International Journal of Obesity (2015) 39, 888–892

www.nature.com/ijo

OPEN

ORIGINAL ARTICLE

Analysis of variants and mutations in the human winged helix *FOXA3* gene and associations with metabolic traits

DC Adler-Wailes[1], AT Alberobello[1], X Ma[1], L Hugendubler[1], EA Stern[2], Z Mou[2], JC Han[2], PW Kim[3], AE Sumner[4], JA Yanovski[2] and E Mueller[1]

BACKGROUND/OBJECTIVES: The forkhead factor Foxa3 is involved in the early transcriptional events controlling adipocyte differentiation and plays a critical function in fat depot expansion in response to high-fat diet regimens and during aging in mice. No studies to date have assessed the potential associations of genetic variants in *FOXA3* with human metabolic outcomes.
SUBJECTS/METHODS: In this study, we sequenced *FOXA3* in 392 children, adolescents and young adults selected from several cohorts of subjects recruited at the National Institute of Child Health and Human Development of the National Institutes of Health based on the availability of dual-energy X-ray absorptiometry data, magnetic resonance imaging scans and DNA samples. We assessed the association between variants present in these subjects and metabolic traits and performed *in vitro* functional analysis of two novel *FOXA3* missense mutations identified.
RESULTS: Our analysis identified 14 novel variants and showed that the common single-nucleotide polymorphism (SNP) rs28666870 is significantly associated with greater body mass index, lean body mass and appendicular lean mass (*P* values 0.009, 0.010 and 0.013 respectively). *In vitro* functional studies showed increased adipogenic function for the *FOXA3* missense mutations c.185C>T (p.Ser62Leu) and c.731C>T (p.Ala244Val) compared with *FOXA3*-WT.
CONCLUSIONS: Our study identified novel *FOXA3* variants and mutations, assessed the adipogenic capacity of two novel missense alterations *in vitro* and demonstrated for the first time the associations between *FOXA3* SNP rs28666870 with metabolic phenotypes in humans.

International Journal of Obesity (2015) **39,** 888–892; doi:10.1038/ijo.2015.17

INTRODUCTION

Forkhead box (Fox) proteins are a large family of factors that share a highly homologous Forkhead DNA-binding domain but diverge in the remaining sequences.[1] These factors have been implicated in differentiation and developmental processes in both mice and humans.[2–5] In particular, the three members of the Foxa subfamily, Foxa1, 2 and 3, which are most closely related to the archetype *Drosophila* FORK HEAD protein, have been shown to transcriptionally control early development, organogenesis and metabolism in mice.[6] Through a genetic screen to assess the role of each Forkhead factor family member in adipocyte differentiation, we demonstrated that Foxa3 is the only Forkhead protein positively affecting adipocyte differentiation through regulation of the nuclear receptor peroxisome proliferator-activated receptors γ (PPARγ), in cooperation with the transcription factors CCAAT-enhancer-binding proteins.[7] In addition, we showed that mice with Foxa3 ablation have decreased adipose tissue expansion in response to high-fat diet regimens[7] and during aging,[8] suggesting a new critical role of this factor in fat tissue biology.

Foxa3 functions through two transcriptional activation domains located at the amino- and carboxy-termini, in addition to the centrally located Forkhead DNA-binding motif, through which it binds to the upstream regulatory elements present in the promoters of target genes. Given the newly established importance of Foxa3 in fat metabolism in mice[7,8] and the absence of data on potential associations of genetic variants of *FOXA3* with human metabolic outcomes, we sequenced *FOXA3* in 392 lean, overweight and obese subjects, assessed the potential correlation between genotype and phenotype by functionally analyzing two novel *FOXA3* missense mutations and tested the association of the common single-nucleotide polymorphisms (SNPs) identified with a series of metabolic parameters. Through these analyses we identified 14 new *FOXA3* variants, demonstrated that the missense mutations c.185C>T (p.Ser62Leu) and c.731C>T (p.Ala244Val) have increased adipogenic function *in vitro* and that the *FOXA3* polymorphism rs28666870 is associated with increased appendicular muscle mass.

MATERIALS AND METHODS

Subjects

We performed a cross-sectional study of 392 lean and obese children, adolescents and young adults selected from several NIH-NICHD (National Institutes of Health-National Institute of Child Health and Human Development) cohorts based on the availability of dual-energy X-ray absorptiometry data, magnetic resonance imaging scans and DNA samples (described in details in the following NIH clinical trials: NCT00001195, NCT00758108, NCT00631644, NCT00001522, NCT00001723 and NCT00005669 at clinicaltrials.gov.[9–11] Non-Hispanic black (NHB) and non-Hispanic white (NHW) in the

[1]Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Disease, National Institutes of Health, Bethesda, MD, USA; [2]Section on Growth and Obesity, Program in Developmental Endocrinology and Genetics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA; [3]National Institute of Arthritis and Musculoskeletal and Skin Disease, National Institutes of Health, Bethesda, MD, USA and [4]Diabetes, Endocrinology and Obesity Branch, Section on Ethnicity and Health, National Institute of Diabetes and Digestive and Kidney Disease, National Institutes of Health, Bethesda, MD, USA. Correspondence: Dr E Mueller, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Disease, Building 10, Room 8D12b, 9000 Rockville Pike, Bethesda, MD 20892, USA.
E-mail: elisabettam@mail.nih.gov
Received 22 September 2014; revised 12 November 2014; accepted 23 November 2014; accepted article preview online 12 February 2015; advance online publication, 3 March 2015

NIH-NICHD group were defined by the subject having all four grandparents of the same race. Ethnicity was ascertained by self-identification. All the clinical protocols were approved by the Institutional Review Board of the Eunice Kennedy Shriver National Institute of Child Health and Human Development. Each child and adolescent provided written assent and parents gave written consent for their participation.

Body composition measurements

All participants underwent a screening visit by a pediatric endocrinologist or nurse practitioner. Blood samples were obtained for genomic DNA isolation.[12] Weight and height were measured in the post-absorptive state.[13] Homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated from post-absorptive serum insulin and glucose concentrations.[11,14] Total body fat and lean mass were determined by dual-energy X-ray absorptiometry.[15] Magnetic resonance imaging was employed to measure visceral and subcutaneous abdominal adipose tissue areas at $L_{2\text{-}3}$.[15]

Sequencing of human *FOXA3* gene

The entire *FOXA3* gene (Exons 1 and 2) and 65 base pairs of 5′-UTR and 66 base pairs of the intron were sequenced (Polymorphic DNA Technologies, Alameda, CA, USA) using PCR amplification (ABI) in 392 subjects of the NIH-NICHD group. Bidirectional sequencing was performed (ABI, Grand Island, NY, USA) and variants were identified by comparison to reference sequence number NM_004497. The primers used for sequencing were the following: FOXA3-1: forward (F) 5′-GGTGTCCCGGCTATAA-3′ and reverse (R) 5′-AGCGCTCCCATCCAT-3′; FOXA3-2: F: 5′-GCTTTCTACAGAT AGAGGTA-3′ and R: 5′-TTTCACTCAAGGTCAGCAT-3′; FOXA3-3: F: 5′-CTGG GGCCCACTT-3′ and R: 5′-CTTCTCCTCCAGCTTGA-3′; FOXA3-4: F: 5′-CCCT TACTACCGGGA-3′ and R: 5′-GCTGGTGTCTGTTCTGA-3′; FOXA3-5: F: 5′-C AAGCTGGAGGAGAAG-3′ and R: 5′-AGACCCACCCAGATG-3′.

Plasmids and cell culture

FOXA3-WT cDNA (pCMV6-XL5-FOXA3) and vector (pCMV6-XL5) were obtained from Origene (Rockville, MD, USA). *FOXA3* mutants at nucleotides 185 (c.185C>T) and 731 (c.731C>T) were generated by site-directed mutagenesis of the *FOXA3*-WT plasmid (Origene). 10T1/2 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (CellGro, Manassas, VA, USA) supplemented with Penicillin/Streptomycin and 10% fetal bovine serum (Hyclone, Logan, UT, USA). A total of 5.0×10^5 10T1/2 cells were transfected using the Nucleofector 96-well shuttle system (Amaxa Biosystems, Cologne, Germany) with either 1.2 μg of control plasmids or with 1.2 μg *FOXA3*-WT, *FOXA3* c.185C>T or *FOXA3* c.731C>T. pmaxGFP plasmid (0.3 μg, Amaxa Biosystems) was co-transfected for transfection normalization. Twenty-four hours after nucleofection, cells were treated with 5 μg ml^{-1} insulin and with 10 μM troglitazone for additional 3 days before cells were harvested for RNA analysis.

RNA extraction and real-time PCR analysis

Total RNA was obtained from 10T1/2 cells ectopically expressing either vector, *FOXA3*-WT, *FOXA3* c.185C>T or *FOXA3* c.731C>T, using Triazol and transcribed into cDNA by First Strand cDNA Synthesis Kit, following the manufacturer's instructions (Roche, San Francisco, CA, USA). Quantitative PCR was performed on ABI PRISM 7900HT sequence detector system (ABI) using SYBR green (Roche). The delta-delta Ct method was used for analysis of gene expression levels, after normalization to 36B4 expression. The primer sequences used for real-time PCR were the following: GFP, F: 5′-AAGCTGACCCTGAAGTTCATCTGC-3′ and R: 5′-CTTGTAGTTGCCGTCGTCCTT GAA-3′; *FOXA3*, F: 5′-GAGATGCCGAAGGGGTATCG-3′ and R: 5′-TGATTCTC CCGGTAGTAAGGG-3′; mouse 36B4, F: 3′-GCTTCATTGTGGGAGCAGAC-5′ and R: 5′-ATGGTGTTCTTGCCCATCAG-3′; mouse *PPARγ*, F: 5′-AGTCTGCTGAT CTGCGAGCC-3′ and R: 5′-CTTTCCTGTCAAGATCGCCC-3′; mouse *adiponectin*, F: 5′-TGTTCCTCTTAATCCTGCCCA-3′ and R: 5′-CCAACCTGCACAAGTTCCCTT-3′; mouse *aP2*, F: 5′-ACACCGAGATTTCCTTCAAACTG-3′ and R: 5′-CCATCTAGG GTTATGATGCTCTTC-3′; mouse *perilipin*, F: 5′-GGGACCTGTGAGTGCTTCC-3′ and R: 5′-GTATTGAAGAGCCGGGATCTTTT-3′.

Statistical analysis

Cell culture experiments were repeated at least three times and results are shown as means ± standard errors of the means (s.e.m.) calculated by GraphPad Prism. A Student's *t*-test was used for comparison between two groups and differences between groups were considered significant with $P<0.05$. *FOXA3* association analyses in the NIH-NICHD group were performed using SPSS-version 18 software (IBM, Bethesda, MD, USA). Hardy–Weinberg equilibrium tests were performed on all variants. Associations were evaluated using Analysis of covariance (ANCOVA) for continuous variables and χ^2 analyses for categorical variables. The *P* values were corrected for the four common variants found in the NIH-NICHD cohort and significant associations were called when $P<0.0125$. Heterozygous and homozygous subjects were analyzed together, compared with wild-type subjects and NHB and NHW were analyzed separately, if a significant difference by race was found. Body mass index (BMI), fat mass, lean mass, appendicular muscle mass, trunk muscle mass, subcutaneous abdominal adipose tissue (SAT) and visceral abdominal adipose tissue (VAT) areas were log transformed before ANCOVA. Means and standard deviations were back transformed and adjusted for covariates. BMI was adjusted for sex, race and age. Fat, lean mass, appendicular lean mass and trunk lean mass were adjusted for sex, race, age and height squared. $SAT_{2\text{-}3}$ and $VAT_{2\text{-}3}$ areas were adjusted for sex, race, age and log fat mass. HOMA was adjusted for sex, race, age and fat mass. Given the relatively small number of subjects in the NIH-NICHD group, we used higher stringency in calling significant associations ($P<0.0125$) between SNPs and body composition measurements and adjusted for genotypes rather than for the traits studied.

RESULTS AND DISCUSSION

We sequenced *FOXA3* in 392 children, adolescents and young adults (NIH-NICHD group, Table 1) and identified 22 variants (Table 2), 14 of which had not been reported previously. The four common polymorphisms (http://www.1000genomes.org/) identified in the NIH-NICHD group were in Hardy–Weinberg equilibrium (data not shown). In addition, we detected three novel intronic variants and eight new missense mutations (Table 2), which were each present in heterozygosity only in one individual. The majority of the missense alterations were located in the amino- or carboxy-termini with three out of eight found within the last seven C-terminal amino acids. Sequence homology analysis indicated that the majority of the variants identified occurred at nucleotides highly conserved among species (Supplementary Table 1).

Given that Foxa3 potentiates adipocyte differentiation *in vitro* in 10T1/2 cells,[7] we assessed the functionality of two *FOXA3* mutants, c.185C>T and c.731C>T, in *in vitro* adipogenic assays. We chose to analyze the function of these two mutations because they were the only missense alterations that occurred as the sole genetic alteration in the *FOXA3*-coding sequence in the patients in which they were identified (Supplementary Table 2). Ectopic expression of *FOXA3* c.185C>T and *FOXA3* c.731C>T in 10T1/2 mesenchymal cells at levels comparable to those of *FOXA3*-WT (Figure 1a) was

Table 1. Characteristics of the 392 subjects analyzed

Variable	*Value*	*Range*
Sex	44% Males, 56% females	
Race	55% NHW, 40% NHB, 5% Other	
Age (years)	12.2 ± 3.2	6.1 to 21.3
Weight (kg)	70.5 ± 30.8	17.0 to 146.1
Height (cm)	153.4 ± 16.6	108.3 to 186.4
BMI ($kg\,m^{-2}$)	28.7 ± 9.2	14.4 to 53.5
BMI-z score	1.63 ± 1.14	−2.52 to +3.18
Body fat (%)	35.1 ± 11.3	9.8 to 64.1
Fat mass (kg)	27.0 ± 17.1	3.2 to 83.6
Lean mass (kg)	42.1 ± 15.7	12.9 to 83.9
$SAT_{2\text{-}3}$ area (cm^2)	251.0 ± 238.2	13 to 3217
$VAT_{2\text{-}3}$ area (cm^2)	68.3 ± 59.2	3 to 598

Abbreviations: BMI, body mass index; BMI-z, body mass index standard deviation score; NHB, non-Hispanic black; NHW, non-Hispanic white; SAT, subcutaneous abdominal adipose tissue area in cm^2 from $L_{2\text{-}3}$; VAT, visceral abdominal adipose tissue area in cm^2 from $L_{2\text{-}3}$. Other race/ethnicities included: Asian, Hispanic, and mixed. Unadjusted means are reported with standard deviation of the means (± s.d.).

Table 2. *FOXA3* variants identified in 392 children, adolescents and young adults

Chromosome 19 position (hg19 reference)	*HGVS nucleotide change*	*SNP*	*Location*	*Amino acid change*	*Genotype frequencies (%)*			*BMI- z*		
					Wt (Wt/Wt)	*Het (Wt/SNP)*	*Hom (SNP/SNP)*	*Wt (Wt/Wt)*	*Het (Wt/SNP)*	*Hom (SNP/SNP)*
46367703	c.-12 T>C	rs11667582	5′-UTR	No	71.2	25.7	3.1	1.67 ± 1.14	1.52 ± 1.15	1.83 ± 1.19
46367812	c.69+29 A>G	Novel	Intron	No	99.5	0.5	0.0	1.64 ± 1.14	1.64 ± 0.43	–
46367831	c.69+48G>C	Novel	Intron	No	99.7	0.3	0.0	1.64 ± 1.14	0.61	–
46367832	c.69+49G>T	Novel	Intron	No	99.7	0.3	0.0	1.63 ± 1.14	2.00	–
46375364	c.101 T>C	Novel/N-Syn	Exon 2	p.Met34Thr	99.7	0.3	0.0	1.63 ± 1.14	1.17	–
46375448	c.185C>T	Novel/N-Syn	Exon 2	p.Ser62Leu	99.7	0.3	0.0	1.63 ± 1.14	2.30	–
46375462	c.199C>T	Novel/N-Syn	Exon 2	p.Pro67Ser	99.7	0.3	0.0	1.64 ± 1.14	0.19	–
46375608	c.345C>T	rs144459600	Exon 2	No	98.2	1.8	0.0	1.63 ± 1.15	1.98 ± 0.80	–
46375620	c.357G>A	rs147847262	Exon 2	No	99.7	0.3	0.0	1.63 ± 1.14	1.98	–
46375779	c.516C>T	rs16980091	Exon 2	No	78.8	19.9	1.3	1.65 ± 1.16	1.57 ± 1.09	1.81 ± 1.21
46375974	c.711 T>C	Novel/Syn	Exon 2	No	99.7	0.3	0.0	1.64 ± 1.14	0.94	–
46375994	c.731C>T	Novel/N-Syn	Exon 2	p.Ala244Val	99.7	0.3	0.0	1.63 ± 1.14	1.31	–
46376006	c.743C>A	Novel/N-Syn	Exon 2	p.Thr248Asn	99.7	0.3	0.0	1.63 ± 1.14	0.79	–
46376043	c.780 T>C	rs28666870	Exon 2	No	97.7	2.3	0.0	1.62 ± 1.14	2.61 ± 0.21	–
46376089	c.826C>G	rs140760356	Exon 2	p.Pro276Ala	99.7	0.3	0.0	1.64 ± 1.14	−0.23	–
46376217	c.954C>A	rs3810127	Exon 2	No	86.7	13.0	0.3	1.63 ± 0.16	1.66 ± 1.06	2.31
46376226	c.963C>T	rs146135785	Exon 2	No	99.5	0.5	0.0	1.63 ± 1.14	1.81 ± 1.34	–
46376244	c.981C>T	Novel/Syn	Exon 2	No	99.7	0.3	0.0	1.63 ± 1.14	2.51	–
46376293	c.1030C>T	Novel/N-Syn	Exon 2	p.Arg344Cys	99.7	0.3	0.0	1.63 ± 1.14	2.56	–
46376309	c.1046C>T	Novel/N-Syn	Exon 2	p.Ala349Val	99.7	0.3	0.0	1.63 ± 1.14	2.25	–
46376312	c.1049C>T	Novel/N-Syn	Exon 2	p.Ser350Phe	99.7	0.3	0.0	1.63 ± 1.14	2.81	–
46376397	c.*81_*82insT	Novel	Intron	No	99.5	0.5	0.0	1.63 ± 1.14	2.17 ± 0.23	–

Abbreviations: Ala, alanine; Arg, arginine; Asn, asparagine; BMI-z, body mass index standard deviation score; Cys, cysteine; Het, heterozygous; Hom, homozygous; ins, insertion; Leu, leucine; Met, methionine; N-Syn, non-synonymous; Phe, phenylalanine; Pro, proline; Ser, serine; SNP, single-nucleotide polymorphism; Syn, synonymous; Thr, threonine; Val, valine; Wt, wild type.

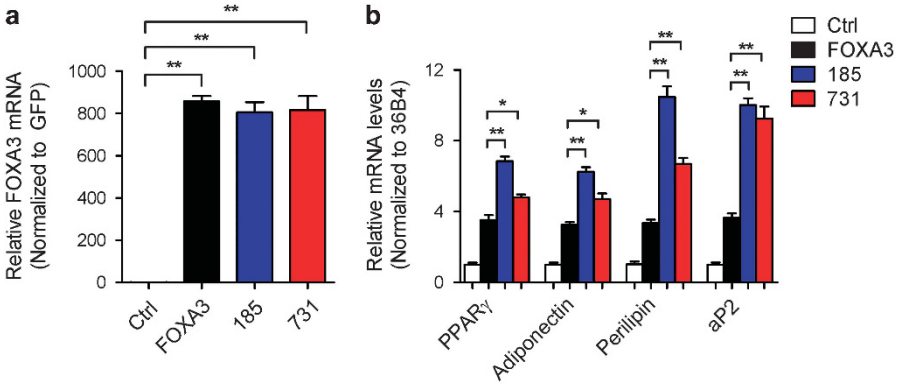

Figure 1. (**a**) mRNA levels of *FOXA3* in 10T1/2 cells ectopically expressing either vector (Ctrl), *FOXA3* wild-type (FOXA3), *FOXA3* c.185C>T (185) or *FOXA3* c.731C>T (731). Data are presented as mean ± s.e.m. $^{**}P < 0.01$ compared with control group. (**b**) mRNA levels of markers of adipocyte differentiation in 10T1/2 cells ectopically expressing either vector (Ctrl), *FOXA3* wild type (FOXA3), *FOXA3* c.185C>T (185) or *FOXA3* c.731C>T (731). Data are presented as mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with *FOXA3* wild type (FOXA3).

associated with increased mRNA levels of classic adipocyte differentiation markers, such as PPARγ, adiponectin, perilipin and aP2, in comparison to *FOXA3*-WT-expressing cells (Figure 1b). These data suggest that these two missense mutations confer increased adipogenic capacity to *FOXA3*.

We next assessed whether the *FOXA3* variants identified in the NIH-NICHD group were associated with metabolic parameters. This analysis revealed a significant association between the common variant rs28666870 and increased total lean body mass (Table 3). A significant association with race/ethnicity was also identified in subjects heterozygous for this variant (χ^2 *P* of 0.002; Table 3). To further confirm that the association identified between rs28666870 and lean mass was not confounded by race, we re-evaluated the association strictly within the subgroup of 158 NHB subjects present in the NIH-NICHD group. Demographics of the NHB cohort re-evaluated are shown in Supplementary Table 3. This analysis confirmed that variant rs28666870 was significantly associated with greater total lean body mass ($P < 0.035$), increased BMI ($P < 0.032$) and appendicular lean mass ($P < 0.044$). No significant associations with body mass parameters were identified for the additional three common variants identified in the NIH-NICHD group, rs11667582, rs3810327 and rs16980091. We subsequently assessed the association between these four SNPs and HOMA-IR. As shown in Table 3, none of the four common

Table 3. Associations of *FOXA3* SNPs with race/ethnicity and metabolic parameters

	Wt	*Het & Hom*	P
rs11667582	n = *279*	n = *113*	
Race (%)			0.964
NHW	55.2	53.1	
NHB	39.8	41.6	
Other	5.0	5.3	
Sex, females (%)	54.8	59.3	0.433
BMI (kg m^{-2})	27.5 ± 1.4	26.4 ± 1.3	0.205
Fat mass (kg)	21.1 ± 1.9	20.2 ± 1.9	0.556
Lean mass (kg)	39.3 ± 1.2	38.2 ± 1.2	0.128
SAT_{2-3} area (cm^2)	158.8 ± 1.4	155.6 ± 1.4	0.303
VAT_{2-3} area (cm^2)	50.3 ± 1.8	48.9 ± 1.8	0.625
HOMA-IR	3.45 ± 2.7	3.80 ± 2.7	0.295
rs16980091	n = *308*	n = *84*	
Race (%)			**0.011**
NHW	58.1	41.7	
NHB	38.0	48.8	
Other	3.9	9.5	
Sex, females (%)	55.2	59.5	0.379
BMI (kg/m^2)	27.4 ± 1.3	26.4 ± 1.3	0.290
Fat mass (kg)	21.4 ± 1.9	19.1 ± 1.9	0.161
Lean mass (kg)	38.9 ± 1.2	39.2 ± 1.2	0.774
SAT_{2-3} area (cm^2)	157.4 ± 1.4	160.3 ± 1.4	0.896
VAT_{2-3} area (cm^2)	50.0 ± 1.8	49.8 ± 1.8	0.959
HOMA-IR	3.50 ± 2.7	3.73 ± 2.7	0.484
rs28666870	n = *384*	n = *8*	
Race (%)			**0.002**
NHW	55.7	0.0	
NHB	39.1	100.0	
Other	5.2	0.0	
Sex, females (%)	56.3	50.0	0.731
BMI (kg m^{-2})	27.0 ± 1.4	35.8 ± 1.3	**0.009**
Fat mass (kg)	20.7 ± 1.9	30.5 ± 1.9	0.091
Lean mass (kg)	38.8 ± 1.2	45.3 ± 1.2	**0.010**
Appendicular lean mass (kg)	17.3 ± 1.2	20.9 ± 1.2	**0.013**
Trunk lean mass (kg)	18.1 ± 1.2	20.7 ± 1.2	0.045
SAT_{2-3} area (cm^2)	159.1 ± 1.4	154.5 ± 1.4	0.940
VAT_{2-3} area (cm^2)	50.0 ± 1.8	49.5 ± 1.8	0.973
HOMA-IR	3.58 ± 2.7	2.16 ± 2.7	0.144
rs3810327	n = *340*	n = *52*	
Race (%)			0.129
NHW	53.8	59.6	
NHB	41.8	30.8	
Other	4.4	9.6	
Sex, females (%)	55.9	57.7	0.789
BMI (kg m^{-2})	27.1 ± 1.3	27.4 ± 1.3	0.797
Fat mass (kg)	20.6 ± 1.9	22.2 ± 1.9	0.460
Lean mass (kg)	39.0 ± 1.2	39.0 ± 1.2	0.960
SAT_{2-3} area (cm^2)	157.0 ± 1.4	163.7 ± 1.4	0.581
VAT_{2-3} area (cm^2)	49.3 ± 1.7	54.4 ± 1.8	0.244
HOMA-IR	3.57 ± 2.7	3.44 ±2.7	0.750

Abbreviations: BMI, body mass index; het, heterozygous for variant allele at one or both loci; HOMA-IR, homeostasis model assessment-estimated insulin resistance; hom/hom, homozygous for variant alleles; NHW, non-Hispanic white; NHB, non-Hispanic black; SAT, subcutaneous adipose tissue area in cm^2 from L_{2-3}; VAT, visceral adipose tissue area in cm^2 from L_{2-3}; Wt/Wt, wild-type alleles at both loci. Chi-square tests for categorical variables (Wt/Wt, $n = 279$; Het & Hom/Hom, $n = 113$) except when cell sizes were < 5. Fisher's exact test was used instead. Categorical percentages were reported. Other category for race includes: Asian, Hispanic and mixed. ANCOVAs were used for continuous variables. For ANCOVAs, Het and Hom/Hom were analyzed together as described in the Methods, and *n* for each variant studied is reported in parentheses. BMI, fat mass, lean mass, appendicular muscle mass, trunk muscle mass, SAT, VAT and SAT/VAT ratios were log transformed before ANCOVA. Adjusted means ± s.d. are reported. BMI was adjusted for sex, race and age. Fat and lean mass were adjusted for sex, race, age and height squared. SAT and VAT were adjusted for sex, race, age and log fat mass. HOMA-IR was adjusted for sex, race, age and log fat mass. $P < 0.05$ is considered significant and shown in bold.

alleles identified showed a significant association ($P > 0.10$) with HOMA-IR.

On the basis of the demonstration that FOXA3 is a novel transcriptional regulator of adipocyte differentiation and fat tissue expansion,[7,8] we took a candidate gene approach to identify possible novel, naturally occurring, mutations in *FOXA3*, assessed their adipogenic capacity and tested the potential association of the variants identified with metabolic traits in human subjects. Our analysis revealed eight novel missense mutations in the NIH-NICHD group analyzed and demonstrated that two pyrimidine transitions C to T resulting in non-synonymous alterations (p. Ser62Leu and p.Ala244Val) conferred increased adipogenic activity to FOXA3 *in vitro*. Detailed mechanistic studies will further define whether phosphorylation at the serine residue 62 may alter FOXA3 nuclear localization and decrease its function, as demonstrated for other Forkhead factors,[16] or whether posttranslational modifications alter FOXA3 interactions with components of the transcriptional machinery. Interestingly, the proadipogenic missense mutation c.185C > T (p.Ser62Leu) was identified in an obese subject (BMI-z score of 2.3) and c.731C > T (p.Ala244Val) was identified in an overweight individual (BMI-z of 1.3), suggesting a potential correlation between genotype and phenotype. In light of the role of Foxa3 in adipose tissue expansion in mice,[7,8] our data suggest that the two novel *FOXA3* missense alterations identified in this study may have an effect on the fat storage capacity of the adipocytes, possibly leading to increased fat tissue expansion.

Our analysis also revealed an association of the *FOXA3* variant rs28666870 with increased lean mass in the NIH-NICHD group analyzed. Given that the majority of lean mass measured by dual-energy X-ray absorptiometry (DEXA) scan is represented by muscle mass, we determined whether the variant rs28666870 was associated with appendicular or trunk muscle mass. Despite an association of rs28666870 with increased appendicular muscle mass, no significant association was found between this variant and increased insulin sensitivity. It is possible that this polymorphism may influence the development of insulin resistance during the course of aging and that its protective effects may not yet be evident, given that the NIH-NICHD cohort is mainly constituted by young subjects. Interestingly, the HOMA-IR value of 2.16 ± 2.7 (mean ± s.d.) for the heterozygous subjects carrying the rs28666870 variant appeared to be below the cutoffs for insulin resistance reported by Kurtoglu[17] in pre-pubertal and pubertal boys (2.67 and 5.22, respectively) and girls (pre 2.22 and pubertal, 3.82), whereas the HOMA-IR values for the subjects carrying the WT allele (3.58 ± 2.7, mean ± s.d.) were higher, suggesting a possible protection from the development of insulin resistance among the subjects carrying the rs28666870 variant.

Our finding that the variant rs28666870 is significantly associated with increased lean mass, may suggest a role for FOXA3 in controlling the differentiation of mesenchymal tissues toward different lineages. This hypothesis is in line with our recent demonstration that Foxa3 plays a role in mouse tissues of mesenchymal origin, such as fat.[7,8] Given that the synonymous variant rs28666870 is predicted by ESEfinder (http://rulai.cshl.edu/tools/ESE) to alter an exon splicing enhancer site for the binding of SR splicing regulators, it is plausible that rs28666870 could give rise to a new spliced isoform of FOXA3, potentially affecting muscle mass. Additional studies employing genome editing and functional assays will determine whether a novel FOXA3 isoform arises from the variant rs28666870 with transcriptional activity in adipose and/or in muscle cell lineages. In addition, further studies in larger cohorts will determine the presence of associations between metabolic parameters and body composition with *FOXA3* variants.

In conclusion, our analysis identified 14 novel *FOXA3* variants, demonstrated a novel association between one *FOXA3* SNP and body mass parameters and showed increased adipogenic function of two missense mutations, each found in individuals with BMI-z

scores greater than 1. These findings support a role for *FOXA3* as a novel critical regulator of fat mass in humans. To our knowledge, this is the first study to report an association between *FOXA3* variants and metabolic parameters, suggesting a potential role of *FOXA3* DNA variants in regulation of body mass.

Supplementary information is available at the International Journal of Obesity's website

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Pasha Sarraf for thoughtful discussions throughout the project and Chen Du and Lingyan Xu for reading the manuscript. We are grateful to the families who participated in the study. JAY is a Commissioned Officer in the United States Public Health Service. This work was supported by the Intramural Research Programs of NIDDK, NICHD, NIMHD and NIAMS of the National Institutes of Health.

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Supplementary Information accompanies this paper on International Journal of Obesity website (http://www.nature.com/ijo)