Replication and Extension of Association Between Common Genetic Variants in *SIM1* and Human Adiposity

Michael M. Swarbrick[1,6], Daniel S. Evans[1], Maria. I. Valle[1], Hélène Favre[1], Shi-Hsuan Wu[1], Omer T. Njajou[1], Rongling Li[2], Joseph M. Zmuda[3], Iva Miljkovic[3], Tamara B. Harris[4], Pui-Yan Kwok[5], Christian Vaisse[1] and Wen-Chi Hsueh[1]

Haplo-insufficiency of the bHLH (basic helix-loop-helix) transcription factor single-minded 1 (SIM1) causes severe obesity in mice and humans. We hypothesized that common genetic variations in/near *SIM1* could exert more subtle effects on its function and associate with human adiposity. First, *SIM1* coding regions were sequenced in severely obese subjects, and two common nonsynonymous single-nucleotide polymorphisms (nsSNPs) in complete linkage disequilibrium (LD) were identified: Pro352Thr (rs3734354) and Ala371Val (rs3734355). We next carried out a SNP association study of five adiposity traits (BMI, % body fat, abdominal visceral and subcutaneous fat, and leptin concentrations) in 1,699 whites and 1,173 blacks. TagSNPs covering *SIM1* and nearby conserved regions, and the only common nsSNP in SIM1's binding partner aryl-hydrocarbon receptor nuclear translocator 2 (ARNT2) (Gly679Ser/rs4072568), were investigated. The effects of rs3734355/4 on SIM1 activity were tested using an *in vitro* reporter assay. We replicated previous observations that homozygosity for the 371Val allele was associated with higher BMI in white males ($P = 0.003$). Together with previous findings in white males (combined $n = 3,479$), BMI was increased by 1.10 kg/m^2 in 371Val homozygotes (95% confidence interval (CI): 0.25–1.95 kg/m^2, $P = 0.01$). *In vitro*, the 352Thr-371Val haplotype impaired SIM1 transcriptional activity by 22% ($P < 0.0001$). TagSNP analysis of *SIM1* revealed two SNPs in the 3′ region (rs9390322 and rs7746743) and another in intron 5 (rs3734353) to be significantly associated with various adiposity measures in ethnicity- and sex-specific manners after multiple testing correction. In white males, rs4072568 in *ARNT2* was also associated with BMI ($P = 9 \times 10^{-4}$) and % body fat ($P = 0.001$). Our findings implicate heritable defects of the SIM1-ARNT2 axis in the predisposition to human obesity.

Obesity (2011) **19,** 2394–2403. doi:10.1038/oby.2011.79

INTRODUCTION

Single-minded 1 (*SIM1*), a bHLH/PAS (basic helix-loop-helix/Per-Arnt-Sim) transcription factor, is essential for the terminal differentiation of second order hypothalamic paraventricular nucleus (PVN) neurons involved in the central regulation of food intake and energy homeostasis (1,2). SIM1 heterodimerizes with another bHLH/PAS factor, aryl-hydrocarbon receptor nuclear translocator 2 (ARNT2) to activate transcription (3). While *Sim1* homozygous null mice die shortly after birth, heterozygotes are viable and develop hyperphagia and obesity (4,5). In humans, rare cases of severe, early onset obesity have been linked to haplo-insufficiency at the *SIM1* locus (6–9). Given the extreme obesity phenotype observed when *SIM1* function is reduced by half, it was of interest to determine the phenotype of individuals with common mutations in *SIM1* that may exert more subtle effects on its function.

Homozygosity for a common *SIM1* haplotype encompassing two linked nonsynonymous single-nucleotide polymorphisms (nsSNPs) within exon 9, Pro352Thr (rs3734354) and Ala371Val (rs3734355), has been previously associated with a higher BMI in white men (10). However, this association was not replicated in either of two subsequent studies of *SIM1*, one of 6,194 full- and mixed-heritage Pima Indians (11) and another of 1,275 obese children/adults and 1,395 lean controls from France (12).

The first two authors contributed equally to this work.

[1]Diabetes Center and Department of Medicine, University of California, San Francisco, California, USA; [2]Department of Preventive Medicine, University of Tennessee Health Science Center, Memphis, Tennessee, USA; [3]Department of Epidemiology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; [4]Laboratory of Epidemiology, Demography and Biometry, National Institute on Aging, Bethesda, Maryland, USA; [5]Cardiovascular Research Institute, University of California, San Francisco, California, USA; [6]Present address: Diabetes and Obesity Program, Garvan Institute of Medical Research, Sydney, Australia; School of Medical Sciences, Wallace Wurth Building, University of New South Wales, Sydney, Australia. Correspondence: Christian Vaisse (vaisse@medicine.ucsf.edu) or Wen-Chi Hsueh (wen-chi.hsueh@ucsf.edu)

Received 17 August 2010; accepted 5 March 2011; published online 21 April 2011. doi:10.1038/oby.2011.79

In Pima Indians, the most significant results for association between *SIM1* SNPs and BMI were obtained within a block of noncoding SNPs extending from the 5′ region of *SIM1* to intron 8 (11). Differences in mean BMI between homozygotes for the major allele and homozygotes for the minor allele were ~2.2 kg/m^2. Two SNPs displaying the strongest associations with BMI in Pima Indians (rs3734353 and rs3213541) were not associated with BMI in French Caucasians, suggesting the presence of genetic heterogeneity. Further investigation of *SIM1* in French Caucasians (12) did not yield any significant evidence for association with obesity after correction for multiple hypothesis testing. Moreover, neither (11) nor (12) reported sex-specific associations between *SIM1* SNPs and adiposity, and neither study assumed a recessive mode of inheritance.

Here, we provide evidence to support the initial association between the Pro352Thr-Ala371Val haplotype and adiposity in white males (10), and demonstrate its biological relevance by showing that it impairs SIM1 activity *in vitro*. Moreover, by analyzing tagSNPs covering *SIM1* and its flanking regions, we establish that the 352Thr-371Val haplotype, as well as another SNP (rs9390332, in the 3′ noncoding region) in moderate linkage disequilibrium (LD) with these two variants, provides the strongest association with adiposity in white males. Other SNPs in/near *SIM1* were also associated with measures of adiposity in black subjects. Finally, we also find an association between the only common nsSNP in *ARNT2* (Gly679Ser/rs4072568) and adiposity in white males, but there was no evidence for an interaction between the nsSNPs in *SIM1* and *ARNT2*. Together, these results support the hypothesis that dysregulation of the SIM1-ARNT2 axis contributes to human obesity.

METHODS AND PROCEDURES

Subjects for *SIM1* variant identification

Identification of nonsynonymous *SIM1* variants was initially performed in severely obese subjects from an ongoing UCSF (University of California, San Francisco) study (13). The UCSF Committee on Human Research approved the protocols, and informed written consent was obtained from all participants.

Subjects and phenotype measurement in the Health, Aging, and Body Composition (Health ABC) Study

The Health ABC study is a population-based prospective study of 3,075 men and women (48.5% male; 41.7% black) aged 70–79 years, from Pittsburgh, PA and Memphis, TN. All participants were physically active at the time of entry into the study and provided informed consent. The study protocol was approved by the institutional review boards at the University of Pittsburgh (Pittsburgh, PA) and the University of Tennessee (Memphis, TN). The present study used data obtained from the baseline examination, during 1997–1998. Measurement of adiposity traits in these subjects has been described previously (14,15). Measures of global adiposity included BMI, the percentage of total body fat (% body fat, assessed by dual-emission X-ray absorptiometry) and fasting leptin (measured using the Sensitive Human Leptin RIA Kit from Linco Research, St Charles, MO). Regional adiposity, including abdominal visceral fat area (visceral fat, in cm^2) and abdominal subcutaneous fat area (subcutaneous fat, in cm^2), was assessed using computed tomography.

Sequencing of the SIM1 coding regions and genotyping of rs3734355

Genomic DNA was extracted from buffy coats using a Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN). *SIM1* exons and splice junctions were amplified using the primers listed in **Supplementary Table S1** online. PCR products were bidirectionally sequenced on an ABI 3700 sequencer (Applied Biosystems, Foster City, CA). Sequences were analyzed using Sequencher software (Gene Codes, Ann Arbor, MI), using the *SIM1* GenBank sequence (accession number NM_005068.2) as a reference. The Ala371Val SNP (rs3734355) was genotyped using fluorescently labeled allele-specific primer extension assayed by fluorescence polarization template-directed dye incorporation (16). Quality control criteria are described in **Supplementary Methods and Procedures** online.

Selection and genotyping of tagSNPs and rs3734354 in *SIM1* and rs4072568 in *ARNT2*

SNPs were selected using HapMap data Phase II (release 20) and genotyped using the Golden Gate Assay (Illumina, San Diego, CA). By considering conservation between human and mouse genomes at ≥75% identity for sequences of ≥100 bp using VISTA (17), the program *Tagger* (18) was used to select tagSNPs ($r^2 \geq 0.8$, minor allele frequency (MAF) ≥ 0.05) in a region spanning 45 kb upstream of the 5′ end to 60 kb downstream of the 3′ end of *SIM1*. SNP rs3734354, which is in complete LD with rs3734355, was included in all tagSNP selections and was used to validate the accuracy of genotypes for rs3734355. The tagSNPs selected in the CEU (European) population were forced to be included while picking tagSNPs in the YRI (Yoruban) population, and all (87) of these tagSNPs were genotyped in 2,982 subjects from the Health ABC cohort. Of the 87 genotyped SNPs, 77 SNPs in whites and 68 SNPs in blacks passed quality control (QC) criteria (see **Supplementary Methods and Procedures** online). Ethnicity-specific tagSNPs were then selected ($r^2 \geq 0.8$) among genotyped SNPs passing QC criteria using the genotype data from the Health ABC Study population. For *SIM1*, 38 SNPs (37 tagSNPs and rs3734355) in whites and 55 SNPs (54 tagSNPs and rs3734355) in blacks were tested for association. The only common nsSNP in *ARNT2* (rs4072568), based on the dbSNP (Build ID: 126 and 130) and HapMap (release 20) databases, was also genotyped.

Statistical analysis

In the severely obese subjects from the UCSF study, differences in BMI values by genotype were examined using the Wilcoxon rank sum test. For the Health ABC Study participants, genetic association analysis of adiposity traits was performed using linear regression analysis. For analyses of BMI, % body fat, and leptin, the effects of age, sex, recruitment site, diabetes status, weekly levels of physical activity, smoking and drinking habits, and education levels were adjusted for in the model. Percentage body fat was included as a covariate in the analysis of leptin, and baseline height and weight were included as covariates for analyses of abdominal subcutaneous and visceral fat. Leptin, visceral fat, and subcutaneous fat were transformed by taking the square-root to approximate normal distributions. For case–control analysis of obesity within Health ABC participants, BMI at the baseline examination was used to define obese cases as BMI ≥ 30 kg/m^2 and nonobese controls as BMI < 30 kg/m^2. The effects of the same covariates used in linear regression of BMI were adjusted for in logistic regression models.

To determine the likely mode of inheritance, mean values of adiposity traits stratified by genotype were examined. All SNPs were modeled with an additive mode of inheritance, except for rs3734355 and rs3734354, which were modeled as recessive. To minimize the effects of population stratification, all analyses were stratified by ethnicity. Analyses were also stratified by sex in order to replicate previously reported results (10). To correct for multiple hypothesis testing, empirical *P* values were obtained by permutation testing using the min-P procedure with 100,000 replicates (19).

Analysis of the overall effect of rs3734355 on BMI was performed using inverse variance weighting for pooling of effect estimates from two population-based cohorts (10) and the Health ABC population-based cohort. Heterogeneity between studies was assessed using the I^2 statistic and the *P* value from the Q-test. The overall effect size and *P* value was determined using a fixed effect model.

Table 1 BMI and genotype frequencies for single-minded 1 (*SIM1*) Ala371Val (rs3734355) in severely obese cases and lean controls

	Genotype				
	Ala/Ala	Ala/Val	Val/Val		
	BMI (kg/m^2) (*n*)	BMI (kg/m^2) (*n*)	BMI (kg/m^2) (*n*)	*P*	Freq (Val)
All cases[a]	48.9 ± 0.6 (297)	47.5 ± 0.8 (98)	51.6 ± 3.0 (14)	NS	0.15
Male cases	48.5 ± 1.1 (83)	44.5 ± 1.1 (26)	54.2 ± 7.4 (2)	0.036	0.14
Female cases	49.0 ± 0.7 (214)	48.6 ± 0.9 (72)	51.2 ± 3.4 (12)	NS	0.16
All controls[b]	22.9 ± 0.1 (379)	23.0 ± 0.1 (166)	22.6 ± 0.4 (19)	NS	0.18
Male controls	23.3 ± 0.1 (126)	23.3 ± 0.1 (47)	23.4 ± 0.4 (8)	NS	0.17
Female controls	22.7 ± 0.1 (253)	22.8 ± 0.1 (119)	22.1 ± 0.5 (11)	NS	0.18

[a]Cases had a BMI >40 kg/m^2 and were obtained from an ongoing study at UCSF (13). BMI is shown as mean ± s.e.m. [b]Sex- and age-matched lean controls (BMI ≤25 kg/m^2) were obtained from the same study.

Values from the *in vitro* transcription assay were removed if the coefficient of variation between two reads was > 0.05. The ratio of the normalized fluorescence of the 352Thr-371Val double mutant *SIM1* and its matched wild-type control was determined for each experiment, and the significance was assessed using a one-sample two-sided *t* test. All statistical analyses were performed using R software (http://www.r-project.org/).

In vitro transcription assay

The *SIM1* coding sequence was amplified from human fetal kidney cDNA and cloned into pCDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA). Mutations were introduced into the vector using QuickChange (Stratagene, La Jolla, CA). Human *ARNT2* was cloned from cDNA from HEK293 cells. The *toll* 4 CME site, consisting of three repeats of the sequence GGAGCATGCGGTACCAGAT(CTAGAAATTTGTACGTGCCACAGA)$_3$GGATCCGTG (20) was cloned into the *Kpn*I/*Bgl*II sites of pLuc-MCS (Invitrogen), which contains a minimal basal promoter followed by the coding sequence for luciferase.

HEK 293 cells were cultured in α-minimum essential medium supplemented with 10% calf serum (Hyclone, Logan, UT), 0.002 mmol/l L-glutamine, nonessential amino acids, and penicillin/streptomycin in an incubator maintained at 37 °C and 5% CO_2. Six hours before transfection, cells were seeded in 24-well plates at a density of 100,000 cells/well. Using Effectene reagent (Qiagen, Valencia, CA), each well was then transfected with pLuc-MCS or the pLuc-MCS containing SIM1 response element (120 ng), 80 ng of SIM1/ARNT2, as well as the plasmid pRL-RSV (Promega, Madison, WI), encoding *Renilla* luciferase, to control for transfection efficiency. Cells were lysed 48 h later, and firefly and *Renilla* luciferase activities were measured in lysates using the Dual Luciferase Reporter Assay System (Promega), according to the manufacturer's standard protocol.

RESULTS

All 11 exons of the human *SIM1* were sequenced in 445 severely obese subjects (74% whites, mean BMI ± s.e.m: 48.0 ± 0.5 kg/m^2)) (13). Five nsSNPs were identified uniquely in five different patients (Thr361Ile, Ser622Pro, His644Arg, Arg665His, Asp707His) as well as two common nsSNPs in complete LD, Pro352Thr and Ala371Val. The rare Thr361Ile and Arg665His variants have been reported previously (11). The common Ala371Val (rs3734355) and Pro352Thr (rs3734354) variants are located in or near the SIM1 nuclear localization signal, respectively (21). Obese males homozygous for the 371Val allele had a higher BMI than obese males of the other two genotypes ($P = 0.036$) (Table 1).

Considering previous findings (10), we investigated the potential relationship between rs3734355 and adiposity in a large population-based cohort, the Health ABC Study. Among whites, those homozygous for the minor (Val) allele at rs3734355 had greater adiposity than individuals with the other two genotypes (Table 2). SNP rs3734355 was more common in whites (MAF = 0.14) than blacks (MAF = 0.03). Sex-stratified analysis showed that in white males but not white females, homozygosity for the 371Val allele was associated with higher BMI ($P = 0.003$) and % body fat ($P = 0.04$) (Table 3). To validate our results, we also genotyped rs3734354 (see Methods and Procedures section), a SNP previously reported to be in complete LD with rs3734355 in whites (10). Among the white Health ABC subjects, these two SNPs were in nearly perfect LD ($r^2 = 0.97$), and the association between rs3734354 and adiposity was nearly identical to that of rs3734355 (see **Supplementary Table S2** online). A case–control analysis of white males from the Health ABC Study confirmed that homozygosity for the 371Val allele of rs3734355 was significantly associated with obesity (BMI ≥ 30 kg/m^2) (odds ratio = 2.8, 95% confidence interval (CI): 1.0–7.9, $P = 0.05$).

A previous report utilizing two population-based cohorts (Ely and EPIC-Norfolk) found the rs3734354/rs3734355 haplotype to be associated with higher BMI among white males using a recessive model (10). Combining these results with those from the Health ABC study (total $n = 3,479$ white males) revealed that homozygosity for the 371Val allele of rs3734355 was associated with an increase in BMI of 1.10 kg/m^2 (95% CI: 0.25–1.95, $P = 0.01$). There was no evidence for heterogeneity between the studies. Sex-specific association results were not provided in the other two reports investigating the relationship between *SIM1* and adiposity (11,12).

Next, common genetic variation in the rest of the *SIM1* locus and the surrounding noncoding conserved regions was surveyed using ethnicity-specific tagSNPs (see **Supplementary Figure S1** online, Figure 1). Using Health ABC genotype data from SNPs passing QC criteria, 38 SNPs (37 tagSNPs and rs3734355) in whites and 55 SNPs (54 tagSNPs and rs3734355) in blacks were selected and tested for association (see **Supplementary Table S2** online). In addition to

Table 2 Adiposity-related traits in the Health ABC cohort by SNP genotype and ethnicity

Traits[a]	*n*	Mean ± s.d.	*n*	Mean ± s.d.	*n*	Mean ± s.d.	*n*	Mean ± s.d.	*n*	Mean ± s.d.	*n*	Mean ± s.d.
	rs3734355 (*SIM1*)											
	Whites						Blacks					
	Ala/Ala		Ala/Val		Val/Val		Ala/Ala		Ala/Val		Val/Val	
BMI (kg/m^2)	1,308	26.5 ± 4.1	418	26.6 ± 4.2	36	28.3 ± 3.8	1,161	28.7 ± 5.4	78	28.3 ± 5.4	1	31.9
% Body fat	1,260	34.6 ± 7.2	398	34.9 ± 7.1	34	37.6 ± 6.2	1,125	35.4 ± 8.6	75	35.5 ± 8.4	1	49.0
Leptin (ng/ml)	1,294	12.5 ± 11.3	413	13.0 ± 11.5	35	16.2 ± 12.9	1,140	17.1 ± 13.9	76	16.4 ± 13.3	1	38.0
VAT (cm^2)	1,255	152.7 ± 69.9	405	147.8 ± 67.1	35	182.9 ± 73.8	1,116	130.0 ± 61.7	76	128.1 ± 57.5	0	—[b]
SAT (cm^2)	1,223	263.4 ± 101.6	402	266.6 ± 103.7	35	303.3 ± 80.6	1,066	314.3 ± 138.7	72	321.7 ± 143.6	0	—[b]
	rs9390332 (*SIM1*)											
	Whites						Blacks					
	A/A		A/T		T/T		A/A		A/T		T/T	
BMI (kg/m^2)	1,004	26.3 ± 4.1	559	26.8 ± 4.2	90	27.6 ± 4.1	1,050	28.7 ± 5.5	122	28.5 ± 5.2	2	29.1 ± 4.0
% Body fat	974	34.3 ± 7.2	528	35.3 ± 7.2	88	36.1 ± 6.8	1,016	35.4 ± 8.6	117	36.3 ± 8.7	2	38.8 ± 14.4
Leptin (ng/ml)	994	12.4 ± 11.4	551	13.4 ± 11.6	88	14.3 ± 13.4	1,030	17.2 ± 13.8	119	18.2 ± 14.8	2	24.9 ± 18.6
VAT (cm^2)	962	149.9 ± 67.2	540	153.6 ± 71.7	86	166.4 ± 72.2	1,007	129.8 ± 61.7	119	134.7 ± 60.3	1	236.3
SAT (cm^2)	942	260.6 ± 102.8	529	271.9 ± 102.7	85	285.6 ± 99.3	961	314.8 ± 140.0	111	323.3 ± 138.7	1	205.3
	rs3734353 (*SIM1*)											
	Whites						Blacks					
	A/A		A/C		C/C		A/A		A/C		C/C	
BMI (kg/m^2)	831	26.6 ± 4.1	686	26.5 ± 4.1	134	26.5 ± 4.5	626	28.7 ± 5.3	465	28.7 ± 5.7	81	28.9 ± 5.2
% Body fat	798	34.6 ± 7.4	660	34.9 ± 6.8	131	34.8 ± 7.4	606	35.5 ± 8.7	447	35.3 ± 8.6	80	36.9 ± 8.2
Leptin (ng/ml)	821	12.6 ± 11.8	679	13.1 ± 11.4	131	12.7 ± 10.9	615	16.8 ± 13.8	456	17.9 ± 14.2	78	18.3 ± 13.6
VAT (cm^2)	796	153.5 ± 71.6	664	150.2 ± 67.0	128	152.9 ± 63.6	604	130.8 ± 62.5	442	130.7 ± 60.9	79	125.4 ± 60.0
SAT (cm^2)	777	265.4 ± 102.2	655	265.1 ± 102.5	124	271.2 ± 107.3	575	312.6 ± 135.9	424	317.8 ± 146.0	72	325.8 ± 134.3
	rs7746743 (*SIM1*)											
	Whites						Blacks					
	A/A		A/T		T/T		A/A		A/T		T/T	
BMI (kg/m^2)	324	26.4 ± 4.4	839	26.6 ± 4.2	491	26.6 ± 3.9	373	29.3 ± 5.6	568	28.6 ± 5.4	234	27.8 ± 5.4
% Body fat	310	34.8 ± 7.5	809	34.9 ± 7.2	472	34.5 ± 7.0	362	36.0 ± 8.9	549	35.4 ± 8.7	225	35.0 ± 8.0
Leptin (ng/ml)	322	13.1 ± 12.0	828	12.9 ± 11.6	484	12.5 ± 11.3	360	17.7 ± 13.8	559	17.0 ± 14.0	233	17.4 ± 14.0
VAT (cm^2)	312	147.1 ± 64.8	804	153.8 ± 71.4	473	152.4 ± 67.6	353	131.4 ± 58.2	552	131.0 ± 62.0	223	127.3 ± 65.9
SAT (cm^2)	307	265.3 ± 107.8	786	267.7 ± 103.1	464	262.6 ± 98.7	336	329.0 ± 145.0	521	315.5 ± 138.8	217	295.0 ± 131.6
	rs4072568 (*ARNT2*)											
	Whites						Blacks					
	Gly/Gly		Gly/Ser		Ser/Ser		Gly/Gly		Gly/Ser		Ser/Ser	
BMI (kg/m^2)	1,043	26.8 ± 4.1	549	26.2 ± 4.2	61	25.6 ± 3.5	877	28.6 ± 5.5	274	28.8 ± 5.5	22	29.4 ± 4.2
% Body fat	1,003	34.9 ± 7.0	526	34.4 ± 7.5	61	34.2 ± 7.1	844	35.5 ± 8.5	269	35.3 ± 9.0	21	37.6 ± 8.5
Leptin (ng/ml)	1,030	13.0 ± 11.5	542	12.7 ± 11.8	61	10.8 ± 10.0	859	17.4 ± 13.8	269	16.9 ± 14.2	22	19.8 ± 16.0
VAT (cm^2)	1,005	155.7 ± 71.0	528	146.3 ± 65.5	55	142.3 ± 60.5	842	129.8 ± 61.3	262	133.1 ± 64.0	22	122.6 ± 42.7
SAT (cm^2)	983	270.0 ± 100.6	518	258.3 ± 107.2	55	259.2 ± 94.3	797	313.5 ± 137.3	254	319.9 ± 145.4	21	347.3 ± 167.2

[a]None of the trait values are transformed or adjusted for covariates. [b]The sample size was zero (measures of regional adiposity were not available for this participant). *ARNT2*, aryl-hydrocarbon receptor nuclear translocator 2; SAT, abdominal subcutaneous adipose tissue; *SIM1*, single-minded 1; SNP, single-nucleotide polymorphism; VAT, abdominal visceral adipose tissue.

the nominally significant associations between rs3734355/4 and adiposity (Table 3), we found three tagSNPs in Health ABC subjects that remained significantly associated with adiposity after correction for multiple testing. The first of these, rs9390332, is located 6.5 kb downstream from the end of the *SIM1* mRNA transcript and is in moderate LD with rs3734355 (r^2 = 0.54, see **Supplementary Figure S1** online). rs9390332 was significantly associated with BMI and % body fat in the

Table 3 Replicated *SIM1* nsSNP association results in white subjects from the Health ABC Study

Dependent variable per SNP[a]	Whites (n = 1,613–1,711)[b]		White males (n = 854–907)[b]		White females (n = 759–804)[b]	
	$\beta \pm$ s.e.	P	$\beta \pm$ s.e.	P	$\beta \pm$ s.e.	P
rs3734355						
BMI (kg/m^2)	1.31 ± 0.67	0.05	2.69 ± 0.91	0.003	0.29 ± 0.99	NS
% Body fat	1.71 ± 0.87	0.05	2.42 ± 1.20	0.04	1.08 ± 1.26	NS
Leptin (ng/ml)	0.35 ± 0.21	NS	0.47 ± 0.26	NS	0.26 ± 0.34	NS
Leptin (ng/ml)[c]	0.06 ± 0.16	NS	0.03 ± 0.20	NS	0.11 ± 0.24	NS
VAT (cm^2)	0.46 ± 0.33	NS	0.16 ± 0.50	NS	0.78 ± 0.42	NS
SAT (cm^2)	–0.09 ± 0.27	NS	0.03 ± 0.38	NS	–0.06 ± 0.37	NS

NS, nonsignificant; SAT, abdominal subcutaneous adipose tissue; *SIM1*, single-minded 1; nsSNP, nonsynonymous single-nucleotide polymorphism; VAT, abdominal visceral adipose tissue.
[a]rs3734355 was studied assuming a recessive mode of inheritance. [b]Sample size range for 6 models, sample sizes for each model are found in **Supplementary Table S2** online. [c]Leptin outcome was adjusted for percentage of body fat.

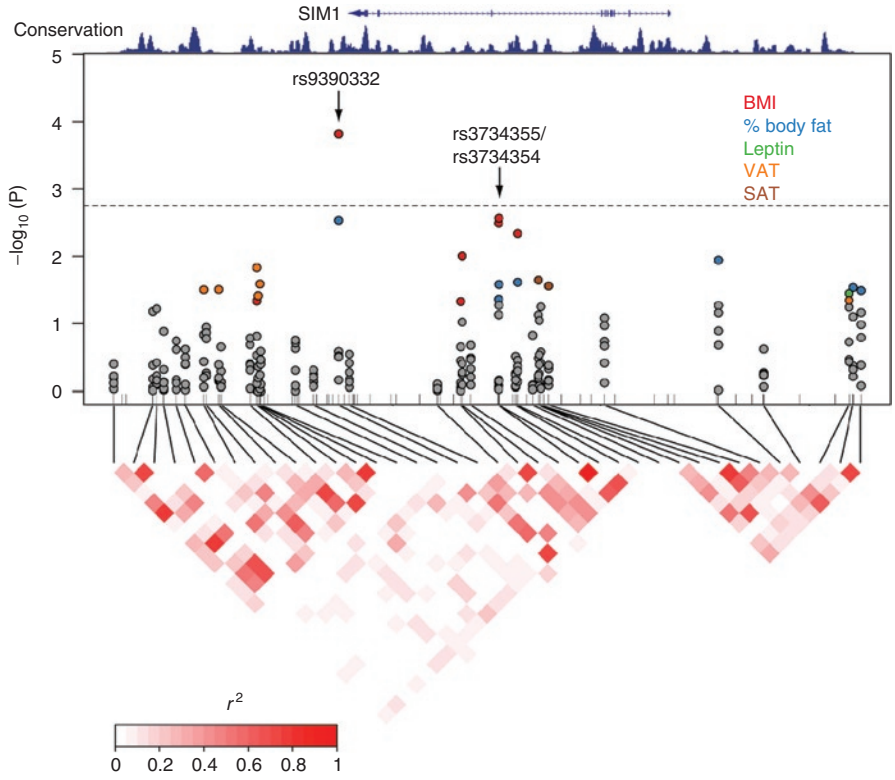

Figure 1 Association analysis and linkage disequilibrium plot for the 38 tagSNPs genotyped in *SIM1* and flanking regions in white males from the Health ABC Study. The upper plot shows the genomic organization of *SIM1* and the degree of conservation among mammalian genomic sequences, with nominally significant P values for associations between various adiposity traits (BMI, % body fat, leptin, VAT, and SAT) at each of 38 tagSNPs shown below. Nonsignificant association P values at the 0.05 level are filled gray, and the colors of the significant ($P < 0.05$) association P values correspond to the colors in the legend for the traits with which they are associated. The dashed line indicates the nominal P value cutoff that results in a significant empirical P value from the permutation test for BMI in white males. Association models assumed an additive mode of inheritance, except for rs3734355/rs3734354, which are modeled using a recessive mode of inheritance. The ticks along the X-axis mark the positions of the 78 genotyped SNPs that passed QC and had an MAF ≥0.05 among whites in the Health ABC Study. The lower plot is of linkage disequilibrium (r^2 units) across *SIM1* in white subjects from the Health ABC Study. MAF, minor allele frequency; SAT, abdominal subcutaneous adipose tissue; *SIM1*, single-minded 1; SNPs, single-nucleotide polymorphisms; VAT, abdominal visceral adipose tissue.

entire group of white subjects and in white males (Figure 1, Table 4). After correction for multiple testing, rs9390332 remained significantly associated with BMI in white males ($P_{unadjusted} = 2 \times 10^{-4}$, $P_{empirical} = 0.005$) under an additive genetic model. The MAF for rs9390332 was 0.22 in whites but only 0.05 in blacks, thus limiting the power to detect an association in the latter group.

The remaining two SNPs were significantly associated with adiposity in black Health ABC subjects after correction for multiple testing. rs3734353, a SNP in intron 5

that was previously associated with BMI in Pima Indians (11), was common in Health ABC whites and blacks (see **Supplementary Table S2** online) and was associated with leptin concentrations in black males ($P_{unadjusted}$ = 0.003). This association became even more significant when leptin concentrations were adjusted for % body fat ($P_{unadjusted}$ = 4 × 10^{-4}, $P_{empirical}$ = 0.02) (**Table 4**). rs7746743, located 35 kb downstream of the *SIM1* polyadenylation signal, was also common in both whites and blacks (see **Supplementary Table S2** online) and was significantly associated with BMI ($P_{unadjusted}$ = 1 × 10^{-4}, $P_{empirical}$ = 0.004) and % body fat ($P_{unadjusted}$ = 5 × 10^{-6}, $P_{empirical}$ = 3 × 10^{-4}) after correction for multiple testing (**Table 4**). When analyzed by sex, these associations were only significant in black females (for BMI: $P_{unadjusted}$ = 7 × 10^{-4}, $P_{empirical}$ = 0.03; for % body fat: $P_{unadjusted}$ = 5 × 10^{-5}, $P_{empirical}$ = 0.002) (**Table 4**).

Table 4 *SIM1* tagSNP association results passing multiple test correction

Dependent variable per SNP[a]	Whites (n = 1,509–1,603)[b]		White males (n = 801–854)[b]		White females (n = 708–749)[b]		Blacks (n = 1,017–1,115)[b]		Black males (n = 435–473)[b]		Black females (n = 582–642)[b]	
	β ± s.e.	P	β ± s.e.	P	β ± s.e.	P	β ± s.e.	P	β ± s.e.	P	β ± s.e.	P
rs9390332												
BMI (kg/m^2)	0.43 ± 0.16	0.009	0.78 ± 0.21	2 × 10^{-4}*	0.01 ± 0.26	NS	0.10 ± 0.48	NS	0.54 ± 0.62	NS	−0.26 ± 0.71	NS
% Body fat	0.49 ± 0.21	0.02	0.80 ± 0.27	0.003	0.19 ± 0.33	NS	1.05 ± 0.54	0.05	1.30 ± 0.77	NS	0.86 ± 0.74	NS
Leptin (ng/ml)	0.05 ± 0.05	NS	0.07 ± 0.06	NS	0.04 ± 0.09	NS	0.21 ± 0.12	NS	0.22 ± 0.16	NS	0.17 ± 0.18	NS
Leptin (ng/ml)[c]	−0.02 ± 0.04	NS	−0.05 ± 0.04	NS	0.01 ± 0.06	NS	0.06 ± 0.09	NS	0.09 ± 0.12	NS	0.00 ± 0.14	NS
VAT (cm^2)	0.01 ± 0.08	NS	0.12 ± 0.12	NS	−0.09 ± 0.11	NS	0.45 ± 0.20	0.02	0.73 ± 0.31	0.02	0.18 ± 0.26	NS
SAT (cm^2)	−0.05 ± 0.07	NS	−0.04 ± 0.09	NS	0.00 ± 0.10	NS	0.35 ± 0.17	0.05	0.28 ± 0.26	NS	0.41 ± 0.24	NS
rs3734353												
BMI (kg/m^2)	0.07 ± 0.15	NS	0.15 ± 0.19	NS	−0.02 ± 0.25	NS	0.07 ± 0.24	NS	0.37 ± 0.33	NS	−0.17 ± 0.35	NS
% Body fat	0.09 ± 0.20	NS	0.42 ± 0.25	NS	−0.29 ± 0.31	NS	0.04 ± 0.27	NS	0.09 ± 0.40	NS	−0.03 ± 0.37	NS
Leptin (ng/ml)	0.04 ± 0.05	NS	0.10 ± 0.05	NS	−0.02 ± 0.08	NS	0.08 ± 0.06	NS	0.25 ± 0.08	0.003	−0.04 ± 0.09	NS
Leptin (ng/ml)[c]	0.02 ± 0.04	NS	0.02 ± 0.04	NS	0.04 ± 0.06	NS	0.07 ± 0.05	NS	0.21 ± 0.06	4 × 10^{-4}*	−0.03 ± 0.07	NS
VAT (cm^2)	0.05 ± 0.08	NS	0.13 ± 0.11	NS	−0.04 ± 0.11	NS	0.01 ± 0.10	NS	−0.14 ± 0.16	NS	0.09 ± 0.13	NS
SAT (cm^2)	0.05 ± 0.06	NS	0.12 ± 0.08	NS	−0.01 ± 0.09	NS	0.07 ± 0.09	NS	−0.04 ± 0.14	NS	0.16 ± 0.11	NS
rs7746743												
BMI (kg/m^2)	−0.04 ± 0.14	NS	0.07 ± 0.18	NS	−0.17 ± 0.23	NS	−0.83 ± 0.21	1 × 10^{-4}*	−0.43 ± 0.28	NS	−1.06 ± 0.31	7 × 10^{-4}*
% Body fat	−0.04 ± 0.18	NS	0.09 ± 0.23	NS	−0.14 ± 0.28	NS	−1.09 ± 0.24	5 × 10^{-6}*	−0.66 ± 0.34	NS	−1.34 ± 0.33	5 × 10^{-5}*
Leptin (ng/ml)	0.00 ± 0.04	NS	−0.02 ± 0.05	NS	0.03 ± 0.08	NS	−0.13 ± 0.06	0.02	−0.08 ± 0.07	NS	−0.14 ± 0.08	NS
Leptin (ng/ml)[c]	0.01 ± 0.03	NS	−0.03 ± 0.04	NS	0.06 ± 0.06	NS	0.01 ± 0.04	NS	0.01 ± 0.05	NS	0.03 ± 0.06	NS
VAT (cm^2)	0.11 ± 0.07	NS	0.21 ± 0.10	0.03	−0.05 ± 0.10	NS	0.11 ± 0.09	NS	0.01 ± 0.14	NS	0.15 ± 0.11	NS
SAT (cm^2)	0.05 ± 0.06	NS	0.04 ± 0.08	NS	0.08 ± 0.09	NS	−0.17 ± 0.08	0.02	−0.29 ± 0.12	0.01	−0.10 ± 0.10	NS

NS, nonsignificant; SAT, abdominal subcutaneous adipose tissue; *SIM1*, single-minded 1; SNP, single-nucleotide polymorphism; VAT, abdominal visceral adipose tissue.
[a]rs9390332, rs3734353, and rs7746743 were studied assuming an additive mode of inheritance. [b]Sample size range for 6 models, sample sizes for each model are found in **Supplementary Table S2** online. [c]Leptin outcome was adjusted for percentage of body fat.
*$P_{empirical}$ < 0.05.

Table 5 *ARNT2* nsSNP association results

Dependent variable per SNP[a]	Whites (n = 1,509–1,602)[b]		White males (n = 802–854)[b]		White females (n = 707–748)[b]		Blacks (n = 1,018–1,113)[b]		Black males (n = 435–472)[b]		Black females (n = 583–641)[b]	
	β ± s.e.	P	β ± s.e.	P	β ± s.e.	P	β ± s.e.	P	β ± s.e.	P	β ± s.e.	P
rs4072568												
BMI (kg/m^2)	−0.51 ± 0.18	0.004	−0.73 ± 0.22	9 × 10^{-4}	−0.21 ± 0.28	NS	0.00 ± 0.31	NS	−0.47 ± 0.41	NS	0.39 ± 0.46	NS
% Body fat	−0.61 ± 0.22	0.007	−0.92 ± 0.28	0.001	−0.17 ± 0.35	NS	−0.04 ± 0.35	NS	−0.76 ± 0.50	NS	0.59 ± 0.48	NS
Leptin (ng/ml)	−0.13 ± 0.06	0.02	−0.13 ± 0.06	0.04	−0.13 ± 0.10	NS	−0.08 ± 0.08	NS	−0.27 ± 0.11	0.01	0.09 ± 0.12	NS
Leptin (ng/ml)[c]	−0.03 ± 0.04	NS	0.01 ± 0.05	NS	−0.10 ± 0.07	NS	−0.04 ± 0.06	NS	−0.12 ± 0.07	NS	0.02 ± 0.09	NS
VAT (cm^2)	0.00 ± 0.09	NS	0.05 ± 0.13	NS	−0.02 ± 0.12	NS	−0.03 ± 0.13	NS	0.08 ± 0.20	NS	−0.09 ± 0.16	NS
SAT (cm^2)	−0.07 ± 0.07	NS	−0.08 ± 0.10	NS	−0.06 ± 0.11	NS	0.09 ± 0.11	NS	−0.09 ± 0.17	NS	0.24 ± 0.15	NS

ARNT2, aryl-hydrocarbon receptor nuclear translocator 2; NS, nonsignificant; nsSNP, nonsynonymous single-nucleotide polymorphism; SAT, abdominal subcutaneous adipose tissue; VAT, abdominal visceral adipose tissue.
[a]rs4072568 was studied assuming an additive mode of inheritance. [b]Sample size range for 6 models, sample sizes for each model are found in **Supplementary Table S2** online. [c]Leptin outcome adjusted for percentage of body fat.

We extended our study by investigating the only common nsSNP, rs4072568, in *SIM1*'s heterodimerization partner, *ARNT2*. rs4072568 was associated with BMI, % body fat, and leptin concentrations in the entire group of white subjects (**Table 5**). Sex-specific analyses indicated that the association was confined to white males (for BMI: $P = 9 \times 10^{-4}$; for % body fat: $P = 0.001$; for leptin concentrations: $P = 0.04$). There was no evidence for a statistical interaction between rs3734355 in *SIM1* and rs4072568 in *ARNT2* in the association with BMI, however.

Finally, we investigated the functional effects of the 352Thr-371Val haplotype on SIM1 activity in an *in vitro* transcriptional assay. When transfected with wild-type ARNT2 into HEK293 cells, the 352Thr-371Val SIM1 double mutant displayed reduced transcriptional activity relative to the wild-type SIM1 (to 78% of wild type, 95% CI: 75–82%, $P < 0.0001$) (**Figure 2**).

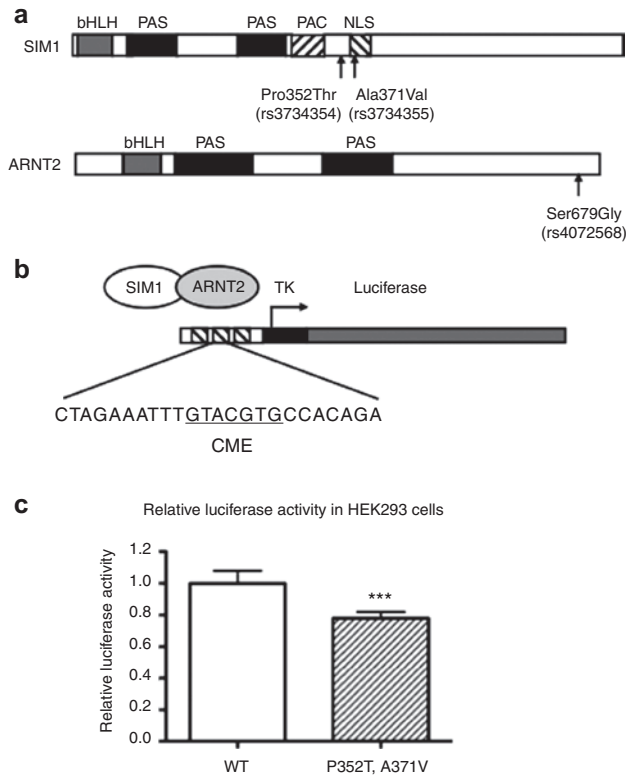

Figure 2 *In vitro* transcriptional assay for SIM1 activity. (**a**) Human *SIM1* and *ARNT2* cDNAs were cloned into the mammalian expression vector pCDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA) and the nsSNPs rs3734354 and rs3734355 were introduced into *SIM1* by site-directed mutagenesis. Functional domains in SIM1 and ARNT2 (and the only common SNP in ARNT2, rs4072568/Gly679Ser) are shown. (**b**) The SIM1 (either WT or 352Thr-371Val) and ARNT2 plasmids were transfected into HEK293 cells using Effectene reagent, along with the pLuc-MCS containing the SIM1 response element (CME) as well as the plasmid pRL-RSV (Promega, Madison, WI), which encodes *Renilla* luciferase, to control for transfection efficiency. (**c**) Relative to the wild-type SIM1, the 352Thr-371Val SIM1 double mutant displayed reduced transcriptional activity (to 78% of WT, 95% CI: 0.75–0.82, $P < 0.0001$) in HEK293 cells. Results shown are the average of seven independent experiments. *ARNT2*, aryl-hydrocarbon receptor nuclear translocator 2; bHLH, basic helix-loop-helix; CI, confidence interval; CME, CNS midline enhancer; NLS, nuclear localization signal; nsSNP, nonsynonymous single-nucleotide polymorphism; PAC, conserved domain occurring C-terminal to a PAS domain; PAS, Per-Arnt-Sim domain; *SIM1*, single-minded 1; WT, wild type.

DISCUSSION

We have confirmed previous findings (10) that homozygosity for a common *SIM1* haplotype consisting of two amino acid changes (Pro352Val/rs3734354 and Ala371Val/rs3734355) is associated with an increase in BMI in white males. We extended these findings in several ways. First, we obtained nominal evidence for an association between homozygosity for this haplotype and a more informative measure of adiposity (% body fat). Second, by systematically scanning the *SIM1* region (from 45 kb upstream of the 5′ end to 60 kb downstream of the 3′ end), we demonstrated that the most significant association with adiposity in white males was at these two nsSNPs, and at a related SNP, rs9390332, in the 3′ flanking region. rs9390332 is located in/near regions of high mammalian conservation 6.5 kb downstream of the *SIM1* polyadenylation signal, and is in moderate LD ($r^2 = 0.54$) with rs3734355/4. Lastly, we strengthened the biological relevance of this association by showing that the 352Thr-371Val haplotype impairs SIM1's ability to activate target gene transcription. Future studies will be required to determine whether this occurs via impaired nuclear localization (as rs3734355 is located in the SIM1 nuclear localization signal (21)), and whether rs9390332 alters SIM1 function in an as yet unidentified manner. It is also unknown whether *SIM1* 352Thr-371Val homozygotes recapitulate the other phenotypes of hyperphagia, hyperinsulinemia, or increased linear growth observed in *SIM1* haploinsufficient mice (5); although milder effects on these phenotypes would be expected based on our functional results. Interestingly, a comparable impairment of SIM1 *in vitro* transcriptional activity (to 67% of wild-type levels, $P = 0.093$) by the 352Thr-371Val haplotype was observed in another recent report (12). The additional association between the only common nsSNP in *SIM1*'s heterodimerization partner *ARNT2* and BMI in white men further highlights the role of this axis in human body weight regulation.

The greater significance of the association between the 352Thr-371Val haplotype and BMI in the present study ($P = 0.002$) compared to the initial report ($P = 0.04$) (10) was in spite of a smaller sample size (907 white men vs. 2,166 white men in the EPIC-Norfolk Study) and was not due to the use of different covariates. It is possible that the effects of this haplotype on adiposity may be age-dependent, as one of the recent studies that did not find an association between rs3734354/5 and adiposity (12) examined subjects who were ~10 years younger than the subjects studied in (10) and ~30 years younger than the subjects in the present study. In addition to its critical role for hypothalamic development, *Sim1* is also known to influence feeding behavior in adult mice (22,23). One interpretation of the available data is that the subtle defect in SIM1 function conferred by the 352Thr-371Val haplotype causes a slight shift toward positive energy balance throughout life. Therefore, it is conceivable that significant differences in

adiposity between genotype groups may not become apparent until well into middle age. This possibility remains to be investigated in longitudinal studies.

Genome-wide association studies (GWAS) for obesity in whites (24–26) have not reported significant associations for BMI at rs3734355, rs3734354, or rs9390332. This could be partly due to the absence of these SNPs from many of the genotyping panels used for the discovery stages of recent GWAS, although their genotypes could be imputed. Moreover, these recent GWAS assumed an additive mode of inheritance for all SNPs, whereas we found a much more significant association between rs3734355 and BMI in white males when using a recessive model ($P_{add} = 0.04$ compared to $P_{rec} = 0.003$). The closest positive result obtained in a GWAS for BMI was obtained at rs2572016, about 1.3 million bp centromeric to rs3734355, ($P = 0.05$ in a meta-analysis of 16,876 individuals) (24). However, this SNP is separated from rs3734354/rs3734355 (and from rs7746743 and rs9390332) by several known human genes (*MCHR2*, *PRDM13*, *CCNC*, *USP45*, *SFRS18*, and *COQ3*). Finally, our results suggest that the effect of rs3734355/rs3734354 on BMI is stronger in males, yet none of these GWAS have performed separate analyses by sex.

We scanned the *SIM1* region with as many or more SNPs than used in current genotyping platforms. In whites from the Health ABC Study, 78 genotyped SNPs passed QC, yielding a density of one genotyped SNP every 2.3 kb. In blacks, 69 of the 88 genotyped SNPs passed QC, yielding a density of one genotyped SNP every 2.7 kb. For this same genomic region, the Affymetrix 500K, 5.0, and 6.0, and the Illumina Infinium HumanHap 370K, 610K, and 1M arrays genotype 37, 34, 62, 27, 46, and 69 SNPs, respectively.

The most plausible mechanism by which genetic variation in *SIM1* might contribute to adiposity is via the regulation of feeding behavior. An individual with a balanced translocation interrupting *SIM1* displayed early onset hyperphagic obesity but no obvious defect in energy expenditure (6). *Sim1*$^{+/-}$ mice exhibit a partial failure of the terminal migration and differentiation of hypothalamic neurons in the PVN, supraoptic, and anterior periventricular nuclei (1,4). *Sim1* is also expressed postdevelopmentally in the PVN, basomedial amygdala, and lateral hypothalamus (27). Adenovirally mediated overexpression or silencing of *Sim1* expression in the PVN of weaned mice inhibits and stimulates food intake, respectively (22).

SIM1's inhibitory effects on feeding are likely to involve a reduction in the expression of specific peptides in the PVN. Secretory PVN neurons produce a variety of peptides, including somatostatin, arginine vasopressin, oxytocin, corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone. In the hypothalamus of a *Sim1*$^{+/-}$ mouse strain, the mRNA expression levels of all of these peptides are reduced by 20–80%, relative to wild-type mice (2). In particular, normal oscillations in *Crh* and oxytocin (*oxt*) mRNA expression in response to feeding and fasting were either markedly attenuated (*Crh*) or completely abolished (*oxt*) in *Sim1*$^{+/-}$ mice. *Crh* and *oxt* mediate the anorectic effects of leptin in the central nervous system (28,29). In particular, oxytocin is thought to facilitate the termination of feeding behavior, as intracerebroventricular infusion of oxytocin produces a dose-dependent reduction in feeding in rats fed *ad libitum* as well as those fed again after a period of food deprivation (30,31). Oxytocin is implicated in the hyperphagia of *Sim1*$^{+/-}$ mice, as intracerebroventricular administration of an oxytocin antagonist increased food intake in *Sim1*$^{+/-}$ mice but not wild-type mice, while central oxytocin administration abolished hyperphagia in *Sim1*$^{+/-}$ mice but had no effect in wild-type mice (2). Intriguingly, oxytocin's effects on feeding cessation may be nutrient-specific, as oxytocin-deficient mice have an increased preference for ingesting excessive amounts of carbohydrates (32). Accordingly, the reduced transcriptional activity of the *SIM1* 352Thr-371Val haplotype could promote weight gain in homozygous individuals through a defect in feeding cessation and/or a change in macronutrient preference. This remains to be investigated in future studies.

It is unclear why the association between 352Thr-371Val haplotype and adiposity is confined to males; such sex-specific effects on adiposity are not observed in *SIM1* haploinsufficient mice (5,27). PVN oxytocin expression is influenced by female sex hormones (33,34); however, the female subjects in the present study were aged 70–79 and therefore postmenopausal. CRH-producing neurons in the human PVN display strong androgen- and estrogen-receptor immunoreactivity (35,36), and expression of androgen receptor in combination with testosterone treatment represses CRH promoter activity in cultured neuroblastoma cells (35). Whether the sex-specific effects of the *SIM1* 352Thr-371Val haplotype involve a greater suppression of PVN oxytocin and/or CRH expression will require further investigation.

Notably, *Sim1*$^{+/-}$ mice are also resistant to the anorexigenic effects of a melanocortin agonist (37), and display a marked reduction in hypothalamic *Mc4r* mRNA expression (23). If the same is true in humans, this suggests that a subtle defect in SIM1 function could recapitulate, at least in part, obesity due to MC4R deficiency (38).

The lack of a significant association between *SIM1* 352Thr-371Val haplotype and adiposity in blacks was most likely due to the low MAF (0.03). However, our results obtained in these subjects do support a relationship between genetic variation in *SIM1* and adiposity: the single black female homozygous for the rs3734355 371Val allele had a much higher BMI than the mean BMI for carriers of the 371Ala allele (**Table 2**), and we obtained independent positive associations at rs3734353 and rs7746743 for global measures of adiposity that remained significant after correction for multiple testing (**Table 4**). rs3734353 is located within intron 5, and rs7746743 is located 35 kb downstream of the *SIM1* polyadenylation signal within a 41 bp conserved region that is 85.4% conserved between mice and humans (39). One could speculate that this region functions as a distal enhancer to regulate *SIM1* expression, as the nearest refSeq gene to rs7746743 other than *SIM1* is *MCHR2*, which is ~300 kb centromerically. The lack of an association between these two SNPs

and adiposity in whites cannot be explained by differences in MAF (see **Supplementary Table S2** online), and therefore indicates the presence of substantial genetic heterogeneity in addition to the sex-specific effects of *SIM1* genotype on adiposity.

A recent investigation of *SIM1* in Pima Indians (11) found that the most significant and reproducible associations with BMI were obtained for SNPs spanning a region from the 5′ untranslated region to intron 8, assuming an additive mode of inheritance. In Pima Indians, neither rs3734355 nor rs3734354 were associated with BMI, however, and attempts to replicate the most significant association results (rs3734353 and rs3213541 in both the full- and partial-heritage Pima Indians) in French whites were unsuccessful (11,12). Marked differences in allele frequency between populations were observed. For example, the frequencies of rs3734355 and rs3734354 were much higher in Pima Indians (MAF ~0.26) than in either the whites (0.14) or blacks (0.03) studied here. Consistent with the findings in French whites (12), we also failed to replicate the rs3734353 result among white Health ABC subjects, although we did obtain robust evidence for association at rs3734353 with leptin concentrations in black males (**Table 4**). We did not investigate rs3213541, as it was excluded due to violation of Hardy–Weinberg equilibrium (see **Supplementary Methods and Procedures** online). Lastly, the SNP rs7746743, where we obtained our most significant results for all black subjects (men and women) and black women, is outside of the region studied by Traurig *et al.* (11).

In conclusion, we have obtained evidence that dysregulation of the SIM1-ARNT2 axis, binding partners required for the differentiation of specific neurons involved in the regulation of appetite and body weight, contributes to adiposity in whites and blacks. This finding reinforces the view that human obesity is influenced by genetic variation in developmental factors (40), many of which are predominantly expressed in the central nervous system (26).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/oby

ACKNOWLEDGMENTS

This work was supported by funding from the National Institutes of Health (K01 AG022782, R01 AG023692, R01 DK60540), the Intramural Research Program of the NIH, National Institute on Aging (N01-AG-6-2101, N01-AG-6-2103, N01-AG-6-2106), the American Diabetes Association (1-05-RA-54), the National Center for Research Resources (M01 RR-00079), and the UCSF Diabetes and Endocrinology Research Center (P30 DK63720). M.M.S. is supported by a National Health and Medical Research Council of Australia (NHMRC) Program Grant "Pathways to Diabetes Prevention." D.S.E. is supported by NIH training grant T32 DK007418. M.I.V. is supported by a Postdoctoral Fellowship from the Spanish Ministerio de Educacion y Ciencia. I.M. is supported by Mentored Research Scientist Development Award from the National Institute of Diabetes and Digestive and Kidney Diseases (K01 DK083029). C.V. is supported by an Established Investigator Award from the American Heart Association. The authors would like to thank all subjects for their participation in the study.

DISCLOSURE

The authors declared no conflict of interest.

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