

Exome Sequencing of 21 Bardet-Biedl Syndrome (BBS) Genes to Identify Obesity Variants in 6,851 American Indians

Samantha E. Day¹, Yunhua L. Muller¹, Cigdem Koroglu¹, Sayuko Kobes¹, Kim Wiedrich¹, Darin Mahkee¹, Hye In Kim², Cris Van Hout², Nehal Gosalia², Bin Ye², Regeneron Genetics Center², Alan R. Shuldiner², William C. Knowler¹, Robert L. Hanson¹, Clifton Bogardus¹, and Leslie J. Baier¹

Objective: In an ongoing effort to identify the genetic variation that contributes to obesity in American Indians, known Bardet-Biedl syndrome (BBS) genes were analyzed for an effect on BMI and leptin signaling.

Methods: Potentially deleterious variants (Combined Annotation Dependent Depletion score > 20) in BBS genes were identified in whole-exome sequence data from 6,851 American Indians informative for BMI. Common variants (detected in ≥ 10 individuals) were analyzed for association with BMI; rare variants (detected in < 10 individuals) were analyzed for mean BMI of carriers. Functional assessment of variants' effect on signal transducer and activator of transcription 3 (STAT3) activity was performed *in vitro*.

Results: One common variant, rs59252892 (Thr549Ile) in *BBS9*, was associated with BMI ($P=0.0008$, $\beta=25\%$ increase per risk allele). Among rare variants for which carriers had severe obesity (mean BMI > 40 kg/m²), four were in *BBS9*. *In vitro* analysis of *BBS9* found the Ile allele at Thr549Ile had a 20% increase in STAT3 activity compared with the Thr allele ($P=0.01$). Western blot analysis showed the Ile allele had a 15% increase in STAT3 phosphorylation ($P=0.006$). Comparable functional results were observed with Ser545Gly and Val209Leu but not Leu665Phe and Lys810Glu.

Conclusions: Potentially functional variants in BBS genes in American Indians are reported. However, functional evidence supporting a causal role for *BBS9* in obesity is inconclusive.

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Introduction

American Indians living in the Southwestern United States suffer from a high prevalence of obesity (1), yet much of what is known about the genetic underpinnings of obesity has come from the Genetic Investigation of Anthropometric Traits (GIANT) (<https://portals.broadinstitute.org/collaboration/giant>) and UK Biobank (<https://www.ukbiobank.ac.uk/>) databases, which do not include this ethnic group. Although obesity in American Indians, as in other ethnic groups, is typically polygenic in nature, we are systematically analyzing the possible contribution of known monogenic obesity genes. For example, missense variation in the melanocortin-4 receptor gene (*MC4R*) is the most common cause of monogenic obesity in humans, yet we have previously found that 2.4% of an American Indian community in Arizona carries one of several rare functional coding mutations in

¹ Phoenix Epidemiology and Clinical Research Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Phoenix, Arizona, USA. Correspondence: Leslie J. Baier (lbaier@phx.niddk.nih.gov) ² Regeneron Genetics Center, Tarrytown, New York, USA.

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Study Importance

What is already known?

- ▶ American Indians are disproportionately affected by obesity, and obesity has a genetic component.
- ▶ Variants in several genes known to cause rare forms of monogenic disease have also been shown to contribute to polygenic obesity, such as the genes known to contribute to Bardet-Biedl syndrome (BBS).

What does this study add?

- ▶ This study provides information on potentially functional BBS variants detected in whole-exome sequence data on 6,851 American Indians, with several rare variants found only among individuals with the most severe obesity.
- ▶ Among the common variants, only rs59252892 in *BBS9* showed a statistical association with BMI, and *in vitro* assessment indicated that the effect on BMI may be explained by altered leptin signaling.

How might these results change the direction of research?

- ▶ Overall, variants in known BBS genes are not a major contributor to obesity among American Indians; however, variation in *BBS9* may have a very modest effect.
- ▶ Our *in vitro* studies indicate that the relationship between the chronic effects of BBS impairment (leptin transport to the cell surface and subsequent signaling) and the development of obesity may be complicated, and future *in vivo* mouse models of impaired *BBS9* should be pursued.

MC4R (2). The leptin-melanocortin pathway is critical for the hypothalamic control of food intake, where *MC4R* mutations have led to hyperphagia and obesity (3,4). Bardet-Biedl syndrome (BBS) is another well-characterized monogenic disease with at least 21 causal genes that all function in primary cilia (5), and some can play a role in the leptin-melanocortin pathway. Obesity is the predominant clinical feature of BBS, and individuals manifesting the full syndrome through recessive inheritance can also present with polydactyly, rod-cone dystrophy, kidney abnormalities, and developmental delay (6). However, heterozygous carriers of BBS mutations have an increased risk of developing obesity but typically lack the other syndromic characteristics (7). Therefore, it is possible that variation in BBS genes may similarly contribute to polygenic obesity in this American Indian population. The leading mechanism by which BBS mutations can lead to obesity is through altered localization of key metabolic receptors in hypothalamic neurons. Multiple BBS proteins (BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9, and BBS18) compose a complex called the BBSome, which mediates protein trafficking to the cilia surface (8). Both the leptin receptor (LEPR) and neuropeptide Y receptor type 2, which contribute to anorexigenic signaling, are shuttled to the cell surface by the BBSome (8-10). Therefore, the objective of this study was to (1) identify variants in known BBS genes that are likely to be functional and to statistically associate with BMI or, if rare, that occur in individuals with severe obesity and (2) assess the impact of these variants on an obesity-related pathway.

Methods

Participants

Participants were from two research protocols of health among American Indians conducted by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) in Phoenix, Arizona. One of these studies was a community-based, longitudinal study (conducted from 1965 to 2007) of American Indians (11), and the other study is an ongoing study (2011 to the present) of American Indians living in urban Phoenix (12). For participants with longitudinal clinical and anthropometric measures, we defined BMI as the participant's maximum BMI recorded at an examination at age ≥ 15 years. For the urban Indian cohort, maximum BMI at age ≥ 15 years was self-reported. In total 6,851 participants had whole-exome sequence (WES) data and were informative for BMI (6,002 from the longitudinal, community-based cohort and 849 from the urban American Indian study; combined participants were 43.0% male, mean [SD] age at maximum BMI = 32.5 [12.8] years; mean [SD] maximum BMI = 36.4 [8.8] kg/m²). Given that the population BMI average is high, we used the Centers for Disease Control and Prevention severe obesity definition of BMI ≥ 40 as a cutoff to focus on variation that may have the greatest genetic penetrance. All participants gave written informed consent before participation in the study. The study protocols were approved by the review board of the NIDDK and/or by the Phoenix Area Indian Health Service Institutional Review Board.

Whole-Exome Sequencing

WES was performed by the Regeneron Genetics Center (Tarrytown, New York) as previously described (13). Sequence data were aligned to the reference human genome (hg38), and $>85\%$ of target regions had 20 \times coverage.

Statistical analysis

Statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina) and SOLAR (Sequential Oligogenic Linkage

Analysis Routines, University of Maryland, Baltimore, Maryland) for variants with a Combined Annotation Dependent Depletion (CADD) score >20 and ≥ 10 carriers out of the 6,851 samples. For the longitudinal protocol participants, BMI association was analyzed by a linear mixed model fitted with a variance components covariance structure to account for genetic relatedness among individuals to avoid bias. The genetic relatedness matrix was estimated as the proportion of the genome shared identical by descent as previously described (14). BMI association from the self-reported data was analyzed by linear regression, and genomic control was used to account for population structure (15). Meta-analysis across both data sets using a fixed-effects inverse variance method was performed for the final BMI association results. *P* values were adjusted for age, sex, birth year, and the first five genetic principal components. The resulting individual variants across all BBS genes from the meta-analysis were considered significant after Bonferroni correction ($P < 0.05/N$ deleterious variants). To identify a gene-level association with BMI, the Meta-Analysis of Gene-level Association (MAGA) software was used with Sequence Kernel Association Test statistics (16).

Functional analyses of five nonsynonymous *BBS9* variants

Expression vectors containing full-length human myc-DDK-tagged *LEPR* and *BBS9* were obtained from Origene (Rockville, Maryland). The QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, California) was used to create five different *BBS9* (NM_198428) constructs with the following amino acid substitutions: exon7:c.G625T:p.V209L, exon16:c.A1633G:p.S545G, exon16:c.C1646T:p.T549I, exon19:c.C1993T:p.L665F, and exon21:c.A2428G:p.K810E. All constructs were confirmed by sequencing.

HEK-293 (American Type Culture Collection, Manassas, Virginia) and mouse N42 hypothalamus cell lines (Cellutions Biosystems Inc., Burlington, North Carolina) were each maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, Maryland) containing 4.5 g/L of glucose, 1.5 g/L of sodium bicarbonate, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin in 5% CO₂ and 95% air atmosphere at 37°C. To assess the effects of *BBS9* variants on LEPR, cells were cotransfected with 500 ng of a *BBS9* construct, 500 ng of *LEPR*, and 500 ng of the reporter mixture of signal transducer and activator of transcription 3 (STAT3)-mediated firefly luciferase construct and constitutively expressed Renilla luciferase construct (40:1) (Qiagen, Hilden, Germany) using Lipofectamine LTX (Invitrogen, Carlsbad, California). Twenty-four hours following transfection, cells were serum starved for 6 hours in Opti-MEM (Thermo Fisher, Waltham, Massachusetts) with 0.5% FBS, then 100 ng/mL of recombinant human leptin (L4146; Sigma Aldrich, St. Louis, Missouri) was added to the medium for 18 hours prior to harvest. For total protein harvest, cells were serum starved 30 hours following transfection in Opti-MEM with 0.5% FBS for 18 hours, then 100 ng/mL of recombinant human leptin (L4146) was added to the medium for 30 minutes prior to harvest.

Luciferase activity was measured in duplicate on a TD-20/20 luminometer (Turner BioSystems, Sunnyvale, California) using the Dual-Luciferase assay kit (Promega, Madison, Wisconsin) per manufacturer's instructions. Relative luciferase activity was calculated as a ratio of the firefly to Renilla luciferase activity to control for transfection efficiency and further normalized to the leptin-treated transfection containing the *BBS9* wild-type (WT) vector. The data from four (N42 cells) or five (HEK-293 cells) separate transfections were averaged and then normalized to the *BBS9* WT. The difference in activity was compared with the *BBS9* WT using a one-sample *t* test.

To further confirm the effects of *BBS9* variants on LEPR-mediated STAT3 phosphorylation, Western blot analysis was performed to detect pSTAT3 using phospho-STAT3 (Tyr705) antibody (Cell Signaling, Danvers, Massachusetts) and normalized to total STAT3 (124H6) (Cell Signaling). The signal intensity was scanned on a ChemiDoc Touch Imaging System (Bio-Rad, Hercules, California) and quantified using Image Lab software (version 5.2.1, Bio-Rad). Data from three (HEK-293 cells) independent experiments were averaged and then normalized to the *BBS9* WT. The difference in activity was compared with the *BBS9* WT using a one-sample *t* test.

Serum leptin measures

Serum leptin was measured using the Human Leptin Quantikine ELISA Kit (R&D Systems, Minneapolis, Minnesota) per the manufacturer's instructions.

Results

Association analysis of potentially damaging BBS variants

Analysis of WES data identified 1,308 variants (Supporting Information Table S1) in the 21 BBS genes (ADP ribosylation factor like GTPase 6 [*ARL6*], BBSome interacting protein 1 [*BBIP1*], *BBS1*, *BBS10*, *BBS12*, *BBS2*, *BBS4*, *BBS5*, *BBS7*, *BBS9*, chromosome 8 open reading frame 37 [*C8orf37*], centrosomal protein 290 [*CEP290*], intraflagellar transport 172 [*IFT172*], intraflagellar transport 27 [*IFT27*], leucine zipper transcription factor like 1 [*LZTFL1*], McKusick-Kaufman syndrome [*MKKS*], Meckel syndrome type 1 [*MKS1*], serologically defined colon cancer antigen 8 [*SDCCAG8*], tripartite motif containing 32 [*TRIM32*], tetratricopeptide repeat domain 8 [*TTC8*], and WD repeat containing planar cell polarity effector [*WDPCP*]), of which 235 variants had a CADD score >20 (top 1% most likely to be deleterious). Among these potentially deleterious variants which had ≥ 10 carriers ($N=50$; Supporting Information Table S2) in the 6,851 participants, only the Thr549Ile (rs59252892) variant in *BBS9* significantly associated with BMI (significance requires a Bonferroni-corrected $P < 0.001$). For this variant, the mean BMI of the 13 Ile-allele carriers was 42.8 (SD 12.6) kg/m² versus 36.3 (SD 8.8) kg/m² for non-carriers ($P=0.0008$, $\beta=0.23$ on log_e scale, which corresponds to a 25% increase in BMI per copy of the risk allele; Figure 1).

Characterization of carriers with uncommon potentially damaging BBS variants

A total of 185 variants were detected that had a CADD >20 but occurred in <10 carriers. Because these variants are too rare to provide robust statistical analysis, we identified those variants that were restricted to participants who met criteria for severe obesity (defined as a mean BMI for the variant carriers [e.g., 1-9 carriers] of ≥ 40 kg/m²). Information on these 39 variants is given in Table 1; all carriers were heterozygous. Among these variants, no individual was homozygous or was a compound heterozygote. One individual with a rare variant in a BBS gene also carried a I269N variant in their *MC4R* gene; this individual was excluded from Table 1 because of confounding effects of *MC4R* on severe obesity status.

BBS9 is prioritized for further study

Among the common variation detected in the BBS genes, only the Thr594Ile variant in *BBS9* provided statistical evidence for an effect on

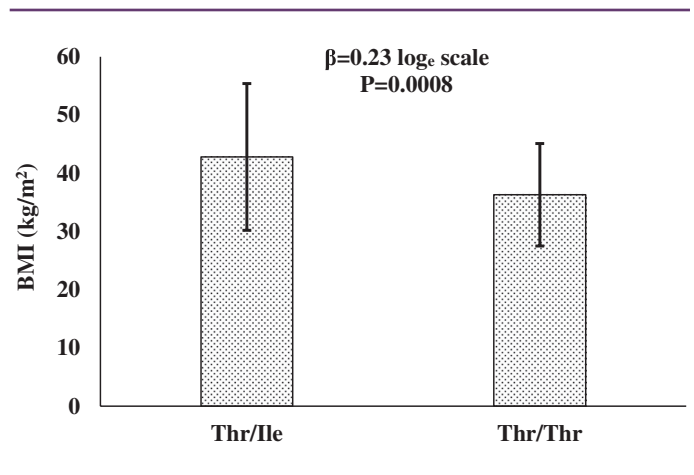


Figure 1 Association of *BBS9* variant rs59252892 (Thr549Ile) and BMI. Bars represent the mean \pm SD for the 13 Thr/Ile heterozygotes and 6,838 Thr/Thr homozygotes. The beta (β) log_e scale corresponds to a 25% increase in BMI per copy of the risk allele. *P* values were adjusted for age, sex, birth year, and the first five genetic principal components.

BMI. *BBS9* also had four uncommon variants detected solely in individuals with severe obesity (Table 1). Therefore, only *BBS9* was further studied both functionally and statistically for a potential role in obesity.

BMI and serum leptin levels in pedigrees informative for Thr594Ile in BBS9. Pedigree information was available for 8 of the 13 Ile-allele carriers for the Thr594Ile variant in *BBS9* (Figure 2). Among full or half siblings, BMI values appeared to segregate by genotype of Thr549Ile, such that Ile carriers had a higher maximum BMI compared with their noncarrier sibling. For example, in Family A, the second-generation Ile carriers had BMI of 41 or 35 kg/m², whereas the noncarrier sibling had BMI of 22 kg/m². Furthermore, serum leptin was measured in available Ile-allele carrier and noncarrier family members. Although women typically have higher leptin levels than men, all Ile-allele carriers had elevated leptin levels compared with their noncarrier siblings when adjusted by age and sex ($P=0.028$, $\beta=17.7$ ng/mL higher in the risk allele carriers). In Figure 2, this can be best observed in the second generation of Family B, in which male and female Ile-allele carriers all had leptin levels >20 ng/mL compared with noncarriers that had <10 ng/mL.

BBS9 variants alter STAT3 activity in leptin signaling. The Thr549Ile and four rare nonsynonymous variants in *BBS9* listed in Table 1 were studied for an effect on leptin signaling using a luciferase reporter assay to detect STAT3 transcriptional activity (Figure 3). In HEK-293 cells, the obesity risk allele (Ile) at Thr549Ile had a 20% increase in leptin signaling activity compared with the nonrisk Thr allele ($P=0.01$). Similarly, the obesity risk allele (Gly) at Ser545Gly had a 24% increase in leptin signaling activity compared with the nonrisk Ser allele ($P=0.02$). A slightly more subtle change was identified for the Val209Leu variant, in which there was a 13% increase in signaling for the obesity risk allele (Leu) compared with the nonrisk Val allele ($P=0.004$). No significant changes were identified for the Leu665Phe and Lys810Glu variants in *BBS9*. The luciferase reporter assay was repeated in the N42 hypothalamus cell line, and a similar trend was observed for all variants, but only Thr549Ile showed a significant difference ($P=0.01$) between the two alleles. Western blot

TABLE 1 Rare variants with a CADD score >20 in known BBS genes, in which the mean BMI of carriers meets the threshold for severe obesity (≥ 40 kg/m²)

Gene	Position	Variant ID	Ref/Alt	Variation type	Carriers (n)	Carrier mean BMI (kg/m ²)	CADD score
BBS9	chr7:33534083	novel	A/G	K810E	3	51.0 ± 10.6	26.6
	chr7:33388022	rs116262072	C/T	L665F	5	43.1 ± 6.5	25.0
	chr7:33264297	novel	G/T	V209L	6	43.2 ± 3.8	25.0
	chr7:33357935	novel	A/G	S545G	1	41.0 ± 0	21.8
BBS12	chr4:122742092	rs767546051	G/A	G67E	1	42.2 ± 0	25.2
	chr4:122742965	novel	T/C	L358P	1	40.3 ± 0	26.8
IFT172	chr2:27465860	rs764302265	C/T	R572Q	1	59.0 ± 0	26.2
	chr2:27453711	rs755179952	C/T	R1247H	1	45.0 ± 0	25.7
	chr2:27457715	novel	T/C	H1051R	1	42.0 ± 0	25.7
	chr2:27478063	rs755816980	G/A	R367C	1	48.0 ± 0	23.2
	chr2:27471067	rs375640327	G/C	S518C	1	45.0 ± 0	23.4
	chr2:27461450	novel	T/C	E754G	4	40.0 ± 7.9	23.3
	chr2:27470935	rs61743977	G/C	T562S	9	40.3 ± 8.9	21.7
	chr2:27481109	novel	C/T	R241Q	1	40.3 ± 0	23.5
TTC8	chr14:88872339	novel	G/T	D412Y	1	50.0 ± 0	29.9
CEP290	chr12:88130283	rs863225185	A/C	Stopgain	2	40.7 ± 2.5	36.0
	chr12:88087886	rs188502327	C/T	R1363Q	1	58.2 ± 0	26.6
	chr12:88060900	rs191613017	A/G	L2151S	5	44.3 ± 7.1	28.2
	chr12:88114508	novel	A/C	L655W	2	48.6 ± 14.6	27.2
	chr12:88093829	rs372918770	G/C	R1084G	3	50.0 ± 12.0	32.0
	chr12:88080352	rs377143954	C/T	E1686K	1	42.3 ± 0	21.2
	chr12:88093829	rs372918770	G/C	R1084G	3	45.4 ± 4.9	22.9
	chr12:88141277	rs185939120	T/C	M11V	1	43.7 ± 0	23.2
BBS4	chr15:72709757	novel	G/A	R45Q	1	48.3 ± 0	22.7
	chr15:72731341	rs34620165	G/A	G250R	1	49.7 ± 0	24.0
WDPCP	chr2:63487446	rs187135801	C/T	Splice donor	1	52.0 ± 0	33.0
	chr2:63259334	rs760742250	C/T	D471N	2	41.0 ± 6.5	24.7
	chr2:63404381	rs769112221	G/A	R368C	1	50.0 ± 0	23.5
	chr2:63439871	rs191796211	G/C	L129V	1	56.0 ± 0	22.0
	chr3:97788082	rs148745414	T/G	C148G	1	66.9 ± 0	20.8
TRIM32	chr9:116699580	rs199704873	G/A	R613Q	2	43.0 ± 6.2	22.8
BBS1	chr11:66514413	novel	T/C	V56A	2	48.6 ± 3.2	24.5
	chr11:66514544	rs769949890	C/T	R100W	1	40.4 ± 0	26.6
	chr11:66530954	rs369653969	C/A	R512S	1	40.4 ± 0	20.4
IFT27	chr22:36762906	rs780844069	C/T	V154M	1	42.0 ± 0	31
	chr22:36775708	rs143443220	G/A	5'UTR	1	58.2 ± 0	20.9
BBS2	chr16:56485706	rs758343294	T/C	Y648C	2	47.2 ± 16.4	24.8
BBS5	chr2:169493769	rs137853921	A/G	N184S	4	40.2 ± 8.8	25.7

"Carriers (n)" represents the number of heterozygous carriers for the alternative allele. Because of longitudinal measures of BMI for most participants, BMI is defined as the maximum BMI measured at an examination at age ≥ 15 years. CADD, Combined Annotation Dependent Depletion score; Ref/Alt, reference and alternative alleles.

analyses to detect the phosphorylated STAT3 mediated by leptin signaling were conducted for both the Thr549Ile and Ser545Gly variants because they each presented a $\geq 20\%$ change in luciferase activity between alleles. Results for both showed a similar increase in leptin signaling in which the Ile allele at Thr549Ile had a 15% increase in STAT3 phosphorylation compared with the Thr allele ($P=0.006$) and the Gly allele at Ser545Gly had an 18% increase in STAT3 phosphorylation compared with the Ser allele ($P=0.07$) (Figure 4).

BBS9 gene-level analysis. Two rare variants, Ser545Gly and a Val209Leu (with only one and six carriers, respectively), in our WES sample provided some experimental evidence of affecting leptin levels. Therefore, we sought to determine whether a gene-based association test of all potentially damaging uncommon variants could lend statistical support for BBS9 having a role in determining BMI. All 26 variants in BBS9 with a CADD >20 and a minor allele frequency (MAF) <0.05 were analyzed using a gene-based meta-analysis (variants shown

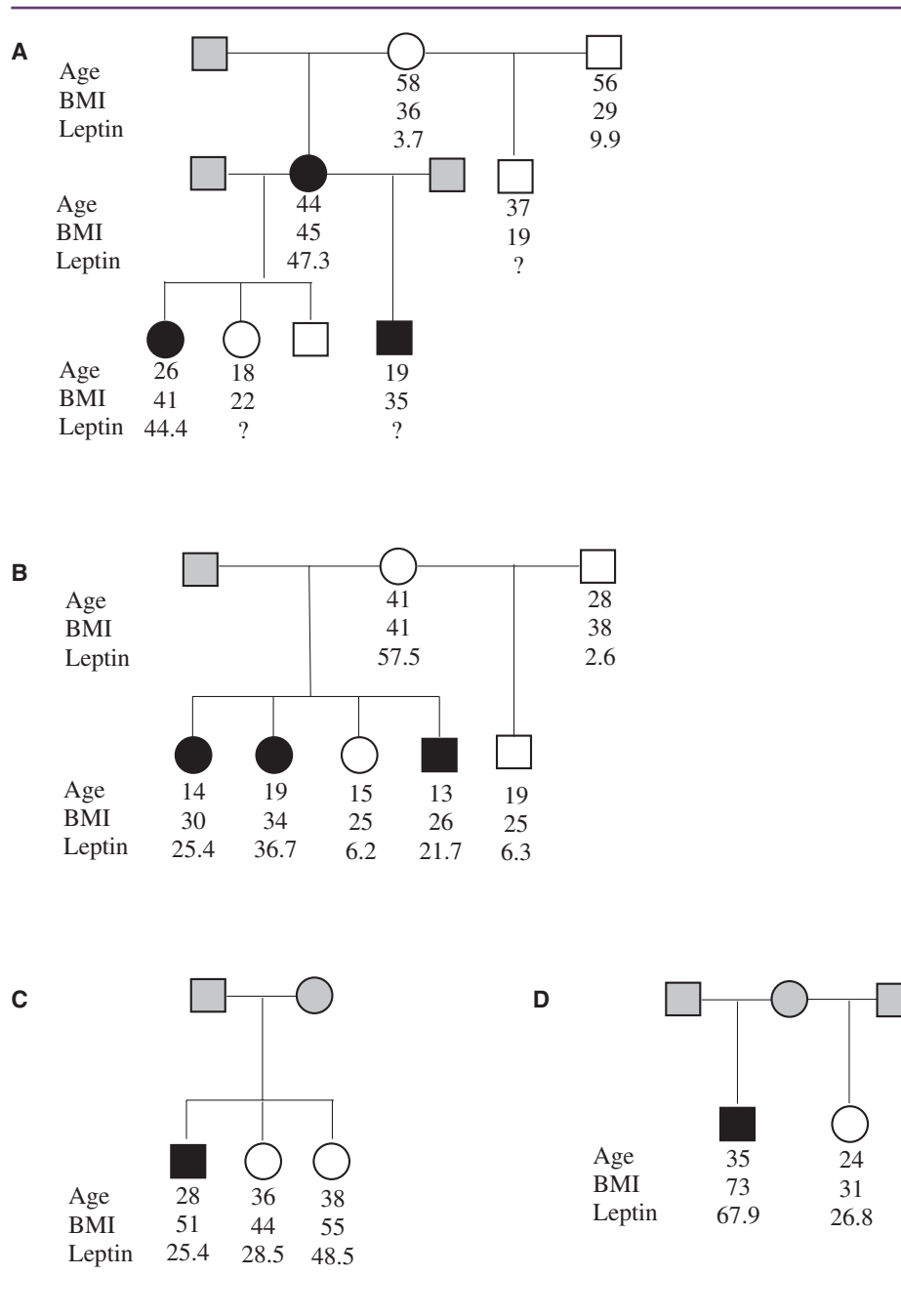


Figure 2 *BBS9* Thr549Ile carrier pedigrees. The black symbols in the pedigrees are heterozygous carriers at Thr549Ile; the white symbols are homozygous at Thr549Thr; the gray symbols are genotype unknown. Maximum BMI (kg/m²), age at maximum BMI, and serum leptin (ng/mL) measures are shown for each participant, if known.

in bold in Supporting Information Table S1). However, a Sequence Kernel Association Test analysis did not provide statistical support that multiple rare variants in *BBS9* affect obesity ($P=0.3$).

Discussion

BBS gene variants have previously been linked to obesity (17,18). More recently, studies have shown that obesity can arise through the

loss of *BBS* subunits, which impairs *LEPR* trafficking and leads to leptin resistance (8). The present study identified a Thr549Ile variant (rs59252892) in *BBS9* that significantly associated with BMI ($P=0.0008$). Although the allele frequency of rs59252892 was uncommon in our study of American Indians (MAF=0.001), it is much rarer in individuals of European descent (MAF=0.00008 in the Exome Aggregation Consortium; <http://exac.broadinstitute.org>). Therefore, it was unexpected that this Thr549Ile variant was detected in one individual in a prior WES study of only 30 Caucasian individuals, but this

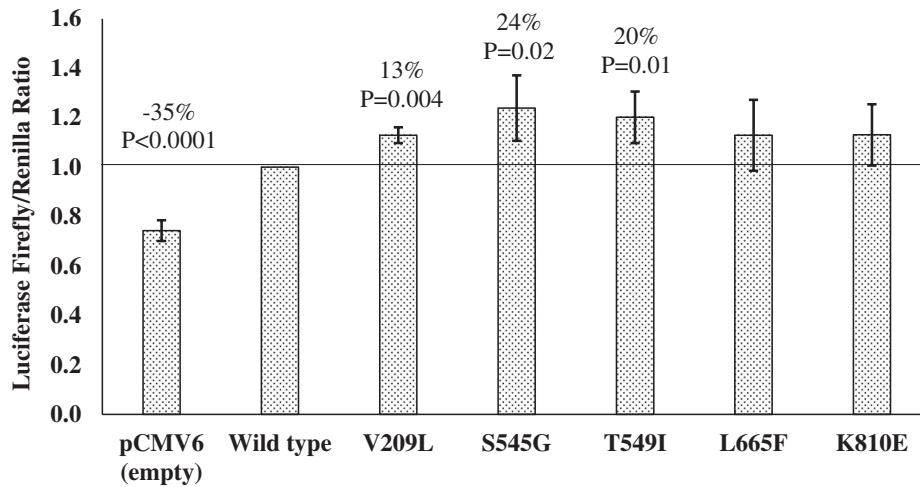


Figure 3 The effect of the *BBS9* variants on STAT3 transcription in HEK293 cells by an *in vitro* luciferase activity assay. Each bar represents the mean \pm SD of luciferase activity, normalized to the leptin-treated (100 ng/mL) *BBS9* WT from five independent experiments. Significant changes (%) in activity from the *BBS9* WT were determined by a one-sample *t* test. WT, wild type.

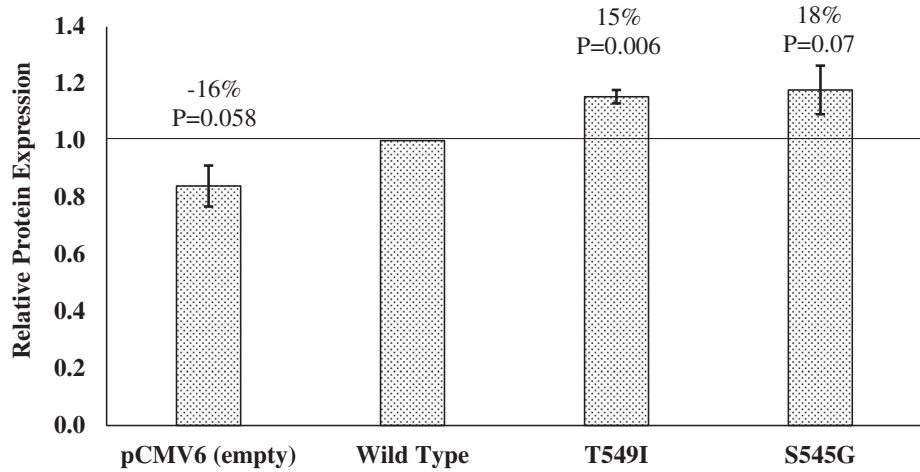


Figure 4 The effect of the *BBS9* variants on STAT3 phosphorylation in HEK293 cells by Western blot analyses. Each bar represents the mean \pm SD of signal intensity normalized to the leptin-treated *BBS9* WT; derived from three independent experiments. Significant changes (%) in activity from the *BBS9* WT were determined by a one-sample *t* test. WT, wild type.

may be explained by the fact that all of the 30 Caucasians had extreme obesity (18). This prior study also concluded that Thr549Ile was contributing to the obesity in this individual. Our result, as well as this prior finding, support the hypothesis that *BBS9*, the central component of the *BBSome*, could affect the transporting capabilities of the protein that may play a role in development of obesity (19).

Our *in vitro* functional analyses of five *BBS9* variants indicate that the BMI risk alleles at Val209Leu, Ser545Gly, and Thr549Ile appear to increase leptin signaling, possibly because of increased LEPR shuttling to the cell surface. The findings of increased leptin signaling in

obesity seem to contradict the conventional mechanism of LEPR deficiency causing obesity. However, several studies have been focused on balanced leptin signaling for normal function (20-22). Zhao et al. had performed a transgenic manipulation of leptin production in obese mice on a high-fat diet and had shown that (1) a subtle reduction of circulating leptin reduced body fat, (2) near-complete absence of leptin increased body fat, and (3) overexpression of leptin increased body fat (22). In a study by Gamber et al., overexpression of LEPR in mice led to increased food intake and weight gain under a high-fat diet (21). Furthermore, these mice became resistant to STAT3 activation and had increased suppressor of cytokine signaling 3 (*Socs3*) expression levels.

It is proposed that increased LEPR at the surface causes overactivity of the signaling pathway, leading to leptin resistance. Here, our study has found increased leptin signaling for obesity-associated *BBS9* variants. Therefore, it is conceivable to suggest that these *BBS9* variants cause a subtle increase in the shuttling of LEPR to the plasma membrane, which, over time, leads to leptin resistance.

The relationship between leptin levels and body weight may also vary over time. For example, a recently published study assessed the effect of hyperleptinemia at various developmental stages on the body weight of mice (23). They found that mice with leptin overexpression at birth initially displayed reduced body weight but that they were more prone to high-fat-diet-induced obesity at later developmental stages than the control littermates. Therefore, our population of American Indians, who on average have high BMI in adulthood, may be showing a similar effect.

Our *in vitro* reporter assay enabled sensitive detection for a subtle change. However, we acknowledge potential limitations. First, cell lines were useful in identifying alterations in leptin signaling from *BBS9* variants in an acute state. But to fully investigate whether these variants directly link to leptin resistance, a mouse model may provide more insight into the chronic impact. Second, our study assessed the downstream effects of leptin signaling but did not directly quantify surface LEPR levels. Therefore, additional studies are required to further confirm long-term mechanistic effects of *BBS9* variants on LEPR surface levels. In conclusion, we have used WES data in a longitudinally studied American Indian community to identify potentially functional missense variants in BBS genes that potentially contribute to obesity. BBS variants have been recognized along with LEPR/proopiomelanocortin (POMC) deficiencies as rare genetic disorders of obesity and have been included in a clinical trial (NCT03013543) using an MC4R agonist developed to restore impaired pathway function. These developments suggest an important role of BBS gene function in the leptin-melanocortin pathway. However, we did not find conclusive functional evidence that supports *BBS9* variants as a contributor to polygenic obesity in this American Indian population, warranting further studies. **O**

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Supporting information: Additional Supporting Information may be found in the online version of this article.

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