# Impact of an Exercise Intervention on DNA Methylation in Skeletal Muscle From First-Degree Relatives of Patients With Type 2 Diabetes

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To identify epigenetic patterns, which may predispose to type 2 diabetes (T2D) due to a family history (FH) of the disease, we analyzed DNA methylation genome-wide in skeletal muscle from individuals with ($FH^+$) or without ($FH^-$) an FH of T2D. We found differential DNA methylation of genes in biological pathways including mitogen-activated protein kinase (MAPK), insulin, and calcium signaling ($P \leq 0.007$) and of individual genes with known function in muscle, including *MAPK1*, *MYO18B*, *HOXC6*, and the AMP-activated protein kinase subunit *PRKAB1* in skeletal muscle of $FH^+$ compared with $FH^-$ men. We further validated our findings from $FH^+$ men in monozygotic twin pairs discordant for T2D, and 40% of 65 analyzed genes exhibited differential DNA methylation in muscle of both $FH^+$ men and diabetic twins. We further examined if a 6-month exercise intervention modifies the genome-wide DNA methylation pattern in skeletal muscle of the $FH^+$ and $FH^-$ individuals. DNA methylation of genes in retinol metabolism and calcium signaling pathways ($P < 3 \times 10^{-6}$) and with known functions in muscle and T2D including *MEF2A*, *RUNX1*, *NDUFC2*, and *THADA* decreased after exercise. Methylation of these human promoter regions suppressed reporter gene expression in vitro. In addition, both expression and methylation of several genes, i.e., *ADIPOR1*, *BDKRB2*, and *TRIB1*, changed after exercise. These findings provide new insights into how genetic background and environment can alter the human epigenome. ***Diabetes*** **61:3322–3332, 2012**

The prevalence of type 2 diabetes (T2D) is rapidly increasing worldwide. Although genome-wide association studies have identified polymorphisms contributing to the risk of T2D, a person's lifestyle is a key factor in the development of the disease (1–3). Indeed, several studies have shown that the risk of T2D can be halved in high-risk groups through nonpharmacological lifestyle interventions involving exercise and diet (4,5). These studies show that the effect is rapid and does not require intensive interventions. Although little is known about the genes that convey the effects in these interventions, changes in DNA methylation have been suggested as a potential molecular mechanism through which exercise and diet mediate their effects on the transcriptome (6). Indeed, dietary factors can affect the degree of DNA methylation (7–11). However, whether an exercise intervention changes DNA methylation genome-wide in skeletal muscle is unknown. A family history (FH) of T2D increases the risk of developing the disease and may also affect the individual's response to physical exercise (2,12–14). Yet, the impact of an FH of T2D on the genome-wide DNA methylation pattern in skeletal muscle is unknown. The objective of this study was therefore to study global DNA methylation patterns in skeletal muscle from individuals with or without an FH of T2D ($FH^+$ and $FH^-$, respectively) before and after an exercise intervention.

## RESEARCH DESIGN AND METHODS

**Cohorts.** Fifteen men with ($FH^+$) and 13 men without ($FH^-$) a first-degree FH of T2D were included in this study (Table 1). All $FH^+$ men had at least one first-degree relative with T2D. At screening, 82 ± 29 days prior to the start of the study, the subjects underwent a physical examination and a 75-g oral glucose tolerance test, in which glucose levels were measured at 0 and 120 min (Table 1). At inclusion, the participants were healthy but sedentary. Based on self-report in which fitness level is rated on a scale of 1–5 (1 is the lowest level), the participants' overall fitness level was 1.75 ± 0.58 prior to inclusion. A total of 25 of the participants were nonsmokers, and 3 were smokers (2 $FH^+$ and 1 $FH^-$). Anthropomorphic measurements and a max biking test using an ergometer bicycle (Marquette-Hellige Medical Systems 900ERG; Milwaukee, WI) were administered at the start of the exercise intervention (Table 1). The $FH^+$ and $FH^-$ groups were groupwise matched for age, sex, BMI, and $V_{O_{2max}}$ at baseline, and there were no significant differences in weight, BMI, waist-to-hip ratio, blood pressure, pulse, and $V_{O_{2max}}$ between the $FH^+$ and $FH^-$ men (Table 1). A muscle biopsy was taken from the vastus lateralis muscle in the fasting state under local anesthesia (1% lidocaine) using a 6-mm Bergström needle (Stille AB, Sweden). The participants were instructed to refrain from vigorous exercise for 48 h prior to the biopsy.

All $FH^+$ and $FH^-$ men participated in a 6-month supervised exercise intervention consisting of mainly endurance exercise. The participants were enrolled in a group training program including one session of 1-h spinning class and two sessions of 1-h aerobic class per week led by a certified instructor. On average, the participation level was 44.3 ± 3.5 sessions, which is slightly less than two sessions per week. After a 6-month exercise intervention and 48 h after the last bout of exercise, a second muscle biopsy and anthropomorphic measurements were taken, and $V_{O_{2max}}$ was analyzed with a max biking test (Table 1). Participants were invited 30 ± 11 days after the intervention for a second oral glucose tolerance test (follow-up).

Nine monozygotic twin pairs discordant for T2D were identified from the Swedish Twin Registry. They underwent clinical examinations, and muscle biopsies were taken in fasting state. Their characteristics are described in Supplementary Table 1.

The studies were approved by the local ethics committee, and written informed consent was obtained from all participants.

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TABLE 1
Clinical characteristics of men with or without an FH of T2D ($FH^+$ and $FH^-$, respectively) before and after a 6-month exercise intervention

| | $FH^-$ | | $FH^+$ | |
|---|---|---|---|---|
| | Before | After | Before | After |
| *N* | 13 | | 15 | |
| Age (years) | 37.5 ± 5.2 | | 37.5 ± 4.0 | |
| Birth weight (g) | 3,498.5 ± 819.4 | | 3,613.3 ± 563.7 | |
| Weight (kg) | 87.7 ± 12.5 | 87.0 ± 11.6 | 93.1 ± 12.6 | 90.5 ± 11.9* |
| BMI ($kg/m^2$) | 27.3 ± 3.0 | 27.1 ± 2.8 | 28.4 ± 2.8 | 27.6 ± 2.8* |
| Waist circumference (cm) | 96.1 ± 9.1 | 93.3 ± 9.7* | 98.2 ± 7.9 | 94.2 ± 7.0* |
| Waist-to-hip ratio | 0.94 ± 0.05 | 0.91 ± 0.06 | 0.94 ± 0.04 | 0.92 ± 0.05* |
| Fat percentage (%) | 21.0 ± 6.0 | 21.5 ± 7.7 | 21.4 ± 5.3 | 21.8 ± 5.0 |
| Systolic BP (mmHg) | 128.2 ± 16.4 | 126.9 ± 13.1 | 135.8 ± 14.1 | 131.5 ± 14.1 |
| Diastolic BP (mmHg) | 83.9 ± 12.9 | 74.2 ± 11.8* | 90.6 ± 7.3 | 77.7 ± 10.9* |
| Pulse (bpm) | 73.5 ± 12.0 | 65.8 ± 9.0* | 71.9 ± 7.6 | 63.5 ± 10.0* |
| $Vo_{2max}$ (mL/kg/min) | 33.0 ± 5.3 | 37.1 ± 6.0* | 32.0 ± 3.5 | 36.9 ± 4.6* |
| $HbA_{1c}$ (%) | 4.3 ± 0.3 | 4.3 ± 0.3 | 4.3 ± 0.3 | 4.2 ± 0.3 |
| Fasting plasma glucose (mmol/L) | 4.32 ± 0.6 | 4.69 ± 0.3 | 4.19 ± 0.4 | 4.81 ± 0.7* |
| Fasting insulin (μU/mL) | 6.73 ± 3.1 | 6.68 ± 2.7 | 7.83 ± 3.9 | 8.84 ± 5.7 |
| Triglycerides (mmol/L) | 1.30 ± 1.1 | 1.68 ± 1.7 | 1.69 ± 1.4 | 1.07 ± 0.6* |
| LDL (mmol/L) | 3.26 ± 0.8 | 2.96 ± 0.5 | 3.49 ± 0.7 | 3.41 ± 1.0 |
| HDL (mmol/L) | 1.05 ± 0.2 | 1.14 ± 0.3 | 1.03 ± 0.2 | 1.12 ± 0.2* |
| LDL/HDL | 3.18 ± 0.9 | 2.67 ± 0.8* | 3.42 ± 0.9 | 3.19 ± 1.3 |
| Testosterone (mmol/L) | 15.8 ± 5.8 | 14.4 ± 5.8 | 14.0 ± 4.3 | 13.1 ± 2.8 |
| SHBG (nmol/L) | 22.1 ± 9.6 | 22.3 ± 7.9 | 18.5 ± 6.9 | 22.7 ± 7.7* |
| Testosterone/SHBG | 0.74 ± 0.2 | 0.66 ± 0.2 | 0.82 ± 0.3 | 0.64 ± 0.3* |
| Tumor necrosis factor-α (pg/mL) | 4.20 ± 0.71 | 4.68 ± 0.90 | 4.49 ± 0.77 | 5.08 ± 0.91 |
| Leptin (ng/mL) | 19.3 ± 13.6 | 18.1 ± 12.0 | 19.4 ± 7.5 | 12.4 ± 4.2* |
| Adiponectin (ng/mL) | 9.4 ± 5.3 | 8.5 ± 3.7 | 9.3 ± 6.0 | 8.3 ± 3.5 |
| Folate (nmol/L) | 12.75 ± 5.91 | 13.67 ± 5.19 | 10.13 ± 2.97 | 10.47 ± 3.62 |
| Homocysteine (μmol/L) | 11.25 ± 2.96 | 11.25 ± 2.70 | 14.13 ± 6.00 | 15.67 ± 8.57 |
| | Screening | Follow-up | Screening | Follow-up |
| Fasting plasma glucose (mmol/L) | 5.30 ± 0.4 | 4.98 ± 0.3# | 4.88 ± 0.6 | 4.96 ± 0.7 |
| Glucose at 120 min (mmol/L) | 5.27 ± 1.1 | 5.25 ± 1.4 | 6.00 ± 1.2 | 5.37 ± 1.4 |
| Systolic BP (mmHg) | 133.9 ± 10.8 | | 134.4 ± 13.5 | |
| Diastolic BP (mmHg) | 77.5 ± 7.7 | | 82.5 ± 8.4 | |

Data are expressed as mean ± SD. The $FH^+$ and $FH^-$ groups were matched for age, sex, BMI, and $Vo_{2max}$ at baseline, and there were no significant differences in weight, BMI, waist-to-hip ratio, blood pressure, pulse, and $Vo_{2max}$ between the $FH^+$ and $FH^-$ men. A screening was performed 82 ± 29 days prior to the start of the intervention measuring glucose levels and blood pressure, and glucose levels were measured again at a follow-up 30 ± 11 days after finishing the exercise intervention. The impact of exercise was analyzed using nonparametric paired tests, Wilcoxon signed-rank tests. The impact of an FH of T2D was analyzed using two-sample Mann-Whitney *U* tests, both at baseline and after exercise. BP, blood pressure; SHBG, sex hormone-binding globulin. *$P < 0.05$ for before vs. after; #$P < 0.05$ for screening vs. follow-up.

**MeDIP-Chip analysis of muscle.** A total of 1.4 μg of genomic DNA was sonicated to an average of 500 bp by 13 cycles of 30 s on and 30 s off at high frequency with the BioRuptor (Diagenode, Liege, Belgium). For immunoprecipitation of methylated DNA, the mc-green-03 kit was used (Diagenode). A total of 1 μg of sonicated DNA was immunoprecipitated using the 5meC antibody with Sepharose beads overnight at 4°C. Immunoprecipitated DNA was purified with the QIAquick-PCR purification kit (Qiagen, Heidelberg, Germany) prior to whole-genome amplification of the DNA with the WGA-kit (Sigma-Aldrich, Stockholm, Sweden). A total of 15 ng of sonicated but not immunoprecipitated DNA (input) was also subjected to whole-genome amplification. A total of 6 μg of whole-genome amplified DNA was hybridized to the human 2.1 promoter DeLuxe tiling array (version 081229_HG18_Promoter_MeDIP_HX1) at the Roche-Nimblegen facility (Roche, Nimblegen, Iceland). Input and immunoprecipitated samples were labeled with Cy3 and Cy5, respectively, and hybridized to the same array. The human 2.1 promoter DeLuxe tiling array covers 10,000 bp of all known genes: 7,500 bp upstream of the transcription start sites (TSS) and 2,500 bp downstream of the TSS and all annotated cytosine guanine dinucleotide (CpG) islands. The total number of probes is 2.1 million per array. A GFF annotation file provided by Nimblegen was used for localization of the probes in relation to gene TSS, and the annotation file is based on build HG18 of the University of California Santa Cruz database. The output data from the MeDIP-Chip analysis consist of log2 ratios of immunoprecipitated (Cy5) versus input (Cy3) signals for each individual probe. The log2 ratio is computed and scaled to center the ratio data around zero. Scaling is performed by subtracting the biweight mean for the log2-ratio values for all features on the array from each log2-ratio value.

**Normalization and statistical analysis of MeDIP-Chip data.** Within-array normalization was performed using model-based analysis of two-color arrays (MA2C), a normalization method for two-color tiling arrays incorporating sequence-specific probe effects (15). As MA2C standardizes probe intensities, dye bias and other nonbiological variations originating from array processing are removed. The normalized log2 ratios of immunoprecipitated versus input data were used for statistical comparisons using R software (16). The impact of an FH of T2D was analyzed using two-sample Mann-Whitney *U* tests for all probes, both at baseline and after exercise. The impact of exercise was analyzed for all probes using nonparametric paired tests: Wilcoxon signed-rank tests. The impact of exercise training was analyzed for the whole cohort ($n$ = 28) and for each FH group separately.

To examine if an FH of T2D or exercise affects the degree of DNA methylation of individual genes, we calculated the mean level of DNA methylation for respective gene only including probes with $P \leq 0.01$ due to either an FH of T2D or exercise. The impact of an FH of T2D or exercise on the mean level of methylation for respective gene was then analyzed using Mann-Whitney *U* tests or Wilcoxon signed-rank test, respectively. *P* values were then corrected for multiple testing using Bonferroni corrections and false discovery rate (FDR) analyses. Genes exhibiting differential DNA methylation with $P \leq 0.05$ after Bonferroni corrections are presented in individual supplementary tables. Moreover, genes exhibiting differential DNA methylation with $Q \leq 0.005$ after FDR were included in pathway analyses using Webgestalt (http://bioinfo.vanderbilt.edu/webgestalt/). Benjamini-Hochberg correction was used to determine the *P* values for the pathways within the Kyoto Encyclopedia of

Genes and Genomes (KEGG) database. For each comparison, the top significant KEGG pathways within Metabolism (1.1–1.8), Signal transduction (3.2), and Endocrine systems (5.2) are presented.

**Microarray analysis.** RNA was isolated from muscle with the RNA fibrous tissue kit (Qiagen). Biotin-labeled cRNA was synthesized and hybridized to the Affymetrix Custom-Array NuGO-Hs1a520180-GeneChip (http://www.nugo.org; Affymetrix), which contains 23,941 probe sets. Images were analyzed using the GeneChip Operating-System (Affymetrix), and data were normalized using the robust multiarray average algorithm (17). The impact of an FH of T2D on expression was analyzed using a two-sample Mann-Whitney *U* test, and the impact of exercise training was tested using nonparametric paired tests. Genes showing nominally significant differences in expression with $P \leq 0.01$ were included for further analysis. We next tried to identify genes displaying changes in DNA methylation ($P \leq 0.01$) and expression ($P \leq 0.01$) in the opposite direction (i.e., increased DNA methylation is associated with decreased expression or vice versa) in $FH^+$ compared with $FH^-$ men or after compared with before exercise. Moreover, a mean centroid expression value was calculated for each of the biological pathways that are among the most significant. We first normalized the expression levels on the arrays to values between 0 and 1 across all analyzed samples, in which the highest expression value on the arrays is normalized to 1. The mean centroid expression value is then calculated as the mean expression of all genes included in respective pathway. Additionally, we examined if expression correlates negatively with DNA methylation of individual probes for respective gene by Spearman correlations.

**Genetic analyses.** Single nucleotide polymorphisms (SNPs) were genotyped using HumanOmniExpress arrays according to the manufacturer's instructions (Illumina, San Diego, CA). SNP data were extracted 7.5 kb upstream and 2.5 kb downstream of the TSS for each gene exhibiting differential DNA methylation in $FH^+$ versus $FH^-$ men after Bonferroni corrections (Supplementary Table 2). SNPs were associated with DNA methylation of the respective gene based on an additive genetic model. A genetic risk score was generated for each individual by counting the number of risk alleles for SNPs previously associated with T2D (Supplementary Table 13).

**Biological validation.** DNA methylation was analyzed in muscle of monozygotic twin pairs discordant for T2D using Infinium HumanMethylation450 BeadChip (Illumina) according to the manufacturer's recommendations.

**Technical validation.** Genes were selected for technical validation of the MeDIP-Chip data based on either their inclusion in biological pathways with differential DNA methylation, or that they show differential expression, and/or play a role in T2D and/or muscle physiology. For technical validation, DNA methylation levels were determined with bisulfite conversion and EpiTYPER (Sequenom, San Diego, CA) according to Sequenom's protocol. The assays were designed with EpiDesigner (Supplementary Table 14). EpiTYPER data were generated for a subset of the included muscle samples because there was not enough DNA from some samples for technical validation. The EpiTYPER data were considered validated when both EpiTYPER and MeDIP show either increased or decreased DNA methylation of a gene with $P \leq 0.05$. To technically validate the microarray expression data, quantitative RT-PCR was used to analyze expression of *MSI2* with an ABI7900HT system and Assays-on-Demand (Hs00292670_m1; Applied Biosystems).

**Luciferase assay.** Human promoter fragments containing 2,580 bp of *THADA*, 2,460 bp of *MEF2A*, 2,700 bp of *RUNX1*, or 2,500 bp of *NDUFC2* were inserted into a CpG-free luciferase reporter vector (pCpGL-basic) provided by Klug and Rehli (18). These promoter fragments cover the gene regions analyzed in the MeDIP-Chip assay. The constructs were either mock-methylated or methylated using two different DNA methyltransferases: SssI and *Hha*I (New England Biolabs, Frankfurt, Germany). Human embryonic kidney (HEK) 293 cells were cotransfected with 100 ng pCpGL-vector either without (control) or with respective insert together with 2 ng of pRL renilla luciferase control reporter vector (pRL-CMV vector; Promega) as a control for transfection efficiency using the FuGENE-HD transfection reagent (Promega) according to the protocol. The luciferase signal was measured with the TD-20/20-Luminometer (Turner Designs).

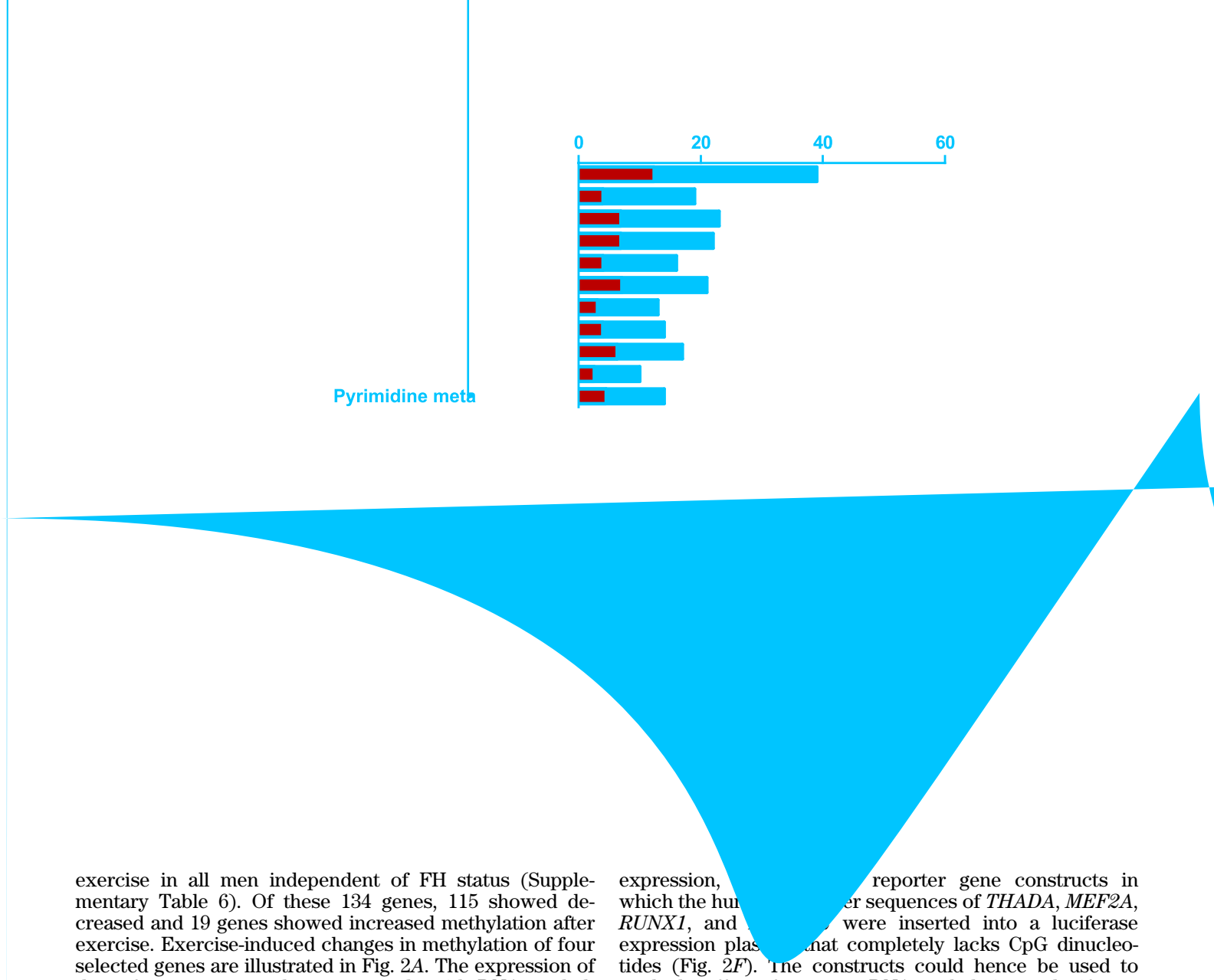
**Analyses of mitochondrial density and lipid content.** Muscle biopsies from 5 $FH^-$ and 10 $FH^+$ men, taken before and after exercise, were sectioned and fixed in 4% formaldehyde. Immunostaining was performed as previously described (19) using a primary mouse monoclonal mitochondria marker antibody, dilution 1:200, code MTCO2 (Abcam), and a secondary mouse-IgG antibody coupled to Cy2 (Jackson ImmunoResearch Laboratories, West Grove, PA). Lipid content was analyzed by staining with Oil Red O (20). Images were captured with a digital camera (Nikon-DS-2Mv; Nikon, Tokyo, Japan). Areas of immunostaining and Oil Red O staining in digitized images were analyzed using Biopix iQ software (BioPix AB, Gothenburg, Sweden).

## RESULTS

**Impact of an FH of T2D on DNA methylation.** DNA methylation was analyzed in skeletal muscle of 15 $FH^+$ and 13 $FH^-$ men using MeDIP-Chip (Table 1). The Chip comprised 2.1 million probes covering gene regions 7.5 kb upstream and 2.5 kb downstream of TSS. We identified 65 individual genes exhibiting differential DNA methylation in muscle of $FH^+$ compared with $FH^-$ men at baseline after Bonferroni correction (Supplementary Table 2). Of these 65 genes, 60 genes had decreased and 5 genes had increased DNA methylation levels in the $FH^+$ men. We next performed KEGG pathway analysis using Webgestalt (http://bioinfo.vanderbilt.edu/webgestalt/) to identify biological pathways with genes exhibiting different DNA methylation patterns in muscle of $FH^+$ compared with $FH^-$ men. A total of 2,085 genes with decreased and 603 genes with increased DNA methylation in $FH^+$ men at $Q \leq 0.005$ were included in the pathway analysis. The top biological pathways of differentially methylated genes in $FH^+$ men are shown in Fig. 1*A* and *B* and Supplementary Table 3. The mitogen-activated protein kinase (MAPK) and insulin-signaling pathways contain genes that exhibit both decreased and increased methylation (Fig. 1*A* and *B*). In contrast, the Wnt-signaling and adipocytokine-signaling pathways only include genes that exhibit decreased methylation, whereas the starch and sucrose metabolism, calcium signaling, as well as sphingolipid metabolism pathways contain genes that exhibit increased methylation in $FH^+$ versus $FH^-$ men (Fig. 1*A* and *B* and Supplementary Table 3).

DNA methylation has been associated with transcriptional silencing (21). We hence examined if any of the genes with differential DNA methylation in $FH^+$ compared with $FH^-$ men also showed different levels of expression. Using microarray data, we identified 46 genes in which differences in DNA methylation ($P \leq 0.01$) were also associated with differential expression ($P \leq 0.01$) (Supplementary Table 4). We further examined if there is any concordance between the top biological pathways of genes that are differentially methylated in $FH^+$ compared with $FH^-$ men and differential expression of the mean centroid expression value of these pathways. The mean centroid expression value of adipocytokine signaling pathway showed differential expression in muscle of men with an FH of T2D ($P = 0.007$). Moreover, DNA methylation correlated negatively with the expression level for 534 genes at $P \leq 0.001$ in the whole cohort at baseline.

We next addressed whether genetic variation could infl



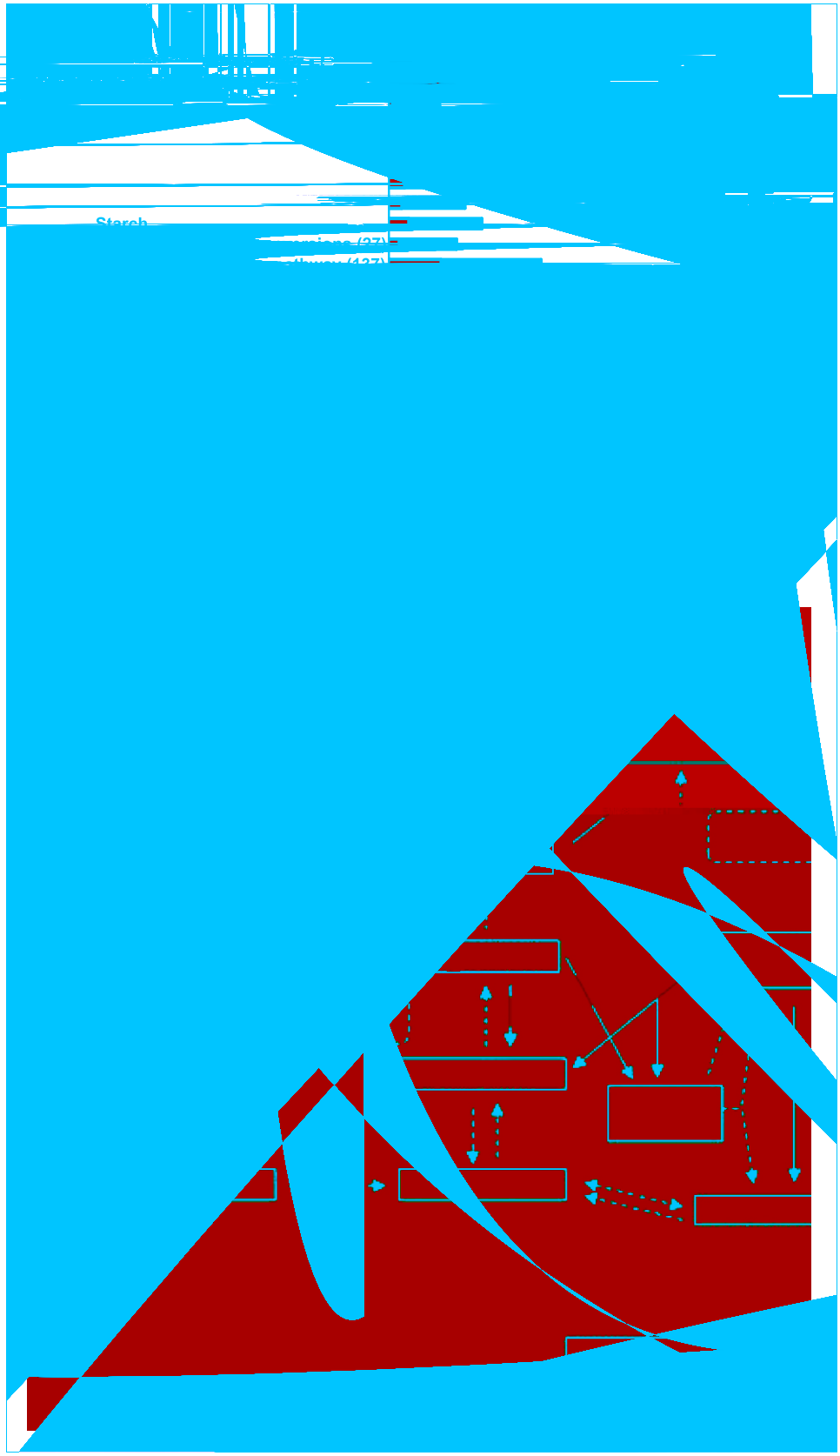

exercise in all men independent of FH status (Supplementary Table 6). Of these 134 genes, 115 showed decreased and 19 genes showed increased methylation after exercise. Exercise-induced changes in methylation of four selected genes are illustrated in Fig. 2*A*. The expression of these four genes correlates negatively with DNA methylation (Fig. 2*B*–*E*). To functionally test if promoter DNA methylation of these genes is associated with reduced expression, reporter gene constructs in which the hu er sequences of *THADA*, *MEF2A*, *RUNX1*, and were inserted into a luciferase expression plas hat completely lacks CpG dinucleotides (Fig. 2*F*). The constructs could hence be used to study the effect of promoter DNA methylation on luciferase activation in transfection assays. Each construct was mock-methylated or methylated with two methyltransferases.

While SssI methylates all CpG sites, HhaI only methylates the internal cytosine residue in a G**CG**C sequence. Hence, SssI results in totally methylated constructs, and Hha1 gives point methylated constructs in which only a fraction of the CpG sites are methylated. The number of CpG sites that may be methylated in a respective construct is shown in Fig. 2*F*. HEK293 cells were transfected with the mock-methylated or methylated constructs. The highest reporter gene expression was generated by the mock-methylated constructs including the promoter regions (Fig. 2*F*). Furthermore, methylation of the human *THADA*, *MEF2A*, *RUNX1*, and *NDUFC2* promoter regions suppressed reporter expression. While total methylation of the promoter regions by SssI suppressed reporter gene expression to 3.6 ± 2.7%, $P = 0.004$ for *THADA*; 0.8 ± 0.01%, $P = 0.006$ for *MEF2A*; 20.4 ± 9.4%, $P = 0.005$ for *RUNX1*; and 16.3 ± 3.7%, $P = 0.009$ for *NDUFC2*, point methylation by Hha1 suppressed the reporter expression to 32.8 ± 4.5%, $P = 0.01$ for *THADA*; 5.9 ± 1.5%, $P = 0.007$ for *MEF2A*; and 62.2 ± 15.4%, $P = 0.08$ for *RUNX1* (Fig. 2*F*). The *NDUFC2* promoter contains no G**CG**C sequence and could thus not be methylated by Hha1.

We next tried to identify some functional changes related to the genes that exhibit differential DNA methylation in muscle after exercise (Supplementary Table 6 and Fig. 2*A*). Because NDUFC2 is part of the respiratory chain, we studied mitochondria in muscle. With morphological analyses, we found that mitochondrial density and lipid content increased in muscle after exercise (Fig. 2*G*). Moreover, because *IL7* belongs to the genes that exhibit decreased methylation after exercise (Supplementary Table 6), we analyzed mRNA expression in muscle and serum levels of interleukin-7 (IL-7). Both muscle expression and serum levels of IL-7 increased after exercise (Fig. 2*G*).

To identify biological pathways among genes that exhibit differences in DNA methylation after exercise, we performed a KEGG pathway analysis. A total of 2,051 genes with decreased and 766 genes with increased methylation at $Q \leq 0.005$ after exercise were included in the pathway analysis. The most significant pathways of genes with altered DNA methylation due to exercise are shown in Fig. 3*A* and *B* and Supplementary Table 7. Genes involved in retinol metabolism, calcium-signaling pathway, starch and sucrose metabolism, and the insulin-signaling pathway exhibit decreased methylation after exercise. The genes in the starch and sucrose metabolism pathway exhibiting decreased DNA methylation in muscle after exercise are further shown in Fig. 3*C*. Moreover, genes involved in purine metabolism, glycine, serine, threonine metabolism, insulin signaling, and glycolysis/gluconeogenesis exhibit increased methylation after exercise.

We further identifi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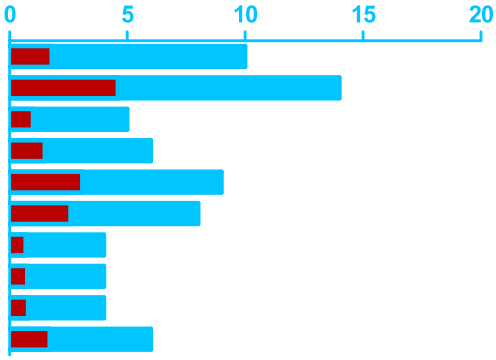


**Technical validation**

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## DISCUSSION

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calcium signaling and of individual genes including *PRKAB1* and *MAPK1*. The protein encoded by *PRKAB1* is a regulatory subunit of AMP-activated protein kinase, which is an enzyme that monitors cellular energy status and regulates metabolism in muscle (30). MAPK1 is also known to have important physiological and metabolic roles in human muscle (31). Pathway analyses provide overviews, not detailed descriptions of individual members. The KEGG analyses summarize our genome-wide methylation data in biological pathways. These results were followed up, and mean centroid expression values were calculated showing that with decreased methylation, there was increased overall expression of some pathways.

To test if DNA methylation in muscle of $FH^+$ men is

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netic differences we find between $FH^+$ and $FH^-$ individuals may be due to genetic factors, future studies are needed to determine whether the observed differences are inherited or if they are simply due to a shared environment within families. Although the epigenome may be dynamic and change due to environmental exposures, once epigenetic modifications are introduced they may be both stable and inherited (29,35–37). Although epigenetics is strongly linked to certain disease states, including Retts syndrome, Prader-Willi syndrome, and transient neonatal diabetes (38–40), there are few studies that describe associations

TABLE 4
Technical validation of MeDIP-Chip data using EpiTYPER

| | Impact of an FH of T2D on DNA methylation at baseline | | | | | | |
|---|---|---|---|---|---|---|---|
| | EpiTYPER data | | | | | MeDIP data | |
| | Assay location | | | | | Difference in DNA methylation | |
| Gene | Start | End | $FH^+$ (%) | $FH^-$ (%) | *P* value | $FH^+$ vs. $FH^-$ | *P* value |
| *AKT1* | −3,071 | −3,409 | 94.1 ± 2.8 | 91.3 ± 2.7 | 0.039 | 0.50 ± 0.15 | $8.4 \times 10^{-4}$ |
| *PPARG* | −375 | 20 | 32.8 ± 8.8 | 25.8 ± 7.3 | 0.035 | 0.44 ± 0.21 | 0.028 |
| *AHCTF1* | −2,250 | −1,841 | 87.0 ± 6.9 | 92.1 ± 0.8 | 0.011 | −0.55 ± 0.13 | 0.0006 |
| *ADCY6* | −2,743 | −2,357 | 20.2 ± 5.4 | 25.7 ± 7.7 | 0.043 | −0.58 ± 0.19 | 0.007 |
| | Impact of exercise on DNA methylation in all men | | | | | | |
| | EpiTYPER data | | | | | MeDIP data | |
| | Assay location | | Before exercise | After | | Difference in DNA methylation | |
| | Start | End | (%) | exercise (%) | *P* value | after vs. before exercise | *P* value |
| *PPARGC1A* | −336 | −2 | 6.3 ± 4.5 | 2.6 ± 2.9 | $4 \times 10^{-4}$ | −0.49 ± 0.13 | $9 \times 10^{-4}$ |
| *PHTF2* | −5,421 | −5,141 | 48.8 ± 8.7 | 53.7 ± 10.0 | 0.036 | 0.31 ± 0.10 | 0.005 |
| *MSI2* | −3,230 | −2,917 | 22.1 ± 9.9 | 16.4 ± 7.6 | 0.011 | −0.53 ± 0.18 | 0.006 |
| | Impact of an FH of T2D on DNA methylation after the exercise intervention | | | | | | |
| | EpiTYPER data | | | | | MeDIP data | |
| | Assay location | | | | | Difference in DNA methylation | |
| | Start | End | $FH^+$ (%) | $FH^-$ (%) | *P* value | $FH^+$ vs. $FH^-$ | *P* value |
| *GRB10* | −3,714 | −3,360 | 91.9 ± 3.9 | 95.6 ± 2.5 | 0.018 | −0.63 ± 0.08 | $1 \times 10^{-5}$ |
| *ADCY6* | −2,743 | −2,357 | 52.8 ± 1.9 | 56.6 ± 3.2 | 0.001 | −0.48 ± 0.14 | 0.0040 |
| *MSI2* | −3,230 | −2,917 | 13.1 ± 8.2 | 20.7 ± 3.8 | 0.038 | −0.53 ± 0.15 | 0.0016 |

Data are mean ± SEM. EpiTYPER data were generated for a subset of the included muscle samples. The difference in DNA methylation for the MeDIP data was generated by subtracting the log2 ratio of $FH^-$ men from the log2 ratio of $FH^+$ men or by subtracting the log2 ratio before exercise from the log2 ratio after exercise.

between epigenetic modifications and metabolic disease in humans (6). Epigenetic modifications have, however, been linked to metabolic disorders in animal models (29,37,41).

This is also the first study examining the impact of an exercise intervention on DNA methylation genome-wide in human muscle. We demonstrate that exercise for 6 months is associated with epigenetic changes, e.g., decreased DNA methylation of *RUNX1* and *MEF2A*, two key transcription factors involved in exercise training (42–44), of *THADA*, previously associated with T2D (1), and of *NDUFC2*, which is part of the respiratory chain (45) was observed after exercise. MEF2A is a transcription factor involved in the exercise-induced regulation of *GLUT4* expression, and hence it may influence glucose uptake in muscle (46). Moreover, exercise changed both DNA methylation and expression of a number of genes, including *ADIPOR1*, *ADIPOR2*, and *BDKRB2*, encoding receptors for adiponectin and bradykinin, respectively, which both regulate metabolism in muscle (47,48). Interestingly, IL-7 was recently found to be expressed and secreted from human skeletal muscle cells, and expression of IL-7 increased during differentiation of human myotubes (49). In this study, we found decreased DNA methylation in parallel with increased mRNA and serum levels of IL-7 in muscle after exercise, further supporting a role for IL-7 in human muscle. Although we find associations between increased DNA methylation and decreased expression for some genes in vivo and increased methylation was associated with reduced transcriptional activity in vitro, we cannot draw a conclusion as to whether differential expression is a consequence rather than a cause of changes in methylation (50).

Our group has previously shown that ageing is associated with increased DNA methylation and decreased expression of genes involved in oxidative phosphorylation in human muscle (23,24). In this study, we found that a gene from the respiratory chain *NDUFC2* exhibited decreased methylation after exercise. We further showed that increased methylation of the *NDUFC2* promoter reduced its transcriptional activity in vitro, indicating a role for DNA methylation in the regulation of *NDUFC2* expression. Moreover, exercise increased $V_{O_{2max}}$ and the mitochondrial density in muscle.

Our study may point to some of the molecular mechanisms explaining the results seen in previous exercise intervention studies (4,5). It is further possible that the epigenetic modifications induced by exercise reduce the future risk of T2D among $FH^+$ men. The two FH groups were matched for age, sex, BMI, and $V_{O_{2max}}$ at baseline in order to reduce the impact of lifestyle factors on our study. However, while exercise significantly improved a number of phenotypes, including waist circumference, diastolic blood pressure, and $V_{O_{2max}}$ in both FH groups, weight and BMI were only significantly reduced in $FH^+$ men. A possible explanation for this phenomenon could be that the participation in the intervention study reminded the $FH^+$ men that they are at greater disease risk and although they were requested not to change their overall lifestyle during the exercise intervention, one cannot exclude that they have changed their diet or other parts of their lifestyle.

Overall, this study provides novel insights into how exercise can induce genome-wide epigenetic changes in human muscle and that the response may differ in people with different genetic predispositions to metabolic disease.

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M.D.N. and C.L. were responsible for study design, researched data, performed data analyses, and wrote the manuscript. T.D., E.H., E.N., and T.R. researched data, performed data analyses, and reviewed and edited the manuscript. P.V., B.T.Y., S.L., H.P., H.W., J.A., and P.A. performed data analyses and reviewed and edited the manuscript. T.E., K.-F.E., and L.G. were responsible for study design, collected clinical material, and reviewed and edited the manuscript. Y.W. collected clinical material, performed data analyses, and reviewed and edited the manuscript. M.A., N.W., P.-A.J., and O.H. researched data and reviewed and edited the manuscript. C.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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