Evaluation of A2BP1 as an Obesity Gene

Running Title: A2BP1 and Obesity

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Submitted 30 October 2009 and accepted 8 August 2010.

Additional information for this article can be found in an online appendix at
http://diabetes.diabetesjournals.org

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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. Based on 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 cases/controls \((n = 1,392)\) (1). Selected variants were genotyped in 2 additional samples of Caucasians (Amish, \(n = 1,149\) and German children cases/controls, \(n = 998\)) and 1 additional Native American \((n = 2,531)\) sample. Small interfering RNA (siRNA) 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 body mass index (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 \((rs8054147, P = 9.2 \times 10^{-6})\) cases/controls. 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.

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 another 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 non-risk 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 non-diabetic 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 non-diabetic, 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 \( \geq 2 \) 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 75g 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 “non-overlapping 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 ¼ 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 (controls, mean age =
11.7 ± 2.7 years, BMI between 16th and 85th percentile) and 283 obese subjects (cases, mean age = 11.5 ± 3.7 years, BMI >90th percentile) (29). French adult and children cases/controls 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 ≥40kg/m² and evidence of familial obesity and control adults had a repeated BMI measure of <25 kg/m².

**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 non-obese), 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 Supplemental Table 2 in the online appendix available at [http://diabetes.diabetesjournals.org](http://diabetes.diabetesjournals.org). Linkage disequilibrium plots (D’ and r²) were generated using the Haploview program (Haploview, [http://www.broad.mit.edu/mpg/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 statistical analysis system of the SAS Institute (Cary, North Carolina). 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 (GEE) procedure was used to account for family membership since 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 chi-square value was used to obtain the inflation parameter (λ) and the corrected P value was calculated from an F test with 1 df 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 individuals 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 Amerindians and Caucasians (34). Tests for 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 generalized linear model after adjusting for age, sex, and family membership. Comparison of genotype frequencies between the lean and obese German school children cases/controls were performed by logistic regression analysis adjusted for age, sex, pubertal stage and height (SDS). Since parameter estimates for case-control 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% fetal bovine serum (ATCC, Manassas, VA) and 20mM glucose (EM Science, Cincinnati, OH) at 37 °C with 5% CO2. Prior to small interfering RNA (siRNA) transfection, N-41 cells were seeded in six-well plates at a density of ~10^5 cells per well. For each
transfection, 9 ul of siPORT Amine Transfection Agent (Silencer siRNA Transfection II Kit, Ambion, Austin, Texas) and A2bp1 siRNA were resuspended separately in 100ul 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 200ul) and siRNA:transfection agent complexes were allowed to form for 10 min. Following the 10 minute incubation, the 200ul transfection mixture was added to each well and after 8-24 hrs the media was replaced. The transfected cells were incubated for 48 hrs and then harvested for RNA extraction. SiRNA (sense, 5’-GAUUUGGUUUCGUAACUUUt3’ and antisense, 5’-AAAGUUA CGAAACCAAUCcc-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 6 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’s 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, non-diabetic 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; Supplemental Figure 1 and designated in bold in Supplemental Table 1). 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 Supplemental 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 population” sample ($P = 1.9 \times 10^{-7}$) and remained significant after excluding all subjects that over-lapped with the initial GWAS study (“non-overlapping 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 utilized the family structure of these samples and performed a within-family analysis, where rs10500331 was again
-associated with BMI in the initial “GWAS” sample \( (P = 5.3 \times 10^{-2}, \text{Supplemental Table 1}) \), the entire “full-heritage Pima population” sample and the population sample after excluding the overlapping GWAS subjects \( (P = 7.4 \times 10^{-3} \) and \( 3.2 \times 10^{-2} \), respectively) \( \) (Supplemental 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 (Supplemental 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 non-obese. 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 Supplemental Table 2). One hundred and eight additional variants spanning A2BP1 were then selected for genotyping in the “full-heritage Pima population” sample \( (n = 3,234) \). The 108 variants consisted of 66 tag SNPs (minor allele frequency \( \geq 0.15 \) and a pair-wise \( r^2 \geq 0.8 \)) from our previous 100K GWAS, 34 variants selected from our ongoing 1M GWAS, and 8 variants identified by sequencing including the 2 novel variants. Linkage disequilibrium (LD) plots \( (r^2 \) and \( D' \)) for all 109 variants \( (\text{including rs10500331}) \) are shown in Supplemental Figure 2. 2. Associations for all 109 variants with BMI, using both general and within-family analytical models, are shown for the entire “full-heritage Pima population” sample to preserve power \( (n = 3,234) \), as well as for the 2,843 “non-overlapping with GWAS” sample \( \) (Supplemental Table 2). Several variants displayed modest associations \( (\text{general and within-family analyses}) \) in both samples, however, none of the associations was as strong as rs10500331.

Two variants, rs10500331 and rs12924838, which are in high LD 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; Figure 1B and 1C) were significantly and reproducibly associated with BMI in Pima Indians \( \) (Table 2). These variants were further evaluated in 4 additional cohorts of European ancestry including French adult cases/controls \( (1) \), French children cases/controls \( (1) \), Amish families \( (28) \), and German school children cases/controls \( (29) \). There was no evidence of association of rs10500331 with obesity in the French adult or children cases/controls nor was there an association of this variant with BMI in the Amish or German school children cases/controls \( \) (Table 2); however, rs12924838 was nominally associated with obesity the French adult case/control sample \( (P = 0.03) \) 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 samples 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 stage 1 samples, rs8054147 displayed the strongest association with obesity \( (P = 9.2 \times 10^{-6}, \text{Fig. 2}) \), whereas in the French adult case/control stage 1 samples, rs4786847 had the strongest association \( (P = 1.9 \times 10^{-10}, \text{Fig. 2}) \). 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, 5 variants (rs9302818, rs10500331, rs8052357, rs12924838 and rs1946127) with the strongest associations with BMI in the full-heritage Pima Indians, and 1 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 cases/controls ($P = 1.9 \times 10^{-10}$) modestly replicated ($P = 0.02$, Table 3). Combining the full-heritage Pima and mixed-heritage Native American population-based samples provided strong associations with BMI for all 6 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 sample (Table 3). European genotypic data was additionally available on 3 of these variants making an “all sample” (i.e., subjects in Table 1) analysis possible. “All sample” $P$ values for these 3 variants ranged from 0.02-0.0002 (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 5 target genes were assessed by RT-PCR. These 5 genes included $Atxn2$, which encodes the binding partner of $A2bp1$ and has been implicated in human hyperphagia (23) and 4 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, while 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 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 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...
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 re-sequencing 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 re-sequencing 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 (44). *A2BP1* binding to UGCAUG elements downstream of the exon enhances exon inclusion, while binding to UGCAUG elements upstream of the exon represses exon inclusion (44). The UGCAUG motif is highly enriched in brain-specific intronic regions flanking exons and alternative exons (45). 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* (5 elements), *ATXN2* (2 elements), and *INSR* (2 elements) (45). In addition to binding the UGCAUG splicing motif, *A2BP1* is a binding partner for the ataxin 2 (*ATXN2*) protein (21) which also contains predicted RNA binding and RNA splicing motifs and is thought to be involved in mRNA degradation and regulating translation (46,47).

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 (48,49). Following 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. Since both *ATXN2* and *INSR* have predicted UGCAUG splicing elements (45), mRNA splicing for the two genes may be affected by a decrease in *A2BP1* leading to unstable transcripts which are subsequently degraded. Unlike *ATXN2* and *INSR*, the transcript for *Mc4R* codes for only one exon and does not appear to contain bindings 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 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 re-sequencing of \textit{A2BP1} and further \textit{in vivo} studies are necessary to confirm the biological role of this gene in the pathogenesis of obesity.

\textbf{Author contributions.} L.M. researched data, contributed to discussion, wrote manuscript. R.H. researched data, contributed to discussion, review/edited manuscript. M.T. reviewed/edited manuscript. Y.M. contributed to discussion, review/edited manuscript. B.K. researched data. J.P. researched data. D.M. researched data, review/edited manuscript. M.F. researched data, review/edited manuscript. A.K. researched data, contributed to discussion, review/edited manuscript. P.F. contributed to discussion, review/edited manuscript. W.K. researched data, review/edited manuscript. S.K. researched data, review/edited manuscript. W.C.K. researched data, review/edited manuscript. P.K. researched data, review/edited manuscript. P.F. researched data, review/edited manuscript. A.S. researched data, review/edited manuscript. C.B. researched data, contributed to discussion, review/edited manuscript. L.J. B. researched data, contributed to discussion, wrote manuscript, review/edited manuscript.

\textbf{ACKNOWLEDGMENTS}  
This work was supported by the intramural research program of NIDDK, NIH, and NIH Grants R01 DK54261 (to A Shuldiner) NIH P30 DK072488 (Clinical Nutrition Research Unit of Maryland) and NIH P60 DK079637 (Baltimore Diabetes Research and Training Center). Grant support was also provided by the American Diabetes Association (individually to C. Bogardus and A Shuldiner). Additional support was provided by grants from the Deutsche Forschungsgemeinschaft (DFG) KFO 152: project KO 3512/1–1, 1264/10–1 and the European Community integrated project grant “PIONEER.”

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FIGURE LEGENDS
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.
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 samples. Gray circles, Association results for obesity in the French adult case/control samples.
FIG. 3. Expression profile for A2BP1 in different human tissues.
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 non-specific siRNA sequence.
TABLE 1. Subjects analyzed in association studies.

| Sample | n (M/F)* | Sample type | Age (yrs) | 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 population” | 3,234 (1,350/1,884) | Population | 36.9 ± 14.6‡ | 33.5 ± 7.9‡ | N.A. |
| Full-heritage Pima population “non-overlapping with GWAS” | 2,843 (1,130/1,713) | Population excluding GWAS | 38.1 ± 13.4‡ | 33.4 ± 7.8‡ | N.A. |
| Caucasians | | | | | |
| French adult case/control | 695 (147/548) | cases | 44.1 ± 12.0 | 47.3 ± 7.6 | N. A. |
| French children case/control | 731 (181/550) | controls | 55.2 ± 8.2 | 21.8 ± 1.9 | N. A. |
| German School Children case/control | 685 (310/375) | cases | 10.9 ± 3.3 | 29.5 ± 6.5 | N. A. |
| Old Order Amish | 707 (332/375) | controls | 11.9 ± 2.3 | 17.6 ± 2.3 | N. A. |
| “Mixed-heritage” Native American | 1,149 (592/557) | family-based | 49.6 ± 16.8 | 30.0 ± 4.7 | N. A. |

*M, males; F, females. Data are means ± SD. N. A., information on percent body fat was not available. †Age, BMI, and percent body fat at first visit. ‡Age and BMI averaged over all visits.
| Population sample                  | n   | AF (T)† | P       | Z    | AF (A)† | P       | Z    |
|-----------------------------------|-----|---------|---------|------|---------|---------|------|
| Full-heritage Pima Indian “GWAS”  | 413 | 0.49    | $8.1 \times 10^{-5}$ | 3.94 | 0.50    | $3.0 \times 10^{-4}$ | 3.61 |
| “Full-heritage Pima population”   | 3,234 | 0.49 | $1.9 \times 10^{-7}$ | 5.20 | 0.50    | $3.3 \times 10^{-6}$ | 4.65 |
| Full-heritage Pima population “non-overlapping with GWAS” | 2,843 | 0.49 | $1.9 \times 10^{-5}$ | 4.28 | 0.50    | $4.2 \times 10^{-5}$ | 4.10 |
| French adult case/control         | 1,426 | 0.13   | 0.98    | 0.025 | 0.27    | 0.03   | 2.20 |
| French children case/control      | 1,392 | 0.13   | 0.96    | -0.06 | 0.27    | 0.90   | 0.13 |
| German School Children case/control | 998 | 0.12   | 0.80    | 0.26  | N. D.   | N. D.  | N. D. |
| 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 |

Association of rs10500331 and rs12924838 with BMI or obesity in different populations. *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 $D^2 = 0.95$, $r^2 = 0.95$ and Caucasians $D^2 = 0.83$, $r^2 = 0.25$. N. D., not determined.
### 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)

| 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 | N. D. |
| 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 | 0.00007 | 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 | N. D. |
| 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 | N. D. |
| 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 cases/controls GWA study. ‡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 cases/controls was not available for variants rs12924838 and rs4786847. N. D., not determined.
Figure 1

A2BP1 and Obesity

A

B

C

Shading indicates $D'$
Numbers indicate $r^2$

Shading indicates $D'$
Numbers indicate $r^2$
Figure 2
Figure 3

![Image of gel electrophoresis with bands for different tissues: kidney, islet, pancreas, adipocyte, pituitary, hypothalamus, fetal liver, adult liver, skeletal muscle, heart, total cDNA control, and 100bp ladder.]

Figure 4

![Graph showing mRNA expression levels for different genes: A2bp1, Atxn2, Insr, Mc4r, Lepr, Npy1r. Bars for Negative control siRNA and A2bp1 siRNA with p-values: P = 0.02, P < 0.0001, P = 0.005, P = 0.002, P = 0.52, and P = 0.18.]

A2BP1 and Obesity