts for thousands of protein-coding genes in adipose tissue correlated with the Matsuda index, a well-validated estimate of insulin sensitivity measured by physiologically relevant OGTT (29). Thus, IR involves activation and repression of multiple pathways. We found that expression levels of a subset of these IR-correlated transcripts in adipose tissue are regulated by DNA sequence variants. The identification of eQTLs for IR-correlated transcripts in adipose tissue suggests that the expression of these transcripts is at least partially genetically-driven and causal, and not just the result of altered insulin sensitivity. Comparing two cohorts, one of African Americans and one of European ancestry, provides independent validation and generalizability of our findings. The causal role of the ECHDC3 gene in IR, a candidate gene identified through variation-based statistical genetic analyses, was validated through genetic silencing and functional studies in an adipocyte model.

In this study, we compared gene expression profiles from subcutaneous adipose tissues in an African American and a European ancestry cohort, primarily to identify trans-ancestral genetically-regulated mechanisms of IR. We identified 3,755 genes with transcript levels significantly correlated with the Matsuda index and in the same direction of effect in both cohorts. These data support the concept of common molecular mechanisms of insulin resistance in both European and African ancestry. We further focused on these replicated insulin sensitivity-correlated genes and their regulation mediated by QTLs to define common causal mechanisms of IR. However, the genetic regulatory architecture of a subset of IR-correlated genes differs between ethnic groups, and comparison of ethnicities may be instrumental in identifying putatively ancestry-specific biological mechanisms of IR.

We identified 654 transcripts significantly correlated with the Matsuda index in our African American cohort (AAGME, FDR p \(\leq 0.01\)), but probes representing corresponding transcripts in the European ancestry cohort (METSIM) showed no significant correlation (p >0.05); whether these transcript-Matsuda index
correlation represents true African-derived mechanisms or false statistical correlations (i.e., type 1 errors) remains to be determined. As examples, expression of SFFV proviral integration oncogene-1 (SPI1) in adipose tissues of AAGMEx participants were significantly inversely correlated with Matsuda index (ILMN_1696463, NM_001080547, β = -1.19, p = 2.97 x 10^{-13}; ILMN_2392043, NM_003120, β = -1.13, p = 1.21 x 10^{-11}), but its expression was not significantly correlated in the METSIM cohort (represented by probe 11723165_a_at). The SPI1 gene encodes for PU.1, a key transcription factor involved in monocyte to macrophage differentiation (30), and also plays a role in adipocyte inflammatory mechanisms causing IR (31;32). Despite the larger size of the METSIM cohort, this lack of replication may suggest that the effect of SPI1 gene predominates in individuals of African ancestry. However, we also noted an inverse correlation between SPI1 expression in adipose tissue and insulin sensitivity (Spearman’s semi partial ρ = -0.40, p<0.0001 for ILMN_1696463) in another small cohort (N=99) of European American individuals recruited from Arkansas in a previously published study (33). Gene expression data in the METSIM cohort were generated using Affymetrix U219 array, while Illumina HumanHT-12 v4 Expression BeadChips were used for the AAGMEx cohort. Probes may have quantified different transcript isoforms or may have different efficiencies in the quantification of this gene. Thus, lack of replication, at least for a subset of these genes, may reflect technical rather than true biological difference between ethnicities, and may be explained by such differences in probes. Our future studies will utilize alternative strategies that focus on ancestry-specific or ethnically-predominant mechanisms of IR.

This study identified cis-eQTLs for 587 Matsuda index-correlated transcripts in both the AAGMEx and METSIM cohorts. Thus, in line with the anticipated polygenic nature of the insulin sensitivity phenotype, genetic regulation of a large subset of genes in adipose tissue may, at least in part, be causally involved in the pathogenesis of IR in African and European ancestry populations. These IR-correlated cis-eGenes are enriched for cellular functions and biological pathways. However, the precise role of many of these genes in IR is not known. To prioritize and begin to understand the role of genes in modulating cellular and molecular mechanisms causing IR, we ranked this large list of transcripts based on their statistical significance of correlation with the Matsuda index and association with genotype. ECHDC3, macrophage migration inhibitory factor (MIF), centromere protein V (CENPV), D-dopachrome tautomerase (DDT), and apolipoprotein B
were among the top Matsuda index-correlated *cis*-eGenes. Some of these genes have a known role in adipose tissue biology and IR. For example, macrophage migration inhibitory factor superfamily gene members *MIF* and *DDT* bind to the CD74/CD44 receptor complex and have distinct roles in adipogenesis. *MIF* positively correlates with insulin resistance and contributes to adipose tissue inflammation by modulating adipose tissue macrophage functions, while *DDT* reverses glucose intolerance (34;35).

The *ECHDC3* gene emerged as the most significant Matsuda index-correlated *cis*-eGene. Multi-tissue expression data from the GTEx study showed high *ECHDC3* expression in fatty acid metabolizing tissues, including liver, muscle, and adipose. Strong *cis*-eQTL associations were detected for *ECHDC3* expression in subcutaneous adipose tissue in GTEx_V7-P2 (rs10906007, $p= 1.40 \times 10^{-10}$, https://gtexportal.org/) and STARNET (36) (rs718641, $p= 4.78 \times 10^{-10}$) studies. However, its role in adipose or other tissue is unknown. Based on protein sequence homology, peroxisomal enoyl CoA hydratase (*ECH1*) and short-chain acyl-CoA dehydrogenase (*ECHS1*/SCHE) are the closest human homologs. This suggests that *ECHDC3* may act as enoyl-CoA hydratase. The enoyl coenzyme A hydratases is a key mitochondrial enzyme involved in fatty acid β-oxidation, and catalyzes the addition of H$_2$O across the double bond of trans-2-enoyl-CoA, resulting in formation of a β-hydroxyacyl-CoA (37). Biochemical analyses suggest that human *ECHDC1*, a likely homolog of *ECHDC3*, lacks enoyl-CoA hydratase activity (38). A recent study suggested that *ECHS1*, a close homolog of *ECHDC3*, senses nutrient signals and acts as the converging enzyme for fatty acid and branched chain amino acid oxidation (39).

Supporting a connection between decreased *ECHDC3* expression in adipose tissue and IR, *in vitro* knockdown of *ECHDC3* expression in our study significantly downregulated insulin signaling and insulin-stimulated glucose uptake in human adipocytes. Our unbiased transcriptome-wide analysis suggested that downregulation of *ECHDC3* in human adipocytes may cause IR by significantly inactivating fatty acid biosynthesis, including the γ-linolenate biosynthesis, cholesterol biosynthesis, and LXR/RXR activation pathways. Genetic polymorphisms in the *ECHDC3* regulatory element (eQTL) determine a significant proportion of its variable expression in adipose tissue, likely by their differential ability to interact (as suggested
by SNP2TFBS and RegulomeDb) with transcription factors. A regulatory element in the intron of ECHDC3 (near SNP rs34844369) was observed in both European and African ancestry cohorts, while an additional regulatory element upstream to ECHDC3 (-400bp to -4Kb, near SNP rs3814627) was observed only in European ancestry cohorts. These two regions are not in linkage disequilibrium in African ancestry cohorts (D’= 0.21, R^2=0.007, 1KGP Phase 3_V5 data from LD link: https://ldlink.nci.nih.gov/), but are in strong linkage disequilibrium in European ancestry cohorts (D’= 0.95, R^2=0.63). Future studies will define the transcriptional regulatory mechanisms mediated by these regulatory elements and their interactions that may have ancestry-specific contributions in determining genetic regulatory networks important for the pathogenesis of IR.

In summary, genetic regulation of a large subset of genes in adipose tissue is causally involved in the pathogenesis of IR in African and European ancestry individuals. Our variation-based genomic analysis with concurrent analyses of glucose-homeostasis phenotypes, adipose tissue transcriptome, and in vitro genetic silencing studies identified several genetic regulatory mechanisms, including the novel role of the ECHDC3 gene in causing IR putatively by modulating fatty acid metabolism and other key pathways in adipocytes.

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**Author Contributions**

NKS, performed genomic and cell biological experiments, analyzed data, and reviewed/edited manuscript; CCK, performed cell biological experiments, analyzed data, and reviewed/edited manuscript; MC, analyzed METSIM cohort data, and reviewed/edited manuscript; MW, supplied a critical reagent (SGBS cells) for this study, and reviewed/edited manuscript; MEC, performed statistical genetic analysis, and reviewed/edited manuscript; CDL, supervised statistical genetic analysis, interpreted the data, and reviewed/edited manuscript; JP, supervised cell biological studies, interpreted the data, and reviewed/edited manuscript; SKD, designed the study, analyzed and interpreted the data, and wrote the manuscript.

**Conflict of Interest Statement:** The authors have nothing to disclose

**Guarantor Statement:** S.K.D. and C.D.L. are the guarantors of this work, and as such, had full access to all study data and take responsibility for the integrity of the data and accuracy of data analysis.

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### Table 1: Twenty most significantly correlated expression level of subcutaneous adipose tissue transcripts with Matsuda Insulin sensitivity index in AAGMEx (Top 10 positively and top 10 inversely correlated genes are shown).

| Probe Id     | Symbol | Entrez Gene_ID | $\beta$ | SE of $\beta$ | Adjusted $R^2$ | p-value         | q-value         |
|--------------|--------|----------------|---------|---------------|----------------|-----------------|-----------------|
| ILMN_1721035 | MS4A6A | 64231          | -1.06   | 0.093         | 0.365          | 4.27E-24        | 2.02E-19        |
| ILMN_2397721 | GLB1   | 2720           | -1.94   | 0.174         | 0.361          | 1.01E-23        | 2.39E-19        |
| ILMN_1796409 | C1QB   | 713            | -0.94   | 0.087         | 0.342          | 3.44E-22        | 2.71E-18        |
| ILMN_1722622 | CD163  | 9332           | -1.01   | 0.095         | 0.338          | 6.67E-22        | 4.51E-18        |
| ILMN_1805992 | KIA1598| 57698          | -1.51   | 0.144         | 0.333          | 1.91E-21        | 1.13E-17        |
| ILMN_1745963 | FOLR2  | 2350           | -1.50   | 0.145         | 0.326          | 6.27E-21        | 2.97E-17        |
| ILMN_1792473 | AIF1   | 199            | -1.19   | 0.117         | 0.319          | 2.16E-20        | 7.86E-17        |
| ILMN_1740015 | CD14   | 929            | -1.20   | 0.118         | 0.317          | 2.97E-20        | 9.06E-17        |
| ILMN_1757387 | UCHL1  | 7345           | -0.66   | 0.066         | 0.315          | 4.36E-20        | 1.15E-16        |
| ILMN_1780533 | RNASE6 | 6039           | -2.42   | 0.241         | 0.314          | 5.50E-20        | 1.30E-16        |
| ILMN_1740024 | NAALAD2| 10003          | 1.06    | 0.111         | 0.296          | 1.18E-18        | 1.22E-15        |
| ILMN_1757882 | PPP1R16A| 84988         | 1.51    | 0.157         | 0.296          | 1.20E-18        | 1.22E-15        |
| ILMN_3285959 | LOC645515| 645515        | 2.54    | 0.265         | 0.296          | 1.22E-18        | 1.22E-15        |
| ILMN_1785284 | ALDH6A1| 4329           | 1.22    | 0.124         | 0.305          | 2.79E-19        | 4.41E-16        |
| ILMN_1778104 | ACADM  | 34             | 1.44    | 0.144         | 0.311          | 9.30E-20        | 2.00E-16        |
| ILMN_1694106 | GPD1L  | 23171          | 1.37    | 0.136         | 0.317          | 3.06E-20        | 9.06E-17        |
| ILMN_1718924 | ETFA   | 2108           | 1.76    | 0.175         | 0.317          | 3.02E-20        | 9.06E-17        |
| ILMN_1690040 | TM7SF2 | 7108           | 1.14    | 0.112         | 0.320          | 1.75E-20        | 6.89E-17        |
| ILMN_1678323 | AASS   | 10157          | 2.84    | 0.279         | 0.322          | 1.42E-20        | 6.12E-17        |
| ILMN_1797154 | AZGP1  | 563            | 1.33    | 0.120         | 0.355          | 2.96E-23        | 4.64E-19        |

Results for all significant probes are shown in Supplementary Table-1. $\beta$, beta coefficient value; p-value, significance level of correlation of transcript level with Matsuda index; and adjusted $R^2$, proportion of the variation in Matsuda index (outcome) explained by expression levels (predictor) in linear regression analysis adjusted for age, gender and ancestry proportion/admixture; q-value, FDR (Benjamini-Hochberg false discovery rate) corrected p-value.
Table 2: Top 10 ranked Matsuda Insulin sensitivity-correlated \textit{cis}-eGenes in adipose tissue from African American (AAGMEx cohort) and European ancestry (METSIM) individuals.

| Gene Symbol | Chr | \(p\) | p-value | Probe_Id | Top \textit{cis}-eSNP | A1 | MAF | \(\beta\) | eQTL_\(p\)-value | \(\beta\) | p value | Probeset ID | Top \textit{cis}-eSNP | alt_allele | MAF | \(\beta\) | eQTL_\(p\)-value |
|-------------|-----|-------|---------|----------|-------------------|----|-----|--------|----------------|--------|--------|-------------|-----------------|------------|-----|--------|----------------|
| ECHDC3      | 10  | 0.471 | 5.33E-15| ILMN_2072178 | rs200943982      | T  | 0.401| -0.281| 1.94E-09       | 0.342  | 2.35E-22| 11728810_a_at | rs3814627      | G           | 0.312| -0.755| 8.3E-52 |
| MIF         | 22  | -0.390| 2.26E-10| ILMN_1807074 | rs4822455        | T  | 0.435| -0.188| 4.56E-13       | -0.257| 5.64E-13| 11744470_x_at | rs4822443      | A           | 0.225| -0.989| 1.3E-78 |
| CENPV       | 17  | 0.411 | 1.86E-11| ILMN_3246608 | rs3112521        | T  | 0.427| 0.107 | 5.83E-09       | 0.288  | 4.85E-16| 11722721_a_at | rs3112526      | C           | 0.485| 0.751 | 2.3E-53 |
| DDT         | 22  | 0.362 | 4.71E-09| ILMN_1690982 | rs79966373       | G  | 0.178| -0.302| 2.81E-14       | 0.277  | 5.30E-15| 11731089_a_at | chr22:24334948 | C           | 0.353| -1.055| 1E-125  |
| APOB        | 2   | 0.401 | 6.50E-11| ILMN_1664024 | rs1429974        | T  | 0.241| 0.293 | 5.28E-09       | 0.256  | 7.13E-13| 11758035_s_at | rs11693870     | C           | 0.265| 0.950 | 9E-72   |
| ORM DL3     | 17  | 0.445 | 2.24E-13| ILMN_1662174 | rs28820390       | A  | 0.091| -0.355| 3.37E-07       | 0.392  | 1.52E-29| 11736188_a_at | rs2872516      | T           | 0.495| -0.565| 1.2E-29 |
| GLIPR1      | 12  | -0.458| 3.55E-14| ILMN_1769245 | rs117251563      | A  | 0.013| 0.705 | 8.07E-07       | -0.217 | 1.30E-09| 11721839_at   | rs35619460     | C           | 0.194| -1.445| 1E-158  |
| ACS33       | 12  | 0.449 | 1.32E-13| ILMN_1659885 | rs4842386        | G  | 0.167| -0.111| 6.97E-06       | 0.332  | 2.99E-21| 11759049_at   | rs3794325      | A           | 0.332| -0.919| 5.9E-81  |
| ITIH5       | 10  | -0.434| 9.54E-13| ILMN_1731862 | rs201694044      | G  | 0.375| -0.154| 5.05E-07       | -0.325 | 2.66E-20| 11734677_x_at | rs867490       | G           | 0.312| 0.610 | 6.8E-33  |
| DMRT2       | 9   | 0.401 | 6.05E-11| ILMN_1751785 | rs756145         | G  | 0.315| 0.282 | 2.77E-08       | 0.300  | 1.80E-17| 11727728_a_at | rs10959032     | T           | 0.271| -0.699| 9.4E-37  |

Matsuda insulin sensitivity-correlated transcripts of Entrez_ID genes in adipose tissue associated with a SNP (Q-value<0.04) within ±500kb of the 5' and 3' end of the transcript in AAGMEx are shown. Data for most significantly associated genotyped \textit{cis}-eSNP in AAGMEx are presented. Data on Europeans ancestry males are from METSIM cohort (Civelek et al., 2017). In AAGMEx: \(p\), Spearman partial correlation coefficient; p-value, significance level of correlation of transcript level with Matsuda index in Spearman partial correlation analysis; A1, Minor Allele; MAF, Minor Allele Frequency; \(\beta\), effect size of minor allele (A1); eQTL_\(p\)-value, significance in additive model (in MatrixEQTL analysis). In METSIM: \(\beta\), beta value; and p-value, significance level of correlation of transcript level with Matsuda index in linear regression analysis; alt_allele, alternative allele; \(\beta\), effect size for alt_allele of best \textit{cis}-eSNP in FaST-LMM eQTL analysis. Results for all significant probes are shown in Supplementary Table-4. Top \textit{cis}-eSNPs are not the same in both cohorts, but comparison of their effects are shown in Supplementary Table-4A and 4B and in Supplementary Figure-3.
Figure Legends:

**Figure 1.** Enoyl-CoA hydratase domain-containing-3 (*ECHDC3*) transcript expression in adipose tissue is correlated with Matsuda index of insulin sensitivity and is genetically regulated in African Americans.

The scatter plot shows correlation of *ECHDC3* transcript expression (ILMN_2072178) in adipose tissue with Matsuda index in AAGMEx (A). The box plot shows association of *ECHDC3* transcript expression (ILMN_2072178) in adipose with genotype of the *cis*-eSNP rs200943982 (B). LocusZoom plots show regional association of *ECHDC3* *cis*-eQTL region SNPs (genotyped and imputed) with transcript expression (C). Significance level (-log10 p-values) of genotyped SNPs are indicated as circles and imputed SNPs are indicated as squares in the LocusZoom plot. Linkage disequilibrium (LD) plot below the LocusZoom plot shows LD relationship (r²) between SNPs in the marked region and indicates the location of top imputed and genotyped *cis*-eSNP for ECHDC3, rs34844369 and rs200943982, respectively.

**Figure 2:** In subcutaneous adipose tissue Enoyl-CoA hydratase domain-containing-3 (*ECHDC3*) is primarily expressed in adipocytes and *ECHDC3* expression is induced with adipocyte differentiation.

Results from the qRT-PCR analysis of expression of *ECHDC3* mRNA (normalized to RPLP0 (36B4) endogenous control gene) are shown. A) Relative expression of *ECHDC3* in pooled RNA samples from stromal vascular fractions (SVF) compared to the adipocyte fraction (AF) of subcutaneous adipose tissue in African Americans (data for two technical replicates of the pooled RNA samples from 5-6 individual donors are shown). Expression of ECHDC3 at different stages of in vitro differentiation of B) human adipose-derived stem cells (hADSCs) and C) Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes. Data is shown for independent biological triplicates at each stage of differentiation.

**Figure 3:** Knocking down of Enoyl-CoA hydratase domain-containing-3 (*ECHDC3*) gene in Simpson-Golabi-Behmel syndrome (SGBS) adipocytes affects insulin sensitivity. A) Lentiviral particle-mediated delivery of gene-specific shRNA expression vector stably knocks down *ECHDC3* mRNA levels in SGBS adipocytes. Results from the qRT-PCR analysis of expression of *ECHDC3* mRNA (normalized to RPLP0 (36B4) endogenous control gene) in SGBS cells stably expressing control-shRNA or *ECHDC3*-shRNA at 14
days post initiation of differentiation are shown. Bar graph indicates the mean ± SD of six biological replicates for each condition from two independent experiments. **B)** Basal and insulin stimulated glucose uptake in control-shRNA and ECHDC3-shRNA (ECHDC3-KD) SGBS cells at 14 day of differentiation. Bar graph indicates the mean ± SD of biological triplicates for each condition. Western blot analysis in 14 day of differentiated control-shRNA and ECHDC3-KD SGBS cells using antibodies specific for pAKt, total-Akt and GAPDH at basal and insulin-stimulated (100nM for 15min) conditions (C), and quantified data (mean ± SD of biological triplicates) from scanning of a representative (selected from two independent experiments) western blot (D) is shown.

**Figure 4: Knocking down Enoyl-CoA hydratase domain-containing-3 (ECHDC3) gene in adipocytes modulates many pathways including genes in insulin sensitivity.** A) Bar graph showing results from the qRT-PCR analysis of expression of adiponectin (ADIPOQ), peroxisome proliferator activated receptor gamma (PPARG) and facilitated glucose transporter-4 (GLUT4/SLC2A4) mRNA (normalized to RPLP0) in SGBS cells stably expressing control-shRNA or ECHDC3-shRNA at 14 days post initiation of differentiation are shown. Bar graph indicates the mean ± SD of six biological replicates for each condition from two independent experiments. *, p<0.01. **B)** Heat map from unsupervised hierarchical clustering showing expression of 691 differentially expressed genes in ECHDC3-knockdown cells (E) compared with control shRNA (C) treated SGBS cells at 14 day of differentiation. The expression levels of genes from RNA-seq analysis (FPKM values) were Z-score transformed for clustering analysis. The intensity of purple and blue color in heat map indicates the degree of high and low expression of transcripts, respectively. **C)** qRT-PCR analysis validates downregulation of fatty acid desaturase 1 (FADS1) in ECHDC3-knockdown SGBS cells in six biological replicates for each condition from two independent experiments. **D)** A network diagram from Ingenuity pathway analyses (IPA) that include selected differentially expressed genes (DEGs) in ECHDC3-KD cells, and were significantly enriched for genes involved in the metabolism of triacylglycerol, fatty acid metabolism, and insulin resistance. **E)** Top regulatory network (consistency score =20.85 and 37 nodes ) identified by regulator effects analysis in IPA connected 23 DEGs with 8 putative network regulators in ECHDC3-KD cells and
suggested its impact on multiple biological processes in adipocytes including accumulation of lipid and adipogenesis.
Figure 1. Enoyl-CoA hydratase domain-containing-3 (ECHDC3) transcript expression in adipose tissue is correlated with Matsuda index of insulin sensitivity and is genetically regulated in African Americans.
Figure 2: In subcutaneous adipose tissue Enoyl-CoA hydratase domain-containing-3 (ECHDC3) is primarily expressed in adipocytes and ECHDC3 expression is induced with adipocyte differentiation.

130x182mm (300 x 300 DPI)
Figure 3: Knocking down of Enoyl-CoA hydratase domain-containing-3 (ECHDC3) gene in Simpson-Golabi-Behmel syndrome (SGBS) adipocytes affects insulin sensitivity.
Figure 4: Knocking down Enoyl-CoA hydratase domain-containing-3 (ECHDC3) gene in adipocytes modulates many pathways including genes in insulin sensitivity.
Online Appendix:

Genetic regulation of enoyl-CoA hydratase domain-containing 3 in adipose tissue determines insulin sensitivity in African Americans and Europeans

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**Supplementary figure:**

**Supplementary Figure 1: Plots of Admixture coefficients.** HapMap Phase 3 CEU (Caucasian), YRI (African), and CHB (Chinese) samples were used as reference panels and were merged with AAGMEx study samples and admixture estimates were computed using the software ADMIXTURE (http://software.genetics.ucla.edu/admixture/; Alexander et al., Genome Research, 19:1655–1664, 2009). AAGMEx participants are labeled as INSTUDY in the plots. Three samples with >50% European ancestry proportion were excluded from further analyses.
Supplementary Figure 2: Correlation of adipose tissue transcript levels with Matsuda index and their genetic regulation in African Americans replicates in Metabolic Syndrome in Men (METSIM), an independent European ancestry cohort. A) Heat map from unsupervised hierarchical clustering showing high concordance in the direction of correlation of 3,849 adipose tissue transcripts correlated with Matsuda index (q<0.05) in both AAGMEx and METSIM cohorts. B) The scatter plot shows correlation of $ECHDC3$ transcript expression ($11728810\text{.a\_at}$) in adipose tissue with Matsuda index in METSIM. C) The box plot shows association of $ECHDC3$ transcript expression in adipose with genotype of the top cis-eSNP rs3814627 in METSIM. The box represents the interquartile range, which contains 50% of the values. The whiskers are lines that extend the box to the highest and lowest values, excluding outliers. A line across the box indicates the median. D) LocusZoom plots show regional association of $ECHDC3$ cis-eQTL region SNPs with transcript expression in METSIM cohort.
Supplementary Figure 3: Comparison of the effect of top cis-eSNPs for Matsuda index-correlated eGenes in AAGMEx and METSIM cohort. A different genotyping platform was used in the two cohorts, and based on genotype data availability; of the total 587 common Matsuda index-correlated cis-eGenes, we were able to retrieve results for 498 METSIM top cis eSNPs from the AAGMEx dataset and 363 AAGMEx top-cis-eSNPs from the METSIM dataset. After carefully matching the effect allele we observed that 52% (259/498) of the METSIM top cis-eSNPs were also significant in AAGMEx (p<0.05). (A) METSIM top cis-eSNPs replicated in AAGMEx shows 91.5% concordance in effect direction and strong correlation in effect size (r=0.70). Similarly, 65.8% (239/363) of the AAGMEx top cis-eSNPs were also significant in the METSIM cohort (p<0.01). (B) AAGMEx cis-eSNPs replicated in METSIM shows 86.6% concordance in effect direction and a strong correlation in effect size (r=0.62).
Supplementary Figure 4: Enoyl-CoA hydratase domain-containing-3 (ECHDC3) transcript expression in adipose tissue is correlated with Matsuda index of insulin sensitivity in all genotype groups. The scatter plot shows correlation of ECHDC3 transcript expression (ILMN_2072178) in adipose tissue with Matsuda index in AAGMEx. Results for genotype groups based on top genotyped (rs200943982) and imputed (rs34844369) ECHDC3 cis-eSNP are shown in different colors. Results from linear regression analysis are also shown in a tabular format.

| SNP      | Genotype | N  | β    | SE(β)  | R-square full | Adjusted R-square | P-value  |
|----------|----------|----|------|--------|---------------|-------------------|----------|
| rs200943982 | CC       | 89 | 0.96284 | 0.13725 | 0.3910        | 0.3620          | 5.44x10^{-10} |
|          | CT       | 113 | 0.57119 | 0.14921 | 0.1646        | 0.1337          | 2.17x10^{-4}   |
|          | TT       | 39  | 0.91404 | 0.19380 | 0.4534        | 0.3891          | 3.99x10^{-5}   |
| rs34844369 | GG       | 75  | 0.93036 | 0.14756 | 0.4004        | 0.3662          | 2.25x10^{-8}   |
|          | AG       | 122 | 0.64326 | 0.14759 | 0.1559        | 0.1270          | 2.83x10^{-5}   |
|          | AA       | 46  | 0.78834 | 0.16290 | 0.4760        | 0.4249          | 1.88x10^{-3}   |
Supplementary tables:

(in Microsoft –Excel file: MATSUDA-ECHDC3 paper-Supplementary tables-final-Diabetes.xlsx; can be viewed in [Google drive link:](https://drive.google.com/file/d/1ddJ8xaz4AvsZhsLraPfdIxfyuajScsu/view?usp=sharing)

**Supplementary Table 1: Expression levels of subcutaneous adipose tissue transcripts are correlated with Matsuda Insulin sensitivity index in AAGMEx.** Transcripts of genes with Entrez gene id and correlated (q<0.01) with Matsuda index, based on regression analysis (adjusted for age, gender and ancestry proportion/admixture) are shown. Results in Column L-Q are based on secondary analysis adjusted for age, gender, BMI, and admixture.

**Supplemental Table 2A: Adipose tissue transcripts correlated with Matsuda insulin sensitivity index in AAGMEx are enriched for genes in biological pathways.** Results from Ingenuity Pathway Analysis (IPA) for adipose tissue genes correlated with Matsuda index (q<0.01 in Supplemental Table 1) are shown. Significant pathways (two-sided Fisher's exact test p-value < 0.05) are shown. Predicted direction of activation and number of genes negatively (β < 0) or positively (β > 0) correlated with Matsuda index are also shown.

**Supplemental Table 2B: Adipose tissue transcripts correlated with Matsuda insulin sensitivity index in AAGMEx are enriched for genes in biological pathways.** Results from DAVID v6.9 analysis for adipose tissue genes correlated with Matsuda index (q<0.01 in Supplemental Table 1) are shown. Selected significant pathways (Benjamini-Hochberg adjusted p-value < 0.05) are shown.

**Supplemental Table 3: Comparison of correlation of subcutaneous adipose tissue transcripts with Matsuda insulin sensitivity index in African Americans from AAGMEx cohort and Europeans from METSIM cohort.** Most significantly correlated probes for each gene in METSIM study (from Civelek et al., 2017) are shown. Sorted list of genes significant (FDR-P /q -value <0.05) in both cohort are shown. List sorted on average ranking on significant correlation in both cohorts.

**Supplementary Table 4: Matsuda Insulin sensitivity-correlated transcripts are cis-eQTL in adipose tissue from both African American (AAGMEx cohort) and European ancestry (METSIM) individuals. Sorted list of 587 Matsuda index-correlated cis-eGenes based on average rank of genes on Matsuda index correlation**
and eQTL analysis p-values in AAGMEx and METSIM cohort. Matsuda insulin sensitivity-correlated transcripts of Entrez_ID genes in adipose tissue associated with a SNP (Q-value<0.04) within ±500kb of the 5' and 3' end of the transcript in AAGMEx are shown. Data on Europeans ancestry males are from METSIM cohort (Civelek et al., 2017). Data for most significantly associated genotyped cis-eSNP in AAGMEx and best cis-eSNP in FaST-LMM eQTL analysis in METSIM are presented. In AAGMEx: $\rho$, Spearman partial correlation coefficient; p value, significance level of correlation of transcript level with Matsuda index in Spearman partial correlation analysis; A1, Minor Allele; A2, Major Allele; MAF, Minor Allele Frequency; beta, effect size of minor allele (A1); eQTL p-value, significance in additive model (in MatrixEQTL analysis). Count Of cis-eSNP, Number of genotyped cis-SNPs (MAF>0.01) associated with transcript at Q-value $\leq$0.04. In METSIM: $\beta$, beta value; and p-value, significance level of correlation of transcript level with Matsuda index in linear regression analysis; alt_allele, alternative allele; $\beta$, effect size for alt_allele of best cis-eSNP in FaST-LMM eQTL analysis.

Supplementary Table 4A: Most significantly associated cis-eSNP for Matsuda index-correlated cis-eGenes in METSIM are shown and effect of the same SNP in AAGMEx are shown for comparison.

Supplementary Table 4B: Most significantly associated genotyped cis-eSNP for Matsuda index-correlated cis-eGenes in AAGMEx are shown and effect of the same SNP in METSIM are shown for comparison.

Supplementary Table 4C: Matsuda Insulin sensitivity-correlated transcripts are cis-eQTL in adipose tissue from African American (AAGMEx cohort) individuals in BMI adjusted analyses.

Supplementary Table 5: Expression of ECHDC3 Transcripts levels in adipose tissues of African Americans from AAGMEx participants are genetically regulated. Results for significant (p < 1X10^-4) genotyped or imputed cis-eSNPs for ECHDC3 (ILMN_2072178) from expanded cis-eQTL analysis are presented. MAF, Minor allele frequency; A1, Minor Allele; A2, Major Allele; MAF, Minor Allele Frequency; beta, effect size of minor allele (A1); eQTL p-value, significance in additive model (in MatrixEQTL analysis); TSS, transcription start site. Data in Column L-V shows association statistics of respective cis-eSNPs with
MATSDA insulin sensitivity index in AAGMEx cohort. RAF, reference allele frequency; Pvalue, significance from best genetic model; Add_Pvalue, p-value from additive genetic model.

**Supplementary Table 6:** cis-eQTL for *ECHDC3* in adipose tissue samples of European ancestry individuals from METSIM cohort. eQTL results are from Civelek M et al, Am J Hum Genet 2017;100(3):428-43. *ECHDC3* cis-eSNPs (p<1x10^{-3}) from subcutaneous adipose tissue eQTL analysis in METSIM study is shown. MAF= Minor Allele Frequency; effect_size=allele effect for alt_allele. Data in Column L-N shows association statistics of respective cis-eSNPs with MATSUDA insulin sensitivity index in METSIM cohort. P-values from two linear regression models are shown.

**Supplementary Table 7:** Epigenetic regulatory annotation of *ECHDC3* cis-eSNPs identified in AAGMEx, METSIM and GTEx study.

A) Annotation based on Haploreg-v4.1 [https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php](https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php) and

B) Annotation based on RegulomeDb-v1.1 [http://regulomedb.org/](http://regulomedb.org/)

C) Annotation based on SNP2TFBS (https://ccg.vital-it.ch/snp2tfbs/) web interface aimed at studying variations (SNPs/indels) that affect transcription factor binding (TFB) in the Human genome. ECHDCE3 cis-eSNPs with ScoreDifference for TF binding > or < 0, estimated based on position weight matrix (PWM) models are shown.

**Supplemental Table 8:** RNA-seq analysis showing transcripts for protein coding genes differentially expressed in mature SGBS adipocytes (14^{th} day differentiation) expressing ECHDC3-shRNA compared to SGBS adipocytes expressing control-shRNA. Differentially expressed coding genes (DEGs) with Bayes posterior probability ≥70% in NOISeq and average log2 fold change (log2FC) +/-0.58 are shown.

**Supplemental Table 9:** Ingenuity pathway analyses (IPA) suggest enrichment of canonical pathways among genes differentially expressed in mature SGBS adipocytes (14^{th} day differentiation) expressing ECHDC3-shRNA compared to SGBS adipocytes expressing control-shRNA. Selected categories (-log p-value>1.3) are shown.
Supplemental Table 10: Ingenuity pathway analyses (IPA) suggest enrichment of disease or biological function among genes differentially expressed in mature SGBS adipocytes (14 days differentiation) expressing ECHDC3-shRNA compared to SGBS adipocytes expressing control-shRNA. Selected categories (metabolism or lipid) are shown.

Supplemental Table 11: Considering DEGs in ECHDC3-shRNA expressing SGBS cells, regulator effects analysis in IPA suggest the effect of upstream regulators on expression of downstream target molecules and its impact on biological function and diseases.