Original Article
EPIDEMIOLOGY/GENETICS

Obesity

Genome-Wide Association Study in 3,173 Outbred Rats Identifies Multiple Loci for Body Weight, Adiposity, and Fasting Glucose

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Objective: Obesity is influenced by genetic and environmental factors. Despite the success of human genome-wide association studies, the specific genes that confer obesity remain largely unknown. The objective of this study was to use outbred rats to identify the genetic loci underlying obesity and related morphometric and metabolic traits.

Methods: This study measured obesity-relevant traits, including body weight, body length, BMI, fasting glucose, and retroperitoneal, epididymal, and parametrial fat pad weight in 3,173 male and female adult N/NIH heterogeneous stock (HS) rats across three institutions, providing data for the largest rat genome-wide association study to date. Genetic loci were identified using a linear mixed model to account for the complex family relationships of the HS and using covariates to account for differences among the three phenotyping centers.

Results: This study identified 32 independent loci, several of which contained only a single gene (e.g., *Epha5, Nrg1, Klhl14*) or obvious candidate genes (e.g., *Adcy3, Prlhr*). There were strong phenotypic and genetic correlations among obesity-related traits, and there was extensive pleiotropy at individual loci.

Conclusions: This study demonstrates the utility of HS rats for investigating the genetics of obesity-related traits across institutions and identify several candidate genes for future functional testing.

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Study Importance

What is already known?

- Obesity is influenced by both genetic and environmental factors.
- Rodents can be used to investigate the genetic basis of obesity and related morphometric and metabolic traits.

What does this study add?

- We identified 32 independent loci that are associated with adiposity, body size, BMI, and fasting glucose levels.
- Three of these loci contain only a single gene (e.g., *Epha5, Nrg1, Klhl14*), whereas several others contain just a few genes, simplifying gene identification.
- We identified potentially damaging coding variants within 18 loci. Combining this information with the strain distribution pattern of the peak marker at the quantitative trait locus and with the literature search, we identified five additional candidate genes (*Prlhr, Adcy3, Rnf213, Xbp1, and Ngp*).

How might these results change the direction of research or the focus of clinical practice?

- Our work elucidates the genetic architecture of obesity and therefore has the potential to identify novel therapeutic targets.

Introduction

Obesity is a growing health epidemic; more than one-third of the adult population and almost one-fifth of all children in the United States are considered to have obesity. There has been a steady increase in the prevalence of obesity since the 1970s, and the prevalence

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is continuing to rise (1). Obesity is a major risk factor for multiple diseases, including type 2 diabetes, cardiovascular disease, cancer, and stroke (2), thereby placing a tremendous burden on society. Obesity is caused by an interaction between genetic and environmental factors, with genetic factors accounting for up to 70% of the population variance (3). Although human genome-wide association studies (GWAS) of obesity have been extremely productive (4), much of the heritable variance is still unknown.

Model organism studies of body weight and morphometric and metabolic traits represent a complementary approach to understanding the genetic basis of obesity. However, GWAS in model organisms are often limited by the modest recombination present in laboratory crosses. Heterogeneous stock (HS) rats were created by interbreeding eight inbred founder strains and were subsequently maintained as an outbred population (5), making them ideal for fine-mapping of genetic loci (6). Furthermore, HS founder strains have been fully sequenced (7), such that coding polymorphisms can be rapidly identified (8). Our group previously mapped adiposity traits in HS rats using 743 male HS rats, which identified three genetic loci for visceral adiposity and body weight (8).

As part of a large multisite study (www.ratgenes.org), behavioral traits that are relevant to drug abuse have been assessed in thousands of male and female HS rats. To more fully use these rats, we have also collected phenotypic data on body weight and length (which permit calculation of BMI), fat pad weight, and fasting glucose levels. All animals were also extensively genotyped. This data set provides an unprecedented opportunity to map adiposity-related traits in a large cohort of HS rats.

Methods

Animals

The NMcwi:HS colony (hereafter referred to as HS) was initiated by the NIH in 1984 using the following eight inbred founder strains: ACI/N, BN/SsN, BUF/N, F344/N, M520/N, MR/N, WKY/N, and WN/N (5), which have previously been shown to differ significantly for adiposity traits (8). The rats described in this study were from generations 73 to 80 and were a separate cohort from that in previously published work (8). Breeders were given ad libitum access to a Teklad 5010 diet (Envigo, Madison, Wisconsin).

The rats used for this study are part of a large multisite project focused on genetic analysis of behavioral phenotypes related to drug abuse (www.ratgenes.org). HS rats from the Medical College of Wisconsin (Milwaukee, Wisconsin) were sent to three institutions throughout the United States: University of Tennessee Health Science Center (Memphis, Tennessee), University at Buffalo (Buffalo, New York), and University of Michigan (Ann Arbor, Michigan). Rats were shipped at 3 to 6 weeks of age, and each site received multiple shipments over more than 2 years (from October 27, 2014, to March 7, 2017). There are multiple environmental differences between each site, such that genetic loci are independent of these environmental influences. Rats at the University of Tennessee Health Science Center were fed Teklad Irradiated LM-485 Mouse/Rat Diet (Envigo), rats at the University at Buffalo were fed Teklad 18% Protein Rodent Diet (Envigo), and rats at the University of Michigan were fed irradiated Picolab Laboratory Rodent Diet (LabDiet, St. Louis, Missouri).

Rats were exposed to a different battery of behavioral testing at each site (Supporting Information Table S1), followed by euthanasia, which occurred at different ages at each site. All phenotypes presented in this paper were collected at the time of euthanasia. Briefly, at the University of Michigan, rats were housed in trios, exposed to a single modest dose of cocaine (15 mg/kg) each day for 5 days, and then euthanized 4 to 7 days after the final cocaine exposure (89 ± 6 days of age). At the University at Buffalo, rats were housed in pairs, tested for multiple behaviors over 16 weeks, exposed to a modest dose of cocaine (10 mg/kg) once daily for 3 days, and then euthanized 7 to 10 days after the last dose of cocaine (198 ± 13 days of age). At the University of Tennessee Health Science Center, there were two separate cohorts: breeders (sent from the Medical College of Wisconsin) and experimental rats (bred at the University of Tennessee Health Science Center). Female breeders had mostly one, sometimes two, litters and underwent no behavioral testing. The experimental rats were tested for multiple behaviors, exposed to nicotine (self-administration, resulting in a range of doses) for 12 days, and euthanized 10 days after the final dose of nicotine (73 ± 12 days of age). The numbers of rats phenotyped at each site as well as ages when phenotypes were collected are shown in Table 1.

Phenotyping and tissue collection

Several days after completion of behavioral experiments, rats were fasted overnight (17 ± 2 hours), and body weight was measured. Under anesthesia (phenobarbital at the University of Michigan and University at Buffalo; isoflurane at the University of Tennessee Health Science Center), two measures of body length (from nose to base of tail [body length_NoTail] and from nose to end of tail [body length_Tail]) were collected, allowing us to calculate tail length (TL) and two measures of BMI: BMI without the tail (BMI_NoTail) and BMI with the tail (BMI_Tail). BMI was calculated as (body weight / body length^2) × 10. For animals at the University of Michigan and University at Buffalo, we also measured fasting glucose levels using the Contour Next EZ system (Bayer, Elkhart, Indiana). Several tissues were dissected and weighed, including retroperitoneal fat (RetroFat), epididymal fat (EpiFat; males), and parametrial

TABLE 1 Age and number of rats at time adiposity phenotypes were collected

Phenotyping site	Males, *N*	Females, *N*	Total *N*	Age, d, mean ± SD
University of Michigan	519	514	1,033	89 ± 6
University at Buffalo	449	443	892	198 ± 13
University of Tennessee Health Science Center (experimental)	458	451	909	73 ± 12
University of Tennessee Health Science Center (breeders)	182	157	339	169 ± 34
Total	1,608	1,565	3,173	—

fat (ParaFat; females) visceral fat pads. All protocols were approved by the Institutional Animal Care and Use Committees for each of the relevant institutions.

Genotyping

Genotypes were determined using genotyping-by-sequencing, as described previously (9,10). This produced 3,400,759 single-nucleotide polymorphisms (SNPs) with an estimated error rate < 1%. Variants for X and Y chromosomes were not called. Prior to GWAS, SNPs in high-linkage disequilibrium were removed using PLINK (11) with an r^2 cut-off of 0.95; this produced a set of 128,447 SNPs, which was used for GWAS, genetic correlations, and heritability estimates. The unpruned set of SNPs was used to produce LocusZoom plots (12).

Phenotypic and genetic correlations and heritability estimates

Each trait within each research site was quantile-normalized separately for males and females; this approach is similar to using sex as a covariate. Other relevant covariates (including age, batch number, and dissector) were identified for each trait, and covariate effects were regressed out if they were significant and if they explained more than 2% of the variance (Supporting Information Table S2). Residuals were then quantile-normalized again, after which the data for each sex and site were pooled prior to further analysis. This approach removed mean differences due to sex; however, it did not attempt to model gene-by-sex interactions. By quantile-normalizing the three centers separately, we also addressed the numerous and confounded differences among the three cohorts, such that quantitative trait loci (QTLs) identified in the current study are resistant to environmental influences that differed among the sites. (Supporting Information Table S1). Phenotypic correlations were determined using the Spearman test. Genetic correlations were calculated using bivariate genome-based restricted maximum likelihood analysis (GREML) as implemented by genome-wide complex trait analysis (GCTA) (13,14). GCTA-GREML analysis was used to estimate the proportion of variance attributable to SNPs.

Genetic mapping

GWAS analysis employed a linear mixed model, as implemented in the software GEMMA (15), using dosages and genetic-relatedness matrices to account for the complex family relationships within the HS population and using the leave-one-chromosome-out method to avoid proximal contamination (16,17). Significance thresholds were calculated using permutation; because all traits were quantile-normalized, we used the same threshold for all traits (18). To identify QTLs, we scanned each chromosome to determine whether there was at least one SNP that exceeded the permutation-derived threshold of $-\log_{10} P > 5.6$, which was supported by a second SNP within 0.5 Mb that had a P value that was within $2 - \log_{10} P$ units of the index SNP. This algorithm failed to identify two SNPs, which were then identified using a wider supporting interval (chr10:85082795 for body length_Tail and chr12:5782829 for RetroFat). Then correlation between the top SNP and other SNPs in the 6-Mb vicinity was calculated. Other QTLs on the same chromosome were tested to ensure that they were independent of the first. To establish independence, we used the top SNP from the first QTL as a covariate and performed a second GWAS. If the resulting GWAS had an additional SNP (on the same chromosome) with a P value that exceeded our permutation-derived threshold, it was considered to be a second, independent locus. This process was repeated (including all previously significant SNPs as covariates) until no more QTLs were detected on a given chromosome.

Linkage disequilibrium intervals for the identified QTL were determined by identifying those markers that had a high correlation coefficient with the peak marker ($r^2 = 0.6$). Credible set analysis (19) was also performed for each locus. The credible set analysis uses a Bayesian approach to calculate the posterior probability for each SNP ("probability of being causal"). This method also chooses the credible set of SNPs that is the smallest set of SNPs that can account for 99% of the posterior probability.

Results

Strong phenotypic and genetic correlations among multiple adiposity traits

We observed strong phenotypic and genetic correlations among adiposity traits (Figure 1). Although the phenotypic correlation was weaker, fasting glucose levels correlated with body weight, BMI_Tail, BMI_NoTail, RetroFat, and EpiFat but they were not correlated with body length or ParaFat. Genetic correlations for fasting glucose levels differed from the phenotypic correlations, with a negative correlation seen with body length_NoTail and a positive correlation seen with ParaFat and BMI_NoTail. TL exhibited strong phenotypic and genetic correlations with BMI_NoTail and negative phenotypic and nonexistent genetic correlations with BMI_Tail (Supporting Information Figure S1). TL was also moderately correlated with the adiposity traits, indicating a role for this trait in rat metabolism, possibly through energy expenditure, as rats lose heat through their tail.

Adiposity traits exhibit high heritability

Heritability estimates for adiposity traits ranged from 0.26 ± 0.03 (BMI_NoTail) to 0.46 ± 0.03 (body weight; Table 2), whereas heritability estimates for fasting glucose were lower. We also calculated heritability for males and females separately; in general, the results were similar for the two sexes (Supporting Information Table S3). Although we performed the GWAS separately for both sexes, we have not provided the results of sex-specific GWAS because the reduction in power dramatically reduced the number of genome-wide significant results.

Identification of multiple GWAS hits

We identified a total of 32 independent loci for eight adiposity traits (Figure 2). Specifically, we identified nine QTLs for body weight, seven for RetroFat, three for EpiFat, one for ParaFat, eight for body length_Tail, five for body length_NoTail, three for BMI_Tail, and three for BMI_NoTail. Six of these loci mapped to more than one trait (Figure 2), four of which were likely pleiotropic (Table 3). The most highly significant loci were pleiotropic: chromosome 1:282 Mb (body weight, BMI_Tail, RetroFat, EpiFat, and ParaFat), chromosome 6:27 Mb (RetroFat and EpiFat), and chromosome 7:36 Mb (body weight, body length_NoTail, body length_Tail, and TL). Body weight QTLs consistently overlapped with both fat pad QTLs and body length QTLs, whereas only one BMI_Tail QTL overlapped with adiposity traits. BMI_Tail and BMI_NoTail loci did not overlap. It is difficult to use these data to determine whether the BMI calculation is useful, although the fact that BMI_Tail mapped to the highly significant pleiotropic locus on chromosome 1:282 Mb suggests that

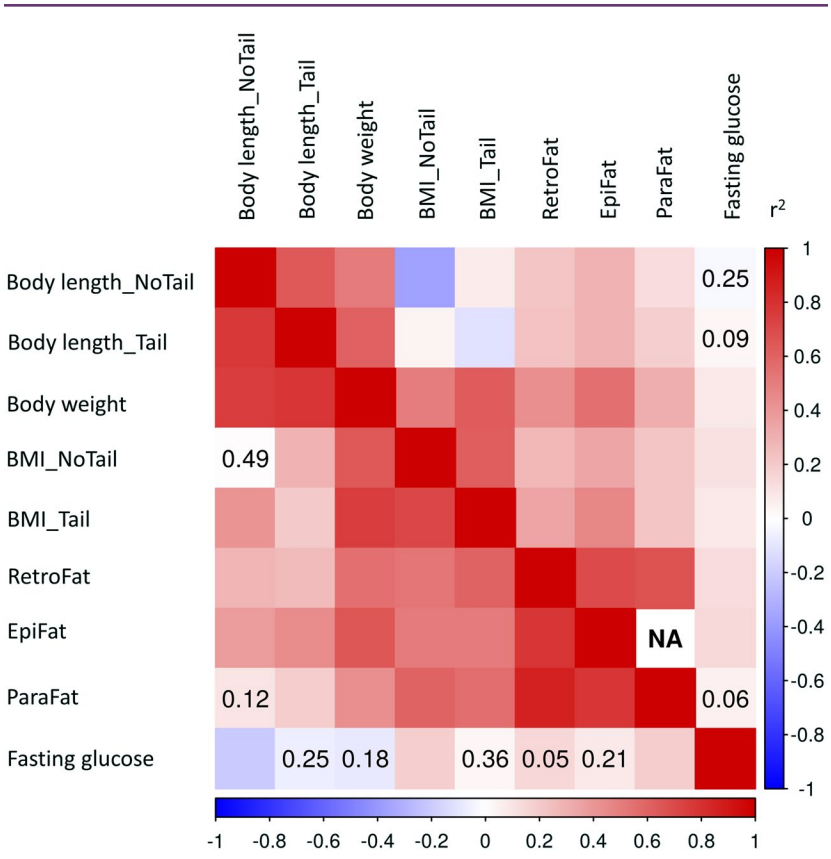

Figure 1 Genetic and phenotypic correlation between adiposity traits and fasting glucose. Phenotypic correlations are depicted in the upper part of the matrix, and genetic correlations are depicted in the lower part of the matrix. Numbers inside squares show *P* values > 0.05. BMI_NoTail, BMI without the tail; BMI_Tail, BMI with the tail; body length_NoTail, body length from nose to base of the tail; body length_Tail, body length from nose to end of the tail; EpiFat, epididymal fat; NA, not available; ParaFat, parametrial fat; RetroFat, retroperitoneal fat. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 SNP heritability estimates

Trait	Heritability ± SE
Body weight	0.46 ± 0.03
Body length_Tail	0.36 ± 0.03
TL	0.30 ± 0.02
Body length_NoTail	0.30 ± 0.03
BMI_Tail	0.31 ± 0.03
BMI_NoTail	0.26 ± 0.03
RetroFat	0.42 ± 0.03
EpiFat	0.37 ± 0.03
ParaFat	0.38 ± 0.04
Fasting glucose	0.15 ± 0.03

BMI_NoTail, BMI without the tail; BMI_Tail, BMI with the tail; body length_NoTail, body length from nose to base of the tail; body length_Tail, body length from nose to end of the tail; EpiFat, epididymal fat; ParaFat, parametrial fat; RetroFat, retroperitoneal fat; TL, tail length.

similar biology may underlie these traits. We identified two loci for fasting glucose, one of which overlapped a body weight locus on chromosome 10, although it is unlikely that these loci are driven by the same variant. Four QTLs were identified for TL, one of which overlapped with previously identified loci (Supporting Information Figure S2). Linkage disequilibrium intervals were 0.2 to 9.2 Mb and contained anywhere from 1 to 96 genes. Table 3 provides a detailed summary of these findings; Supporting Information Table S4 contains all the information from Table 3, with additional information, such as the strain distribution patterns (SDPs) of the founder strains as well as the full list of genes within each interval and credible set analysis results. Manhattan plots for all traits are included in Supporting Information Figure S2.

Pleiotropic loci

To determine whether traits that mapped to the same location were pleiotropic, we considered the mean allele frequencies (MAFs) and the SDPs of the index SNP among the eight founder strains that were used to create the HS strain. In a few cases, we found that loci for two or more phenotypes overlapped in terms of their chromosomal positions but that they had different MAFs and SDPs, suggesting different causal loci that happened to be located in approximately the same genetic location. We also observed a few cases in which the SDPs were similar but not identical (e.g., one strain's genotype

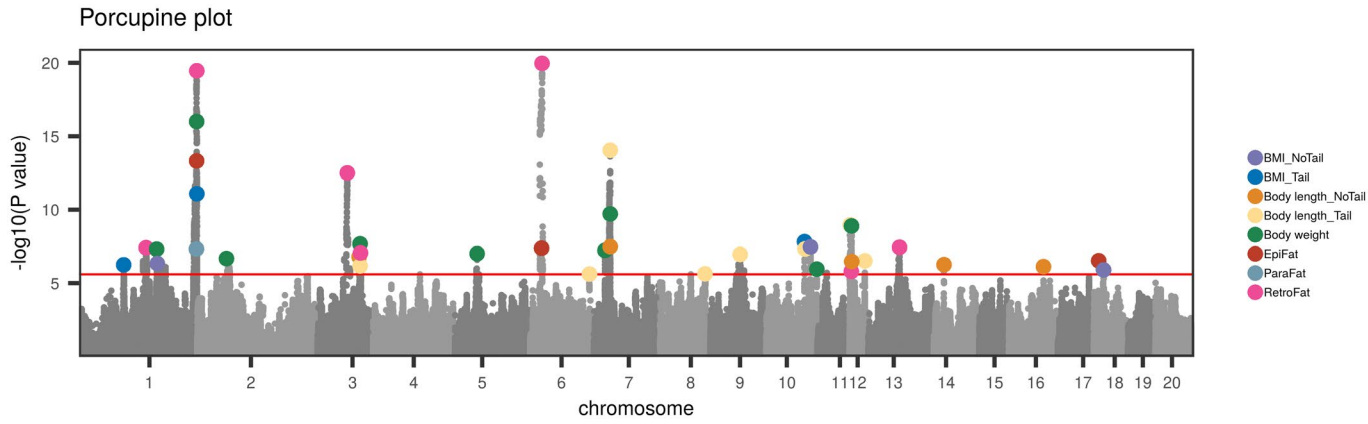

Figure 2 Combined Manhattan plots of genome-wide association study (GWAS) data for eight adipose traits. Genome-wide association results from the GWAS analysis. Chromosomal distribution of all the *P* values ($-\log_{10}$ *P* values) is shown, with top single-nucleotide polymorphisms (SNPs) highlighted. BMI_NoTail, BMI without the tail; BMI_Tail, BMI with the tail; body length_NoTail, body length from nose to base of the tail; body length_Tail, body length from nose to end of the tail; EpiFat, epididymal fat; ParaFat, parametrial fat; RetroFat, retroperitoneal fat.

was dissimilar between the two loci); we assumed that those situations were consistent with pleiotropy. Note that this approach is conceptually similar to the estimation of the phenotype associated with founder haplotypes, which was described in Svenson et al. (20) and in Yalcin et al. (21), but it would not perform well if there were more than two causal alleles.

Using these criteria, we designated four loci as pleiotropic, which are bolded in Table 3. The chromosome 1:281 Mb locus was the strongest, with $-\log_{10}$ *P* values that ranged from 7 to 20 for nearly all of the adiposity traits, including body weight, BMI_Tail, and all three fat pads (RetroFat, EpiFat, and ParaFat). Although BMI_Tail mapped to the pleiotropic locus on chromosome 1:281 Mb, BMI_NoTail did not. In addition, chromosome 6:~27 Mb mapped to both RetroFat and EpiFat, and, finally, chromosomes 3 and 7 mapped to body weight, body length_Tail, and body length_NoTail.

Nearby loci that were not considered pleotropic are italicized in Table 3. We identified two sets of QTLs that mapped to similar regions and therefore might have appeared to be pleiotropic; however, after applying the criteria described earlier, we determined that they were not truly pleiotropic because the MAFs and SDPs of the founder strains (Supporting Information Table S4) were not similar. These loci were chromosome 10:~110 Mb for fasting glucose and body weight and chromosome 12:6 Mb for body weight, RetroFat, and body length_NoTail.

Candidate gene identification

The number of genes within the identified QTLs ranged from 1 to 96 (Table 3). There were three regions that contained a single gene: EPH receptor A5 (*Epha5*) within chromosome 14:26 Mb for body length_NoTail, neuregulin 1 (*Nrg1*) within chromosome 16:64 Mb for body length_NoTail, and Kelch-like family member 14 (*Klhl14*) within chromosome 18:12 Mb for EpiFat. Both *Epha5* and *Nrg1* are known to be involved in growth and development and are therefore logical candidate genes. In contrast, very little is known about *Klhl14*, meaning that if this gene is responsible for the observed QTLs, it would offer novel biological insight. Despite the fact that these loci contained only a single gene, it is possible that the causal allele is a regulatory variant that is located in this interval but that regulates a gene outside of the identified interval.

All other loci contained more than one gene. To identify candidate genes, we used founder sequence information (22) to identify potentially damaging coding variants using the software SnpEff (23). We identified 152 coding variants for 18 QTLs, 2 of which were predicted to have high impact. The SDPs for 63 coding variants matched the SDP at the peak SNP, and they are therefore candidates for future analysis (Supporting Information Table S5). Combining these data with a literature search, we identified plausible candidate genes/variants within six loci (Table 4). Representative LocusZoom plots for select loci are shown in Figure 3, and LocusZoom plots for all other loci are in Supporting Information Figure S3.

Discussion

The current study is the largest rodent GWAS ever reported. This study measured multiple adiposity traits collected at three different sites at multiple ages. Traits measured include body weight, body length, BMI (calculated with and without the tail), fat pad weights, and fasting glucose, all major risk factors for development of type 2 diabetes. We identified 32 significant loci, several of which corresponded to narrow regions that contained logical candidate genes. We replicated previously identified loci and identified many novel loci. The large number of significant associations, the small regions implicated, and the replication of previously reported loci despite age, diet, and other environmental differences all highlight the power of HS rats for GWAS.

This study was part of a large multicenter behavioral study focused on genetic influences on drug abuse–relevant behaviors. As a result, the animals in this study were exposed to a variety of factors that may have altered adiposity. These include brief drug exposure, different

TABLE 3 Summary of QTLs

	Peak marker					LD interval			
Trait	Position	–log *P*	Ref allele	Allele frequency	Effect size	Start (bp)	Stop (bp)	Size (Mb)	Genes in LD interval
BMI_Tail	chr1:106866154	6.05	G	0.29	0.15 ± 0.03	105,730,059	109,396,142	3.67	7
RetroFat	chr1:160530456	7.51	T	0.53	–0.14 ± 0.02	157,254,290	162,857,247	5.60	15
Body weight	chr1:185730317	7.58	C	0.74	0.16 ± 0.02	184,463,432	187,738,111	3.27	6
BMI_NoTail	chr1:187300775	6.72	A	0.66	0.15 ± 0.02	184,772,656	189,346,447	4.57	27
TL	chr1:253524003	5.76	G	0.45	0.13 ± 0.03	253,082,171	254,725,734	1.64	6
ParaFat	**chr1:280924549**	**7.22**	**G**	**0.57**	**0.20 ± 0.03**	**280,876,316**	**282,114,080**	**1.24**	**9**
Body weight	**chr1:281756885**	**16.21**	**C**	**0.58**	**0.21 ± 0.02**	**280,924,333**	**282,114,080**	**1.19**	***Fam204a, Prlhr, Cacul1***
RetroFat	**chr1:281777218**	**20.21**	**A**	**0.57**	**0.24 ± 0.02**	**280,924,333**	**282,114,080**	**1.19**	**9**
EpiFat	**chr1:281802657**	**14.00**	**C**	**0.56**	**0.29 ± 0.03**	**280,924,333**	**282,736,277**	**1.81**	**13**
BMI_Tail	**chr1:282049439**	**11.44**	**C**	**0.55**	**0.18** ± 0.02	**280,924,333**	**282,736,277**	**1.81**	**13**
Body weight	chr2:65816485	6.55	A	0.91	0.20 ± 0.03	62,570,942	71,814,490	9.24	6
RetroFat	chr3:95389621	13.12	A	0.44	–0.19 ± 0.02	92,336,188	97,685,154	5.35	30
Body length_NoTail	**chr3:136021511**	**6.92**	**A**	**0.77**	**–0.16 ± 0.03**	**132,291,573**	**137,146,532**	**4.85**	**10**
Body length_Tail	**chr3:136021511**	**5.97**	**A**	**0.77**	**–0.14 ± 0.03**	**132,291,573**	**137,146,532**	**4.85**	**10**
Body weight	**chr3:136021511**	**7.34**	**A**	**0.77**	**–0.16 ± 0.02**	**132,291,573**	**137,146,532**	**4.85**	**10**
RetroFat	chr3:137537161	8.21	G	0.44	–0.15 ± 0.02	136,161,761	138,849,437	2.69	20
Body weight	chr5:50933779	6.99	A	0.78	–0.16 ± 0.03	49,152,709	50,940,275	1.79	12
EpiFat	**chr6:26266960**	**7.25**	**G**	**0.59**	**–0.20 ± 0.03**	**22,684,886**	**28,223,800**	**5.54**	**55**
RetroFat	**chr6:28148338**	**19.50**	**G**	**0.65**	**–0.26 ± 0.02**	**25,954,450**	**28,752,109**	**2.80**	**58**
Body length_Tail	chr6:137745191	5.89	C	0.54	–0.12 ± 0.02	136,769,305	138,087,183	1.32	24
Body weight	chr7:24886476	7.29	G	0.07	0.26 ± 0.04	24,869,890	25,213,445	0.34	*Tcp11l2, Nuak1, Ckap4*
Body weight	**chr7:36497588**	**9.77**	**C**	**0.14**	**–0.24 ± 0.03**	**34,119,928**	**36,522,260**	**2.40**	**21**
Body length_NoTail	**chr7:36517726**	**7.88**	**T**	**0.12**	**–0.25 ± 0.04**	**34,119,928**	**36,522,260**	**2.40**	**15**
Body length_Tail	**chr7:36517726**	**14.37**	**T**	**0.12**	**–0.33 ± 0.04**	**34,119,928**	**36,522,260**	**2.40**	**21**
TL	chr7:36526715	6.10	A	0.37	0.14 ± 0.03	36,289,478	36,765,903	0.48	5
Body length_Tail	chr8:118711320	5.72	A	0.67	0.13 ± 0.02	116,614,891	119,781,444	3.17	96
Body length_Tail	chr9:65078205	6.81	C	0.76	0.16 ± 0.03	64,013,585	65,670,399	1.66	20
BMI_Tail	chr10:84080794	8.21	G	0.57	–0.16 ± 0.02	83,442,285	85,006,252	1.56	42
TL	**chr10:84263936**	**9.38**	**T**	**0.57**	**0.18 ± 0.03**	**83,442,285**	**85,006,252**	**1.56**	**42**
Body length_Tail	**chr10:85082795**	**7.37**	**C**	**0.72**	**0.17 ± 0.03**	**84,902,901**	**85,239,899**	**0.34**	**10**
BMI_NoTail	chr10:96804258	7.66	T	0.48	–0.16 ± 0.02	96,561,667	98,097,621	1.54	16
Fasting glucose	*chr10:109944213*	*6.33*	*A*	*0.75*	*–0.18 ± 0.03*	*108,350,175*	*110,315,359*	*1.97*	*49*
Body weight	*chr10:111010289*	*6.10*	*T*	*0.41*	*0.13 ± 0.02*	*110,664,101*	*111,022,246*	*0.36*	*Tbcd, Znf750, B3gntl1, Metrnl*
Body length_Tail	chr12:2199384	8.94	C	0.18	–0.22 ± 0.03	846,435	5,587,624	4.74	50
Body weight	*chr12:5738696*	*9.12*	*C*	*0.59*	*0.16 ± 0.02*	*4,938,015*	*6,078,451*	*1.14*	*6*
RetroFat	*chr12:5782829*	*7.12*	*T*	*0.73*	*0.14 ± 0.02*	*455,837*	*6,259,634*	*5.80*	*59*
Body length_NoTail	*chr12:6239515*	*6.26*	*A*	*0.52*	*0.13 ± 0.02*	*6,078,237*	*7,035,028*	*0.96*	*9*

TABLE 3 (continued).

Trait	Peak marker					LD interval			
	Position	−log *P*	Ref allele	Allele frequency	Effect size	Start (bp)	Stop (bp)	Size (Mb)	Genes in LD interval
Body length_Tail	chr12:43060205	6.27	G	0.75	−0.15 ± 0.02	42,024,283	43,190,584	1.17	*Oas1g, Tbx3, Tbx5*
TL	chr13:109566014	7.04	T	0.34	0.15 ± 0.03	108,055,241	111,868,706	3.81	30
RetroFat	chr13:55021887	7.16	C	0.67	0.14 ± 0.02	54,910,667	56,487,438	1.58	7
Body length_NoTail	chr14:26365986	6.54	A	0.73	−0.16 ± 0.03	25,129,356	26,478,660	1.35	*Epha5*
Fasting Glucose	chr14:86029588	6.27	T	0.27	−0.17 ± 0.03	82,473,632	87,575,807	5.10	34
Body length_NoTail	chr16:64014119	6.35	G	0.55	0.13 ± 0.02	64,003,027	64,416,540	0.41	*Nrg1*
EpiFat	chr18:12674871	6.47	G	0.22	−0.22 ± 0.04	12,597,378	12,792,162	0.19	*Klhl14*
BMI_NoTail	chr18:25190274	5.78	T	0.04	−0.34 ± 0.07	23,785,532	25,418,376	1.63	20
TL	chr19:32004685	7.81	C	0.41	−0.15 ± 0.03	31,176,512	32,698,569	1.52	7

Positions for pleiotropic loci are bolded; positions for nearby loci that were not considered pleotropic are italicized.
BMI_NoTail, BMI without the tail; BMI_Tail, BMI with the tail; body length_NoTail, body length from nose to base of the tail; body length_Tail, body length from nose to end of the tail; bp, base pairs; EpiFat, epididymal fat; LD, linkage disequilibrium; ParaFat, parametrial fat; QTL, quantitative trait locus; RetroFat, retroperitoneal fat; TL, tail length; *B3gntl1*, UDP-GlcNAc:BetaGal Beta-1,3-N-acetylglucosaminyltransferase–like 1; *Cacul1*, CDK2-associated, cullin domain 1; *Ckap4*, cytoskeleton-associated protein 4; *Epha5*, EPH receptor A5; *Fam204a*, family with sequence similarity 204 member A; *Klhl14*, Kelch-like family member 14; *Metrnl*, meteorin-like glial-cell differentiation regulator; *Nrg1* , neuregulin 1; *Nuak1*, Nuak family kinase 1; *Oas1g*, 2′-5′-oligoadenylate synthetase 1G; *Prlhr*, prolactin-releasing hormone receptor; *Tbcd*, tubulin folding cofactor D; *Tbx 3*, T-box transcription factor 3; *Tbx 5*, T-box transcription factor 5; *Tcp11l2*, T-complex 11–like 2; *Znf750*, zinc finger protein 750.

behavioral experiences, and different diets and ages when tissues were collected. Despite these differences, we were successful in identifying a large number of adiposity QTLs, indicating that these loci are resistant to multiple environmental influences. Importantly, contribution of these environmental influences were taken into account in the statistical analysis by quantile-normalizing data from each institution individually prior to running GWAS on the combined data. Although this design may have diluted our power to detect loci that were relevant only in one set of conditions, the loci that we did detect are likely to be robust to subtle drug, age, and environmental differences. We view this aspect of the study as a strength, as it allows us to generalize our findings across populations, including to humans, for whom there are multiple uncontrolled environmental influences.

With the exception of fasting glucose, all traits demonstrated strong phenotypic and genetic correlations, indicating that they are caused by overlapping sets of alleles. The pleiotropic loci that we identified further underscore the common genetic basis of these traits. The strongest pleiotropic locus identified falls on chromosome 1:282 Mb and maps five of the adiposity traits, with a second locus for RetroFat and EpiFat at chromosome 6:28 Mb, both replicating previous work by our group (8). We also identified novel pleiotropic loci for body weight and body length (with and without the tail) on chromosomes 3 and 7. Interestingly, we found that not all of the loci that contained multiple traits should be considered pleiotropic. We identified two regions that mapped to more than one trait, but because both the MAFs and the founder SDPs did not match between the traits, these loci are likely driven by different genes and/or variants.

One of the strengths of using a highly recombinant outbred strain such as the HS is the ability to map to relatively small regions of the genome, greatly narrowing the number of potential candidate genes that may drive the QTLs. In the current work, three of the identified loci contained only a single gene. For example, *Epha5* is the only gene within the chromosome 14 locus for body length_NoTail. Ephrin receptors make up the largest subfamily of the receptor protein tyrosine kinases and they are known to be involved in embryonic development, cell migration, and axon guidance. Although *Epha5* has not previously been associated with body weight or height in human GWAS, *Epha5*-knockout mice have increased body weight relative to wild-type mice (24), suggesting a potential role for this gene in determining body length. In addition, *Nrg1* is the only gene that falls within the chromosome 16 locus for body length_NoTail. *Nrg1* mediates cell-cell signaling and plays a critical role in the growth and development of multiple organ systems. Intracerebroventricular injection of *Nrg1* leads to increased food intake and weight gain in rodents (25), and a recent study demonstrated a positive metabolic effect of *Nrg1* on multiple metabolic parameters, including body weight (26). In addition, *Nrg1* has been associated with BMI in a Korean population (27), making *Nrg1* a highly plausible gene within this region. Finally, *Klhl14* is the only gene that falls within the EpiFat locus at chromosome 18. This gene localizes to the endoplasmic reticulum and it has not previously been associated with adiposity traits in human or rat GWAS, suggesting that this finding could offer a novel biological insight. Regions containing a single gene are attractive, but we note that these regions may also contain regulatory variants for neighboring genes or unannotated genes or transcripts that cause the association.

Another strength of using the HS strain is the ability to use the founder sequence to identify potentially damaging coding variants within

TABLE 4 Moderate- and high-impact coding variants that fall within adiposity QTLs and are supported by SDPs and/or literature search

Trait	QTL	Candidate gene	SNP in LD with peak marker	SNP change	Amino acid change	Effect	Impact	ACI	BN	BUF	F344	M520	MR	WN	WKY	Literature support
Fasting glucose	chr10: 109 Mb	*Rnf213*	chr10:108582280	c.9502C>G	p.His3168Asp	missense_variant	Moderate	GG	CC	GG	GG	GG	GG	GG	GG	PMID 23410753
			chr10:108582544	c.9766T>C	p.Trp3256Arg	missense_variant	Moderate	CC	TT	CC	CC	CC	CC	CC	CC	PMID 23410753
			chr10:108604073	c.12263G>C	p.Ser4088Thr	missense_variant	Moderate	CC	GG	CC	CC	CC	CC	CC	CC	PMID 23410753
Fasting glucose	chr14: 86 Mb	*Xbp1*	chr14:85756355	c.397A>G	p.Asn133Asp	missense_variant	Moderate	AA	AA	AA	AA	AA	AA	AA	GG	366 PubMed articles for *Xbp1*, glucose
BMI_Tail	chr1: 282 Mb	*Prlhr*	chr1:281755911	c.3G>A	p.Met1?	start_lost	High	CC	CC	TT	CC	CC	CC	CC	TT	PMID 29193816
Body weight			chr1:281755911	c.3G>A	p.Met1?	start_lost	High	CC	CC	TT	CC	CC	CC	CC	TT	PMID 29193816
ParaFat			chr1:281755911	c.3G>A	p.Met1?	start_lost	High	CC	CC	TT	CC	CC	CC	CC	TT	PMID 29193816
RetroFat			chr1:281755911	c.3G>A	p.Met1?	start_lost	High	CC	CC	TT	CC	CC	CC	CC	TT	PMID 29193816
EpiFat			chr1:281755911	c.3G>A	p.Met1?	start_lost	High	CC	CC	TT	CC	CC	CC	CC	TT	PMID 29193816
RetroFat	chr6: 28 Mb	*Adcy3*	chr6:28572363	c.362T>C	p.Leu121Pro	missense_variant	Moderate	TT	TT	TT	TT	TT	TT	TT	CC	PMID 29193816
Body length_Tail	chr8: 118 Mb	*Ngp*	chr8:118664419	c.316C>T	p.Gln106*	stop_gained	High	TT	CC	CC	CC	CC	CC	CC	CC	none

ACI, BN, BUF, F344, M520, MR, WN, and WKY are the eight inbred founder strains.
BMI_Tail, BMI with the tail; body length_Tail, body length from nose to end of the tail; chr, chromosome; EpiFat, epididymal fat; LD, linkage disequilibrium; ParaFat, parametrial fat; PMID, PubMed identifier; QTL, quantitative trait locus; RetroFat, retroperitoneal fat; SDP, strain distribution pattern; SNP, single-nucleotide polymorphism; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Gln, glutamine; His, histidine; Leu, leucine; Met, methionine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; *Adcy3*, adenylate cyclase 3; *Ngp*, neutrophilic granule protein; *Prlhr*, prolactin-releasing hormone receptor; *Rnf213*, ring-finger protein 213; *Xbp1*, X-box binding protein 1.

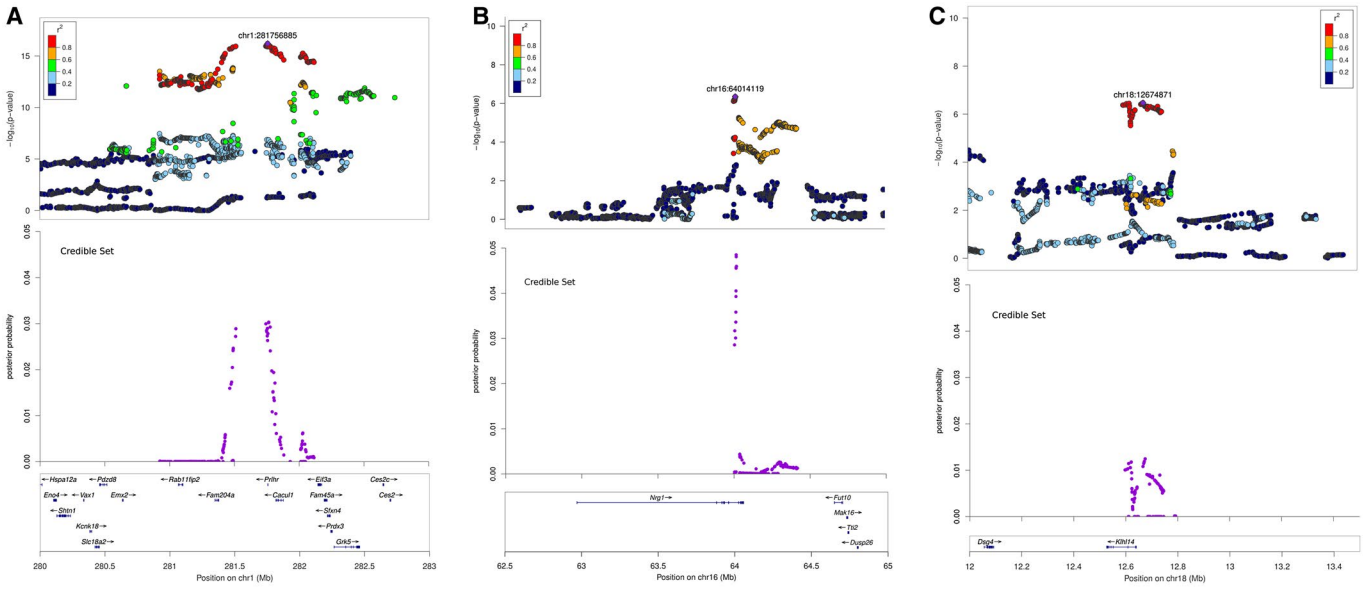

Figure 3 Regional association plots. The x-axis shows the chromosomal position. Top panel: regional association plot. Single-nucleotide polymorphism (SNP) with the lowest *P* value ("top SNP") is shown in purple, and its chromosomal position is indicated on the plot. Color of the dots indicates level of linkage disequilibrium (LD) of each SNP with the top SNP. Middle panel: credible set track shows posterior probability for being causal for the SNPs, which were identified as the smallest set of SNPs accounting for 99% of the posterior probability (see *Methods*). Bottom panel: genes in the region, as annotated by the reference sequence. (**A**) Regional association plot for body weight at chromosome (chr) 1 (280-283 Mb). (**B**) Regional association plot for the body length from nose to base of the tail (body length_NoTail) at chr16 (62.5-65 Mb). (**C**) Regional association plot for epididymal fat (EpiFat) at chr18 (12-13.5 Mb). *Cacul1*, CDK2-associated, cullin domain 1; *Ces2*, carboxylesterase 2; *Ces2c*, carboxylesterase 2C; *Dsg4*, desmoglein 4; *Dusp26*, dual specificity phosphatase 26; *Eif3a*, eukaryotic translation initiation factor 3, subunit A; *Emx2*, empty spiracles homeobox 2; *Eno4*, enolase 4; *Fam204a*, family with sequence similarity 204, member A; *Fam45a*, DENN domain containing 10; *Fut10*, fucosyltransferase 10; *Grk5*, G protein-coupled receptor kinase 5; *Hspa12a*, heat shock protein family A (Hsp70) member 12A; *Kcnk18*, potassium two pore domain channel subfamily K member 18; *Klhl14*, kelch-like family member 14; *Mak16*, MAK16 homolog; *Nrg1*, neuregulin 1; *Pdzd8*, PDZ domain containing 8; *Prdx3*, peroxiredoxin 3; *Prlhr*, prolactin releasing hormone receptor; *Rab11fip2*, RAB11 family interacting protein 2; *Sfxn4*, sideroflexin 4; *Shtn1*, shootin 1; *Slc18a2*, solute carrier family 18 member A2; *Tti2*, TELO2 interacting protein 2; *Vax1*, ventral anterior homeobox 1.

QTLs. We identified 154 coding variants, 63 of which match the SDPs of the founder strains, making them plausible candidates. Replicating previous findings (8), we identified a high-impact variant within prolactin-releasing hormone receptor (*Prlhr*), a gene that falls within the highly significant pleiotropic locus on chromosome 1 and that has previously been shown to play a role in feeding behavior (28,29).

We also identified a variant within adenylate cyclase 3 (*Adcy3*), a gene that falls within the chromosome 6 locus for RetroFat and EpiFat and that has been identified in both HS rats (8) and human GWAS (30-32). We previously demonstrated that multiple genes likely play a role at the chromosome 6 locus, including *Adcy3*, keratinocyte-associated protein 3 (*Krtcap3*), and solute carrier family 30 member 3 (*Slc30a3*) (8). In addition to identifying candidate genes within these pleiotropic loci, we identified candidates within the fasting glucose loci: coding variants that match the SDP were identified in ring-finger protein 213 (*Rnf213*) (chromosome 10 locus), a gene shown to protect beta cells and improve glucose tolerance (33), and X-box binding protein 1 (*Xbp1*) (chromosome 14 locus), a gene shown to have multiple roles in glucose regulation (34). Finally, within the body length_Tail QTL on chromosome 8, we identified a high-impact variant in neutrophilic granule protein (*Ngp*), a gene that has not previously been associated with height or body weight. Future work will investigate the role of these variants in modifying adiposity traits.

This is the largest GWAS ever performed in rodent models and it has identified a large number of significant loci. In spite of our large sample size, however, this study has its limitations. Adiposity measures were collected from both males and females, but we have not presented sex-specific GWAS because the heritabilities for the two sexes were similar and because the reduction in sample size dramatically reduced the number of genome-wide significant results. Therefore, we quantile-normalized the males and females separately and then pooled them, which effectively removed mean differences between the sexes but which did not account for gene-by-sex interactions. We are continuing to collect adiposity traits in additional animals and we plan to use an even larger sample size to test for sex-specific QTLs in the future. A second limitation is that we have not incorporated expression QTL data for some of the most relevant tissues (e.g., liver, fat pads), which would be helpful for identifying candidate genes within the physiological QTLs on the basis of gene expression differences. Our study did not employee haplotype-based analyses, which are often used in highly recombinant populations such as the HS population. Our laboratory is currently addressing several issues that may impact haplotype-based analysis, including generating the genetic relationship matrix and the null distribution as well as the most appropriate tool for determining founder probabilities. Finally, our study was part of a large multicenter behavioral study focused on genetic influences on drug abuse–relevant behaviors. As a result, a subset of the animals was briefly exposed to moderate doses of cocaine or nicotine, both of which could have changed body weight. In addition, the diets, ages, and behavioral experiences of the rats differed across the three study sites. Any contribution of these environmental influences, however, was taken into account

by quantile-normalizing each institution separately prior to analysis. Although this design may have diluted our power to detect loci that were relevant in only one set of conditions, the loci that we did detect are likely to be robust to subtle drug, age, and environmental differences, which can be viewed as a strength if the goal is to generalize our findings across populations, including to humans (35).

Conclusion

The current study is the largest GWAS using a rodent population ever performed. We replicated previously identified loci from smaller GWAS and identified numerous novel loci for multiple adiposity traits. Three of these loci contain only a single gene. Several other loci contain only a few genes, which simplifies the identification of candidate genes. We used prediction tools and founder sequence information to identify candidate variants and genes within five of these loci. This work demonstrates the power of HS rats for fine-mapping and gene identification of adiposity traits, including the power to identify genetic loci across multiple institutions and environmental influences. This work also provides immediate candidate genes for future functional 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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