Evaluation of *A2BP1* as an Obesity Gene

Lijun Ma,[1] Robert L. Hanson,[1] Michael T. Traurig,[1] Yunhua L. Muller,[1] Bakhshish P. Kaur,[1] Jessica M. Perez,[1] David Meyre,[2] Mao Fu,[3] Antje Körner,[4] Paul W. Franks,[5] Wieland Kiess,[4] Sayuko Kobes,[1] William C. Knowler,[1] Peter Kovacs,[6] Philippe Froguel,[2] Alan R. Shuldiner,[3] Clifton Bogardus,[1] and Leslie J. Baier[1]

OBJECTIVE—A genome-wide association study (GWAS) in Pima Indians (n = 413) identified variation in the ataxin-2 binding protein 1 gene (*A2BP1*) that was associated with percent body fat. On the basis of this association and the obese phenotype of ataxin-2 knockout mice, *A2BP1* was genetically and functionally analyzed to assess its potential role in human obesity.

RESEARCH DESIGN AND METHODS—Variants spanning *A2BP1* were genotyped in a population-based sample of 3,234 full-heritage Pima Indians, 2,843 of whom were not part of the initial GWAS study and therefore could serve as a sample to assess replication. Published GWAS data across *A2BP1* were additionally analyzed in French adult (n = 1,426) and children case/control subjects (n = 1,392) (Meyre et al. Nat Genet 2009;41:157–159). Selected variants were genotyped in two additional samples of Caucasians (Amish, n = 1,149, and German children case/control subjects, n = 998) and one additional Native American (n = 2,531) sample. Small interfering RNA was used to knockdown *A2bp1* message levels in mouse embryonic hypothalamus cells.

RESULTS—No single variant in *A2BP1* was reproducibly associated with obesity across the different populations. However, different variants within intron 1 of *A2BP1* were associated with BMI in full-heritage Pima Indians (rs10500331, $P = 1.9 \times 10^{-7}$) and obesity in French Caucasian adult (rs4786847, $P = 1.9 \times 10^{-10}$) and children (rs8054147, $P = 9.2 \times 10^{-6}$) case/control subjects. Reduction of *A2bp1* in mouse embryonic hypothalamus cells decreased expression of *Atxn2*, *Insr*, and *Mc4r*.

CONCLUSIONS—Association analysis suggests that variation in *A2BP1* influences obesity, and functional studies suggest that A2BP1 could potentially affect adiposity via the hypothalamic MC4R pathway. ***Diabetes*** **59:2837–2845, 2010**

From the [1]Phoenix Epidemiology and Clinical Research Branch, National Institute of Diabetes and Digestive and Kidney Disease, National Institutes of Health, Phoenix, Arizona; the [2]CNRS 8090-Institute of Biology, Pasteur Institute, Lille, France; the [3]Division of Endocrinology, Diabetes and Nutrition, University of Maryland School of Medicine, Baltimore, Maryland; the [4]University Hospital for Children & Adolescents, University of Leipzig, Leipzig, Germany; the [5]Clinical Research Center, Malmö General Hospital, Lund University, Malmö, Sweden; and the [6]Interdisciplinary Centre for Clinical Research, University of Leipzig, Leipzig, Germany.

Corresponding author: Leslie J. Baier, lbaier@phx.niddk.nih.gov.

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L.M. is currently affiliated with Wake Forest University Health Sciences, Winston-Salem, North Carolina.

Recent large-scale genome-wide association studies (GWASs) have uncovered common variants in several loci associated with obesity in multiple populations (1–7). Most of these studies have been done in populations of European ancestry. Additional GWASs in diverse ethnic groups could confirm previously identified obesity-associated genes, identify novel ethnic-specific susceptibility genes, or identify ethnic-specific variation within a previously identified gene. Several examples exist of common variation in a gene contributing to obesity in one population but unique, rare variation contributing to obesity in a different population. For example, a common obesity-associated variant, rs17782313, near *MC4R* has been widely replicated in Europeans (3,5,6), but this same variant is nearly monomorphic for the Caucasian nonrisk allele (T allele) in full-heritage Pima Indians, whereas rare coding variants in *MC4R*, one of which is a novel frameshift mutation that has not been reported in other populations, do contribute to obesity in Pima Indians (8,9). Similarly, a common obesity-associated variant in *SIM1* has been reproducibly associated with obesity in Native Americans but not French Caucasians (10), whereas both rare deletions and rare missense variants in *SIM1* have been reported to be associated with severe obesity in Caucasians (11–19).

To search for loci that may be important in determining obesity in Pima Indians, we recently completed a GWAS using the Affymetrix 100K genotyping array in a group of 413 nondiabetic full-heritage Pima Indians who were phenotyped for various measures of body composition including percent body fat. Our most significant association with percent body fat was in the *A2BP1* gene (rs10500331, $P = 6.6 \times 10^{-6}$), which encodes for the ataxin-2 binding protein 1 (also known as FOX-1) and is involved in tissue-specific alternative splicing (20). In addition to containing RNA-binding motifs, A2BP1 also interacts with ataxin-2 (ATXN2) (21), a protein thought to be involved with RNA metabolism (22). ATXN2 has been implicated in the neurodegenerative disorder spinocerebellar ataxia type 2 (SCA2) (22), and hyperphagia and obesity are two major clinical features reported in an Egyptian family with SCA2 (23). Consistent with this observation, ataxin-2 knockout mice ($Sca^{-/-}$) are reported to be much more obese than their wild-type littermates when both are fed a high-fat diet (24,25). Therefore, based on the genetic associations with percent body fat in our GWAS and the obese phenotype of the $Sca2^{-/-}$ mouse, we studied *A2BP1* as a potential candidate gene for human obesity.

RESEARCH DESIGN AND METHODS

Subjects and phenotypes. Descriptions of subjects used in the association analyses are shown in Table 1. The GWAS sample consists of 413 nondiabetic,

TABLE 1
Subjects analyzed in association studies

Sample	*n* (M/F)*	Sample type	Age (years)	BMI	Percent body fat
Full-heritage Pima Indian GWAS	413 (239/174)	Metabolically studied inpatients	26.7 ± 6.2†	34.0 ± 7.5†	33.0 ± 8.5†
Full-heritage Pima Indian population	3,234 (1,350/1,884)	Population	36.9 ± 14.6‡	33.5 ± 7.9‡	NA
Full-heritage Pima population nonoverlapping with GWAS	2,843 (1,130/1,713)	Population excluding GWAS	38.1 ± 13.4‡	33.4 ± 7.8‡	NA
Caucasians					
French adult case/control subjects	695 (147/548)	Case subjects	44.1 ± 12.0	47.3 ± 7.6	NA
	731 (181/550)	Control subjects	55.2 ± 8.2	21.8 ± 1.9	NA
French children case/control subjects	685 (310/375)	Case subjects	10.9 ± 3.3	29.5 ± 6.5	NA
	707 (332/375)	Control subjects	11.9 ± 2.3	17.6 ± 2.3	NA
German school children case/control subjects	283 (136/147)	Case subjects	11.6 ± 3.6	30.5 ± 6.2	NA
	715 (337/378)	Control subjects	11.7 ± 2.7	18.1 ± 2.1	NA
Old Order Amish	1,149 (592/557)	Family-based	49.6 ± 16.8	30.0 ± 4.7	NA
Mixed-heritage Native American	2,531 (1,120/1,411)	Population	29.1 ± 11.7	32.8 ± 8.6	NA

Data are means ± SD. NA indicates information on percent body fat was not available. *M, males; F, females. †Age, BMI, and percent body fat at first visit. ‡Age and BMI averaged over all visits.

full-heritage Pima Indian volunteers who had been metabolically characterized as inpatients in our Clinical Research Center and were informative for quantitative traits related to obesity and diabetes, including percent body fat and BMI. Some of these healthy, metabolically characterized subjects were first-degree relatives (413 subjects came from 264 sibships, 98 of whom consisted of ≥2 siblings). Body composition was estimated by underwater weighing until January 1996 and by dual-energy X-ray absorptiometry (DPX-1, Lunar Radiation Corp, Madison, WI) thereafter. A conversion equation derived from comparative analyses was used to make estimates of body composition equivalent between the two methods (26). Associations were further assessed in a full-heritage Pima Indian population-based sample (full-heritage Pima population, $n = 3,234$) derived from our longitudinal study of the etiology of type 2 diabetes in the Gila River Indian Community in Central Arizona (27). Most of the residents are Pima Indians, and many are related to one another. The study includes biennial exams performed on individuals who provide informed consent and include measurements of height, weight, and a 75-g oral glucose tolerance test, where diabetes was diagnosed according to 1997 American Diabetes Association criteria. BMI was calculated as weight (kg)/height (m^2). Analysis of BMI was restricted to all exams after the subjects reached the age of 15 years (number of BMI measurements for all 3,234 subjects = 15,722). Of these 3,234 subjects, 391 had been included in the GWAS sample; therefore, to assess independent replication of the GWAS associations, the 2,843 "nonoverlapping with GWAS" subjects were additionally analyzed separately (number of BMI measurements for the 2,843 subjects = 13,751). Selected variants were genotyped in a second population-based sample from our study of the Gila River Indian Community, which consisted of all of the remaining longitudinally studied individuals who had a BMI measure after the age of 15 years and a DNA sample available for genotyping ($n = 2,531$). In contrast to the full-heritage Pima sample, no restrictions on heritage were applied for subjects in this second sample. The subjects in this "mixed-heritage," predominately Native American sample (number of BMI measurements for 2,531 subjects = 6,973) self-reported their heritage as, on average, ½ Pima Indian and ¾ Native American, with 59 individuals reporting no Native American heritage.

The Old Order Amish subjects ($n = 1,149$) were from the Amish Family Diabetes Study (28). The German school children consisted of 715 lean subjects (control subjects, mean age = 11.7 ± 2.7 years, BMI between 16th and 85th percentile) and 283 obese subjects (case subjects, mean age = 11.5 ± 3.7 years, BMI > 90th percentile) (29). French adult and children case/control subjects from a GWAS for obesity have been described elsewhere (1). Briefly, case children were in the 97th age-and sex-specific percentile of BMI and had evidence of familial obesity, and control children had a BMI < 90th percentile. Case adults had a BMI ≥ 40 kg/m^2 and evidence of familial obesity, and control adults had a repeated BMI measure of <25 kg/m^2.

Genotyping for GWAS data. Subjects in the Pima GWAS were genotyped using the Affymetrix 100K Human Mapping Array (Affymetrix, Santa Clara, CA), and the methodology and quality control assessment have been described previously (27). Published genotypic data across *A2BP1* for the French Caucasian adults and children were obtained from the Illumina Human CNV370 Duo Array (1).

Sequencing and genotyping. DNA samples from 24 full-heritage Pima Indians (12 obese/12 nonobese), who were not first-degree relatives, were sequenced to identify novel variants in *A2BP1*. Overlapping primers were designed to sequence all 16 exons, 5′- and 3′-untranslated regions, and 2 kb of the adjacent 5′-region. Sequencing reactions were performed using a Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and run on an automated DNA capillary sequencer (model 3730xl, Applied Biosystems, Foster City, CA). Sequence information for the two novel variants (A2BP1E3 and A2BP1E15) is provided at the bottom of supplementary Table 2, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1604/DC1. Linkage disequilibrium plots (D′ and r^2) were generated using the Haploview program (Haploview, http//:www.broad.mit.edu/mpg/haploview). Genotyping of the full-heritage Pima Indian and mixed-heritage samples was performed using SNPlex (Applied Biosystems, Foster City, CA) on an automated DNA capillary sequencer (model 3730xl, Applied Biosystems, Foster City, CA). Selected variants were genotyped in the Old Order Amish subjects (28) and German school children (29) using Taqman genotyping assays (Applied Biosystems, Foster City, CA).

Statistical analysis. Statistical analyses were performed using the SAS program of the SAS Institute (Cary, NC). For the GWAS data, linear regression models were used to assess the association between genotype and percent body fat or BMI, adjusting for covariates including age and sex. The logarithm of BMI was used to reduce skewness. The generalized estimating equations procedure was used to account for family membership because some subjects were siblings. The method of genomic control was used to "correct" the *P* values to their expected distribution. As described by Devlin et al., the mean χ^2 value was used to obtain the inflation parameter (λ) and the corrected *P* value was calculated from an *F* test with one degree of freedom in the numerator and the number of markers in the denominator (30). The λ was 1.14 for percent body fat and 1.16 for BMI. In addition, to provide a test that is robust to stratification (minimizes the false positive rate), a modification of the method described by Abecasis et al. was used where the associations are partitioned into between- and within-family components (31); for the present analyses, these components were represented, respectively, by the mean number of risk alleles for the sibship and each individual's departure from this mean. For the longitudinally studied population-based samples, the association between genotype and BMI was examined using all of the BMI measurements for each individual measured after the age of 15 years. In these analyses, a linear mixed model (PROC MIXED) was fitted that included genotype as a fixed effect along with age, sex, birth year, diabetic status, and duration of diabetes as covariates. For examinations at which an individual did not have diabetes, the duration variable was coded as "0"; this approach can account for the observation that BMI tends to decline after the diagnosis of diabetes in this population (32). In addition, the model included random effects representing sibship (to account for the fact that some individuals were siblings) and individual (to account for multiple examinations within an individual). An autoregressive correlation structure was used to model the relationship between multiple examinations within an individual. To reduce computation time, the random effects were estimated once in the absence of genotypic effects and in subsequent analyses were held fixed at the values estimated in the full data. The likelihood ratio test was used to assess statistical significance. In the second population-based sample where many individuals were of mixed-heritage, the individual estimate of Indian admixture was also used as a covariate. These estimates were derived by using a published method (33) from 32 markers selected for having large differences in allele frequency between Native Americans and Caucasians (34). Tests for

TABLE 2
Association of rs10500331 and rs12924838 with BMI or obesity in the populations described in Table 1

Population sample	n	rs10500331 (C/**T**)*			rs12924838 (G/**A**)*		
		AF (T)†	P	Z	AF (A)†	P	Z
Full-heritage Pima Indian GWAS	413	0.49	8.1×10^{-5}	3.94	0.50	3.0×10^{-4}	3.61
Full-heritage Pima Indian population	3,234	0.49	1.9×10^{-7}	5.20	0.50	3.3×10^{-6}	4.65
Full-heritage Pima Indian population nonoverlapping with GWAS	2,843	0.49	1.9×10^{-5}	4.28	0.50	4.2×10^{-5}	4.10
French adult case/control subjects	1,426	0.13	0.98	0.025	0.27	0.03	2.20
French children case/control subjects	1,392	0.13	0.96	-0.06	0.27	0.90	0.13
German school children case/control subjects	998	0.12	0.80	0.26	ND	ND	ND
Old Order Amish	1,149	0.11	0.80	0.26	0.29	0.12	1.56
All Europeans‡	4,965	–	0.81	0.24	–	0.02	2.25

P values were determined using the additive model. *Bold allele is defined as the risk allele (associated with higher BMI) based on the Pima GWAS data. *Z* scores are calculated from the one-sided *P* value for the alternate hypothesis that the association is in the same direction as that observed in the Pima GWAS. †AF, allele frequency. ‡Combined analysis for all Europeans was performed by combining the *P* values by Stouffer's method (i.e., combining the Z scores). LD between the two variants; Pima Indians $D' = 0.95$, $r^2 = 0.95$ and Caucasians $D' = 0.83$, $r^2 = 0.25$. ND indicates not determined.

genotypic association were undertaken assuming an additive effect of the alleles on the phenotype. To assess the evidence for association when both population-based samples of Native Americans were combined, the coefficients for the genotypic effect were combined and weighted by the inverse of their variance estimates (35). *P* values were not adjusted for multiple comparisons.

The association of BMI versus genotypes in the Amish sample was performed using the generalized linear model after adjusting for age, sex, and family membership. Comparison of genotype frequencies between the lean and obese German school children case/control subjects were performed by logistic regression analysis adjusted for age, sex, pubertal stage, and height. Because parameter estimates for case subject–control subject and quantitative trait analyses are not comparable, results were combined across studies using Stouffer's method of combining *P* values (36).

Tissue profiling for *A2BP1*. Primers located in exons 5 and 6 of the *A2BP1* transcript (accession ID AF107203) were used to amplify cDNA from the following human tissues: adipose, hypothalamus, pituitary (BD Marathon-Ready cDNA; BD Bioscience/Clontech), brain, skeletal muscle, heart, fetal liver, adult liver, kidney, pancreas (BD Human MTC Multiple Tissue cDNA Panels I and II; BD Bioscience/Clontech), pancreatic islets (kindly provided by Dr. Lorella Marcelli at Joslin Diabetes Center), and preadipocytes isolated from Pima Indians. PCR products were sequenced to confirm that they encoded *A2BP1*.

Isolation of preadipocytes and synthesis of cDNA. Subjects for adipose tissue biopsies were admitted as inpatients to our Clinical Research Center and, after an overnight fast, underwent a subcutaneous abdominal needle biopsy under local anesthesia with 1% lidocaine. Collagenase digestion of the subcutaneous abdominal adipose tissue biopsy samples was done as previously described (37,38).

Cell culture and *A2bp1* knockdown. Mouse N-41 hypothalamus cell line (Cat. No. CLU121) was purchased from Cellutions Biosystems, Inc. (Burlington, ON, Canada) and used within 10 passages of the original vial. Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (ATCC, Manassas, VA) and 20 mmol/l glucose (EM Science, Cincinnati, OH) at 37°C with 5% CO_2. Prior to small interfering RNA (siRNA) transfection, N-41 cells were seeded in six-well plates at a density of $\sim 10^5$ cells per well. For each transfection, 9 μl of siPORT *Amine* Transfection Agent (*Silencer* siRNA Transfection II Kit, Ambion, Austin, TX) and *A2bp1* siRNA were resuspended separately in 100 μl of GIBCO Opti-MEM I medium (Invitrogen, Carlsbad, CA). After incubating for 10 min at room temperature, the siRNA and transfection agent mixtures were combined (total 200 μl) and siRNA/transfection agent complexes were allowed to form for 10 min. After the 10 min incubation, the 200 μl transfection mixture was added to each well, and after 8–24 h, the media was replaced. The transfected cells were incubated for 48 h and then harvested for RNA extraction. SiRNA (sense, 5′-GAUUUGGUUUCGUAACUUUtt-3′ and antisense, 5′-AAAGUUACGAAAC CAAAUCcc-3′; assay ID si114129) targeting the mouse *A2bp1* transcript and negative control (scrambled) siRNA (assay ID 4618G) were purchased from Ambion (Austin, TX). Total RNA was extracted from the transfected N-41 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA). To remove any residual DNA, the purified RNA was treated with DNase using an RNase-free DNase set (Qiagen, Valencia, CA). First-strand cDNA was synthesized using an Ambion RT-for-PCR kit (Austin, TX). Gene expression levels for mouse *A2bp1*, *Atxn2*, *Insr*, *Mc4r*, *Lepr*, and *Npy1r* were quantified by real-time PCR using predesigned gene expression assays (assay IDs: *A2bp1*, Mm00480615_m1; *Atxn2*, Mm00485932_m1; *Insr*, Mm00439693_m1; *Mc4r*, Mm00457483_s; *Lepr*, Mm00440181_m1; and *Npy1r*, Mm00650798_g1; Applied Biosystems, Foster City, CA). Real-time PCR was performed using an ABI-7700 sequence detection system (Applied Biosystems, Foster City, CA). Assays were performed in replicates of six, and the mean values were used to calculate expression levels using the relative standard curve method. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, assay ID Mm03302249_g1, Applied Biosystems, Foster City, CA) was used as the endogenous control to obtain normalized values. Each experiment was independently repeated three times. Student *t* test was used to compare the means for cells transfected with *A2bp1* siRNA with those transfected with control siRNA.

RESULTS

Association analyses among 413 metabolically characterized, nondiabetic full-heritage Pima Indians who had been genotyped using the Affymetrix 100K Human Mapping Array identified rs10500331 as the strongest genome-wide signal for percent body fat ($P = 6.6 \times 10^{-6}$ after genomic control, adjusted for age and sex; supplementary Fig. 1 and designated in bold in supplementary Table 1, available in an online appendix). Among these 413 GWAS subjects, rs10500331 was also associated with BMI ($P = 8.1 \times 10^{-5}$ after genomic control, adjusted for age and sex; Table 2 and supplementary Table 1). To validate this GWAS association, rs10500331 was genotyped in a longitudinally studied, full-heritage Pima Indian population-based sample ($n = 3{,}234$) where individuals had multiple measures of BMI (description of the subjects is given in Table 1). Rs10500331 was associated with BMI in this full-heritage Pima Indian population sample ($P = 1.9 \times 10^{-7}$) and remained significant after excluding all subjects that overlapped with the initial GWAS study (nonoverlapping with GWAS full-heritage sample, $n = 2{,}843$; $P = 1.9 \times 10^{-5}$) (Table 2). Although independently replicated, these associations do not quite meet the proposed threshold for genome-wide significance of $P < 7 \times 10^{-8}$ (39). To further substantiate these associations by ruling out potential confounding effects of population stratification, we used the family structure of these samples and performed a within-family analysis, where rs10500331 was again associated with BMI in the initial GWAS sample ($P = 5.3 \times 10^{-2}$, supplementary Table 1), the entire full-heritage Pima Indian population sample, and the population sample after

excluding the overlapping GWAS subjects ($P = 7.4 \times 10^{-3}$ and 3.2×10^{-2}, respectively) (supplementary Table 2).

Rs10500331 maps within intron 1 of the *A2BP1* gene, and several other variants within *A2BP1* also showed significant associations with percent body fat and BMI in the GWAS study (supplementary Table 1). To directly examine *A2BP1* as a candidate gene for obesity in Pima Indians, all exons and 2 kb of the upstream region of this gene were sequenced in 24 Pima Indians selected for being obese or nonobese. Twelve variants were identified of which two were rare novel substitutions (frequencies of the minor alleles = 0.03 and 0.09; sequences are provided in supplementary Table 2). An additional 108 variants spanning *A2BP1* were then selected for genotyping in the full-heritage Pima Indian population sample ($n = 3{,}234$). The 108 variants consisted of 66 tag SNPs (minor allele frequency ≥ 0.15 and a pairwise $r^2 \geq 0.8$) from our previous 100K GWAS, 34 variants selected from our ongoing 1M GWAS, and 8 variants identified by sequencing including the two novel variants. Linkage disequilibrium plots (r^2 and D′) for all 109 variants (including rs10500331) are shown in supplementary Fig. 2, available in an online appendix. Associations for all 109 variants with BMI, using both general and within-family analytical models, are shown for the entire full-heritage Pima Indian population sample to preserve power ($n = 3{,}234$), as well as for the $n = 2{,}843$ nonoverlapping with GWAS sample (supplementary Table 2). Several variants displayed modest associations (general and within-family analyses) in both samples; however, none of the associations was as strong as that for rs10500331.

Two variants, rs10500331 and rs12924838, which are in high linkage disequilibrium in full-heritage Pima Indians but not Caucasians (D′ = 0.96, $r^2 = 0.92$ in Pima and D′ = 0.83, $r^2 = 0.25$ in Caucasians; Fig. 1*B* and *C*) were significantly and reproducibly associated with BMI in Pima Indians (Table 2). These variants were further evaluated in four additional cohorts of European ancestry including French adult case/control subjects (1), French children case/control subjects (1), Amish families (28), and German school children case/control subjects (29). There was no evidence of association of rs10500331 with obesity in the French adult or children case/control subjects nor was there an association of this variant with BMI in the Amish or German school children case/control subjects (Table 2); however, rs12924838 was nominally associated with obesity in the French adult case/control subjects ($P = 0.03$) and when all of the European data were combined ($n = 4{,}965$, $P = 0.02$) (Table 2). Although neither rs10500331 nor rs12924838, which both map within intron 1 of *A2BP1*, appear to be convincingly associated with obesity or BMI in these European cohorts, a prior GWAS for severe obesity using case/control subjects of either French children or French adults identified several other variants within intron 1 of *A2BP1* that were significantly associated with severe obesity (1). In the French children case/control subjects stage 1 samples, rs8054147 displayed the strongest association with obesity ($P = 9.2 \times 10^{-6}$, Fig. 2), whereas in the French adult case/control subjects stage 1 samples, rs4786847 had the strongest association ($P = 1.9 \times 10^{-10}$, Fig. 2). The striking association of rs4786847 with obesity was specific for the stage 1 French adult sample. For the stage 2 sample, which included 519 obese children and 566 lean young adults of French origin, 377 obese children and 731 lean children of German origin, 135 obese adults and 794 lean adults of French origin, 1,036 obese adults and 320 randomly selected adults of Swiss origin, a general population of 5,291 Finnish children, and a general population of 4,417 French adults, the association of rs4786847 with obesity or BMI was nominal (all stage 2 children $P = 0.005$, all stage 2 adults $P = 0.76$, entire stage 2 sample $P = 0.03$) (1).

To determine whether the associations observed with BMI in the Pima Indians represent a signal specific for Native Americans, five variants (rs9302818, rs10500331, rs8052357, rs12924838, and rs1946127) with the strongest associations with BMI in the full-heritage Pima Indians, and one variant (rs4786847) most strongly associated with BMI in the French adults, were genotyped in a second population-based sample of 2,531 individuals who were predominately Native Americans of mixed-heritage. Although none of the five variants most strongly associated with BMI in the full-heritage Pima Indians replicated in this mixed-heritage Native American sample, rs4786847, which had the strongest association in the French adult case/control subjects ($P = 1.9 \times 10^{-10}$), modestly replicated ($P = 0.02$, Table 3). Combining the full-heritage Pima Indian and mixed-heritage Native American population-based samples provided strong associations with BMI for all six variants; however, only the association with rs4786847 was strengthened from the combination of the two samples, whereas the other associations were solely due to the full-heritage Pima Indian sample (Table 3). European genotypic data were additionally available on three of these variants, making an "all-sample" (i.e., subjects in Table 1) analysis possible. All-sample *P* values for these three variants ranged from 0.02 to 0.0002 (Table 3), where significance was largely derived from a single sample.

A2BP1 is expressed in various tissues including hypothalamus, a major tissue in body weight regulation (Fig. 3), and it has also been reported to be expressed in brain, heart, and skeletal muscle tissue (21). To functionally investigate a possible role of A2BP1 in hypothalamic body weight regulation, siRNA that target the mouse *A2bp1* gene were used to knockdown *A2bp1* expression in a mouse embryonic hypothalamic cell line (N-41) and mRNA levels of five target genes were assessed by RT-PCR. These five genes included *Atxn2*, which encodes the binding partner of A2bp1 and has been implicated in human hyperphagia (23), and four additional genes, *Insr*, *Lepr*, *Npy1r*, *and Mc4r*, known to function in key pathways of central regulation of energy balance and known to be expressed in N-41 cells. RT-PCR of *A2bp1* was also used to assess the efficiency of the siRNA knockdown.

We found that a 75% reduction in *A2bp1* expression in the N-41 cells led to a 53, 60, and 75% decrease in gene expression levels for *Atxn2*, *Insr*, and *Mc4r*, respectively, whereas expression levels for *Lepr* and *Npy1r* were largely unaffected (Fig. 4).

DISCUSSION

The associations between variants in *A2BP1* with both percent body fat and BMI in our 100K GWAS and the evidence for association of these variants with longitudinally measured BMI in a large population-based sample of full-heritage Pima Indians, along with the obese phenotypes observed with *Atxn2* knockout mice (24,25) and high expression levels of *A2BP1* in the hypothalamus, leads us to speculate that A2BP1 has a role in body weight

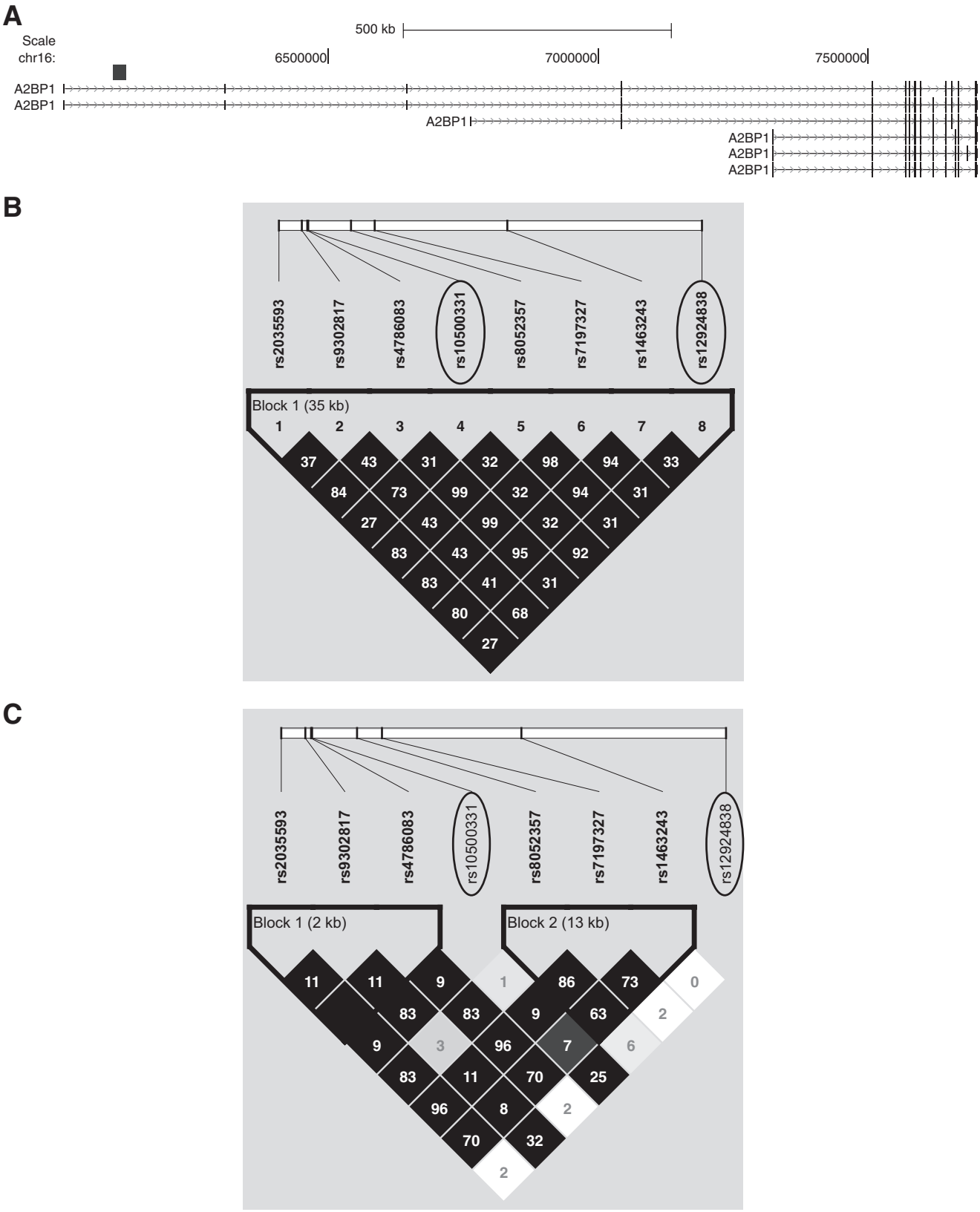

FIG. 1. Linkage disequilibrium plots (D′ and r^2) for the region around rs10500331 and rs12924838. D′ is indicated by the intensity of the shading, and the numbers in the boxes indicate r^2. *A*: Schematic showing *A2BP1* gene structures. Black box indicates the location of the region shown in (*B*) and (*C*). *B*: Linkage disequilibrium pattern for Pima Indians. *C*: Linkage disequilibrium pattern for Caucasians.

regulation. However, the highly significant associations obtained with different variants in each of the Pima Indian, French adult, and French children studies is inconsistent with a single common variant giving rise to this phenotype. Among populations representing different ethnicities, lack of reproducibility with a specific variant may be due to the

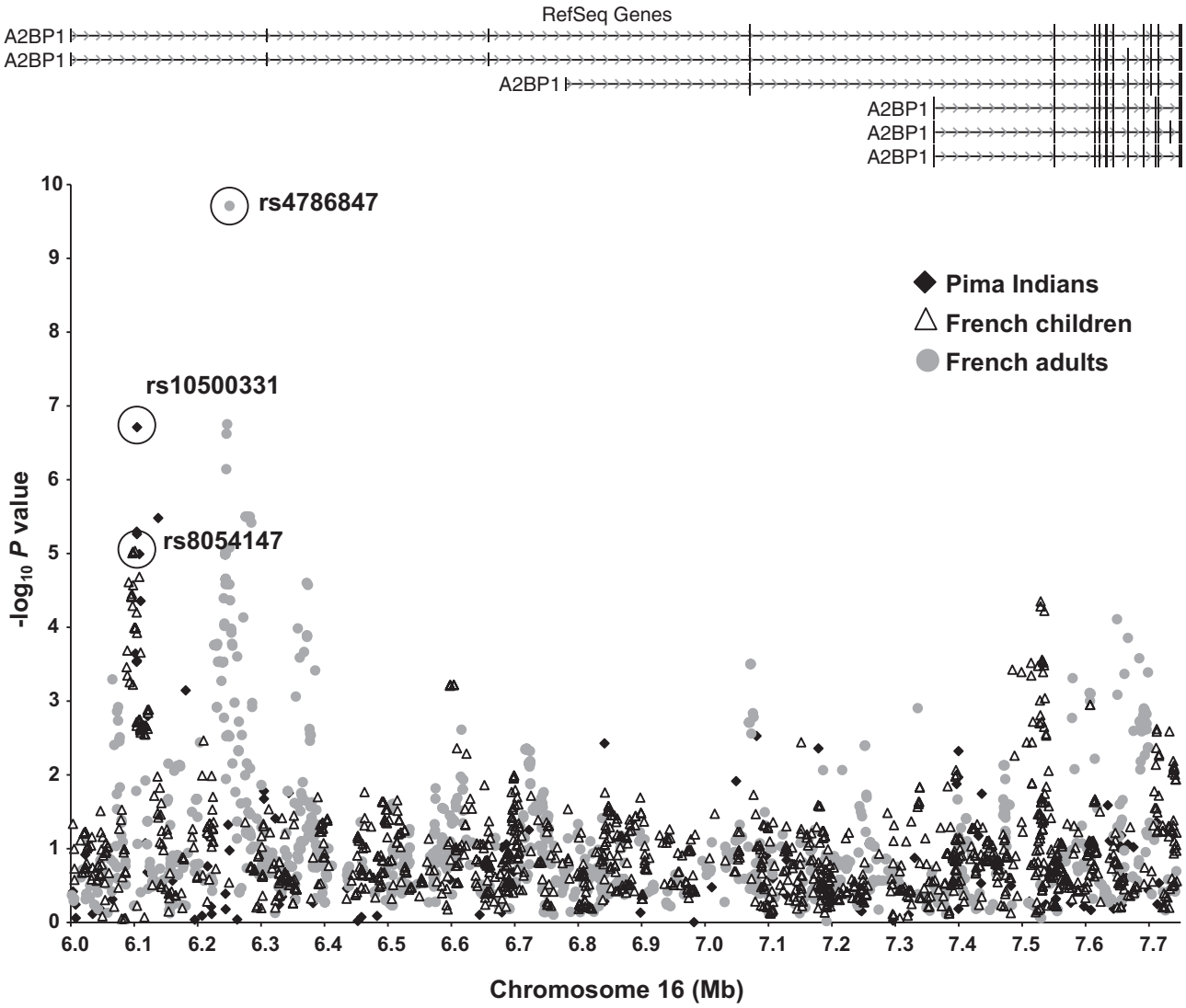

FIG. 2. Association analyses between BMI/obesity and variants spanning 1.2 Mb of *A2BP1*. Black diamonds, association results for BMI in the population-based sample of full-heritage Pima Indians. Open triangles, association results for obesity in the French children case/control subjects. Gray circles, association results for obesity in the French adult case/control subjects.

existence of an untyped causal variant with differing linkage disequilibrium patterns between ethnic groups (40). Pezzolesi et al. describe a similar pattern where different variants across the same locus (*ELMO1*) in different ethnic groups, Caucasians (41), African-Americans (42), and Japanese (43), are associated with diabetic nephropathy (41). They suggest that this allelic heterogeneity is probably the result of different ancestral genetic backgrounds and propose that rare variants in *ELMO1* may be common to each ethnic group and are being tagged by the common variants found in the individual studies (41). This concept, however, cannot explain strong associations of different variants in French adults and in French children. Another hypothesis more consistent with our observations is that strong, yet distinct, associations within a region of a biologically relevant gene would be observed if there are multiple rare causative variants that can occur within a single ethnic group. These rare variants, all of which could affect a potential functionally important region (e.g., intron 1 of *A2BP1*), may be more highly represented by chance in one sample set than in another and, thus, provide association signals with different tag SNPs in one group of individuals than in another. Deep resequencing across intron 1 of *A2BP1* in each of the different populations may be the best approach to test this hypothesis. At present, the costs of deep resequencing in a large number of subjects make this line of investigation difficult. Therefore, we chose to investigate the function of A2BP1 directly based on the current association data and the observation that $atxn2^{-/-}$ mice become obese.

A2BP1 is a RNA-binding protein involved in regulating tissue-specific alternative splicing by binding the RNA *cis*-regulatory element UGCAUG (20). A2BP1 binding to UGCAUG elements downstream of the exon enhances exon inclusion, while binding to UGCAUG elements upstream of the exon represses exon inclusion (20). The UGCAUG motif is highly enriched in brain-specific intronic regions flanking exons and alternative exons (44). A recent genome-wide survey for the UGCAUG element identified 1,103 genes with at least one predicted UGCAUG element. Included in this gene list were *A2BP1* (five elements), *ATXN2* (two elements), and *INSR* (two elements) (44). In addition to binding the UGCAUG splicing motif, A2BP1 is a binding partner for the ATXN2 protein (21), which also

FIG. 3. Expression profile for *A2BP1* in different human tissues.

contains predicted RNA binding and RNA splicing motifs and is thought to be involved in mRNA degradation and regulating translation (45,46).

In the human hypothalamus, LEPR and INSR sense the peripheral leptin and insulin signals to control food intake and energy homeostasis through POMC-MC4R and NPY-Y1R pathways. Central nervous system deficiencies of these pathways are known to affect energy homeostasis and result in severe obesity (47,48). After knockdown of *A2bp1* in the N-41 mouse embryonic hypothalamic cells, we observed a decrease in both *INSR* and *MC4R* expression. Knockdown of *A2BP1* also resulted in a decrease in *Atxn2* gene expression, and it has been shown by others that *Atxn2* knockout mice become obese (24,25). However, the mechanism whereby deficiency of *A2BP1* expression leads to a decrease in mRNA levels for these three genes is unknown. Because both *ATXN2* and *INSR* have predicted UGCAUG splicing elements (44), mRNA splicing for the two genes may be affected by a decrease in A2BP1, leading to unstable transcripts that are subsequently degraded. Unlike *ATXN2* and *INSR*, the transcript for *Mc4R* codes for only one exon and does not appear to contain binding sites for A2BP1. Therefore, instead of affecting mRNA stability, the reduction in *A2BP1* may result in the decrease of *Mc4R* indirectly by affecting some transcriptional regulatory protein involved in the expression of *Mc4R*.

In conclusion, GWAS data in Pima Indians and French Caucasians suggest that multiple variants in *A2BP1* may

FIG. 4. Relative gene expression for *A2bp1*, *Atxn2*, *Insr*, *Mc4r*, *Lepr*, and *Npy1r* in *A2bp1* knockdown N-41 mouse embryonic hypothalamus cells. Gray shaded bars, N-41 cells transfected with a negative control (scrambled) siRNA. Open bars, N-41 cells transfected with *A2bp1* siRNA. Negative control siRNA was a scrambled, randomly selected nonspecific siRNA sequence.

TABLE 3
Association of *A2BP1* variants with BMI in the full-heritage Pima Indians, mixed-heritage Native Americans, full + mixed-heritage combined, and all samples (Native Americans + Caucasians)

			Full-heritage Pima Indians (n = 3,234)				Mixed-heritage Native Americans (n = 2,531)				Full + mixed (n = 5,765)	All samples§ (n = 10,730)
			Mean BMI (kg/m^2)				Mean BMI (kg/m^2)					
Variant	M/m	mAF	M/M	M/m	m/m	P‡	mAF	M/M	M/m	m/m	P‡	P	P
rs9302818*	G/C	0.43	34.0 ± 8.0	33.6 ± 7.9	32.4 ± 7.7	5.5×10^{-6}	0.48	33.4 ± 8.6	32.5 ± 7.9	32.5 ± 8.0	0.75	0.0008	ND
rs10500331*	C/T	0.49	32.6 ± 7.8	33.6 ± 7.9	34.4 ± 8.2	1.9×10^{-7}	0.45	32.4 ± 8.1	32.8 ± 8.2	33.0 ± 8.6	0.90	0.00,007	0.02
rs8052357*	A/G	0.25	34.0 ± 7.9	33.0 ± 7.9	32.4 ± 7.9	1.0×10^{-5}	0.27	32.8 ± 8.1	32.5 ± 8.1	33.0 ± 7.6	0.50	0.0001	ND
rs12924838*	G/A	0.50	32.8 ± 7.8	33.7 ± 7.8	34.7 ± 8.2	3.3×10^{-6}	0.48	32.8 ± 8.2	32.8 ± 8.2	32.5 ± 8.6	0.26	0.006	0.0009‖
rs1946127*	T/C	0.24	34.0 ± 8.0	33.0 ± 7.8	32.8 ± 8.1	7.2×10^{-4}	0.26	33.0 ± 8.2	32.6 ± 8.3	31.3 ± 7.5	0.39	0.002	ND
rs4786847†	A/G	0.31	33.4 ± 8.0	33.7 ± 7.7	34.3 ± 8.4	0.11	0.27	32.0 ± 7.9	33.1 ± 8.3	34.7 ± 8.4	0.02	0.008	0.0002‖

M, major allele; m, minor allele; mAF, minor allele frequency. *Variants displaying the strongest association with BMI in the full-heritage Pima Indians. †Variant with the strongest association with severe obesity in the French adult case/control subjects GWAS. ‡P values were calculated by using a mixed model (see methods). §All samples include all of the subjects listed in Table 1. ‖Data for the German school children case/control subjects was not available for variants rs12924838 and rs4786847. ND indicates not determined.

exist that contribute to human obesity, and *A2bp1* knockdown studies suggest that deficiency of A2bp1 could play a role in the hypothalamic regulation of feeding. However, deep resequencing of *A2BP1* and further in vivo studies are necessary to confirm the biological role of this gene in the pathogenesis of obesity.

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