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Genetic Control of Obesity and Gut Microbiota Composition in Response to High-Fat, High-Sucrose Diet in Mice

Brian W. Parks[1,*], **Elizabeth Nam**[1], **Elin Org**[1], **Emrah Kostem**[2], **Frode Norheim**[1,3], **Simon T. Hui**[1], **Calvin Pan**[4], **Mete Civelek**[1], **Christoph D. Rau**[1], **Brian J. Bennett**[1,5], **Margarete Mehrabian**[1], **Luke K. Ursell**[6], **Aiqing He**[7], **Lawrence W. Castellani**[1], **Bradley Zinker**[8], **Mark Kirby**[8], **Thomas A. Drake**[9], **Christian A. Drevon**[3], **Rob Knight**[6,10], **Peter Gargalovic**[11], **Todd Kirchgessner**[11], **Eleazar Eskin**[2,4], and **Aldons J. Lusis**[1,4,*]

[1]Department of Medicine/Division of Cardiology, David Geffen School of Medicine, University of California, Los Angeles California 90095, USA

[2]Department of Computer Science, University of California, Los Angeles, California 90095, USA

[3]Department of Nutrition, Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo, Norway

[4]Department of Human Genetics, David Geffen School of Medicine, University of California, Los Angeles, California 90095, USA

[5]Department of Genetics, University of North Carolina, Chapel Hill 27599, USA

[6]Department of Chemistry and Biochemistry, University of Colorado Boulder, Colorado 80309, USA

[7]Department of Applied Genomics, Bristol-Myers Squibb, Princeton, New Jersey 08543, USA

[8]Department of Metabolic Diseases-Diabetes Drug Discovery, Bristol-Myers Squibb, Princeton, New Jersey 08543, USA

[9]Department of Pathology and Laboratory Medicine, University of California, Los Angeles, CA 90095, USA

[10]Howard Hughes Medical Institute, Boulder, CO, 80309, USA

[11]Department of Atherosclerosis Drug Discovery, Bristol-Myers Squibb, Princeton, New Jersey 08543, USA

SUMMARY

[*]**Corresponding Authors:** Aldons J. Lusis, Ph.D., Depts. of Human Genetics, Microbiology, Immunology and Molecular Genetics, and Medicine, University of California, Los Angeles, 650 Charles E. Young Dr. South, A2-237 Center for Health Sciences, Los Angeles, CA 90095-1679, jlusis@mednet.ucla.edu, Phone: 310-825-1359, Fax: 310-794-7345; Brian W. Parks, Ph.D., Dept. of Medicine, Cardiology, University of California, Los Angeles, 675 Charles E. Young Dr. South, MRL 3220, Los Angeles, CA 90095, Phone: 310-825-1595, Fax: 310-794-7345, bparks@mednet.ucla.edu.

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All microarray data from this study are deposited in the NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) under the accession number XXX.

Obesity is a highly heritable disease driven by complex interactions between genetic and environmental factors. Human genome-wide association studies (GWAS) have identified a number of loci contributing to obesity; however, a major limitation of these studies is the inability to assess environmental interactions common to obesity. Using a systems genetics approach, we measured obesity traits, global gene expression, and gut microbiota composition in response to a high-fat/high-sucrose (HF/HS) diet of more than 100 inbred strains of mice. Here we show that HF/HS feeding promotes robust, strain-specific changes in obesity that is not accounted for by food intake and provide evidence for a genetically determined set-point for obesity. GWAS analysis identified 11 genome-wide significant loci associated with obesity traits, several of which overlap with loci identified in human studies. We also show strong relationships between genotype and gut microbiota plasticity during HF/HS feeding and identify gut microbial phylotypes associated with obesity.

INTRODUCTION

The dramatic increase in obesity during the past few decades is tightly associated with the increase in obesity-related complications, such as type 2 diabetes, heart disease, and cancer. Energy-rich diets containing high levels of fat and refined carbohydrates along with sedentary lifestyles are believed to be the most significant environmental factors contributing to this epidemic (Finucane et al., 2011; Malik et al., 2010). Understanding the genetic and environmental interactions contributing to obesity is thus crucial for developing novel therapies and preventive strategies. Human genome-wide association studies (GWAS) and studies of rare monogenic forms of obesity, as well as biochemical studies with cells and animal models, have identified relevant genes and pathways important in obesity; however, given the complexity of the obesity phenotype and the small amount of variance that can be explained by known obesity alleles, it is clear that much remains to be discovered (Attie and Scherer, 2009; Bouchard et al., 1990; Knights et al., 2011; Sandholt et al., 2010; Speakman et al., 2011).

Obesity is strongly heritable in humans, with estimates ranging from 50 to 90% (Barsh et al., 2000; Stunkard et al., 1986). Large human GWAS explain less than 3% of this heritable component and environmental interactions with diet composition likely add significant complexity to such studies (Kilpelainen et al., 2011; Sonestedt et al., 2009; Speliotes et al., 2010). Indeed, long-term overfeeding in monozygotic twins promotes striking within-pair similarities in fat mass gain, demonstrating that gene-by-diet interactions may be highly heritable and have a large impact on obesity (Bouchard et al., 1990). In addition to host genetic contributions, the microbial community within the gut has been shown to influence obesity in humans and mice (Turnbaugh et al., 2009a; Turnbaugh et al., 2006). The gut microbiota is a transmissable trait and can undergo dynamic population shifts with varied dietary composition (Benson et al., 2010; Turnbaugh et al., 2009b; Yatsunenko et al., 2012). Obese subjects have an altered gut microbiota compared to lean individuals, which may be an important contributing factor to the obesity epidemic (Turnbaugh et al., 2009a). To date, very little is known about the genetic basis of gene-by-diet and gut microbiota-diet interactions to common obesogenic factors, such as the consumption of energy-rich diets.

Identification of genes contributing to complex traits, such as obesity, in mice has been hampered by the poor mapping resolution of traditional genetic crosses (Bhatnagar et al., 2011; Burrage et al., 2010; Dokmanovic-Chouinard et al., 2008; Ehrich et al., 2005b; Flint et al., 2005; Lawson et al., 2011; York et al., 1999; Zhang et al., 1994). Based on the genome sequencing of many mouse strains, the identification of millions of single nucleotide polymorphisms (SNPs) (Keane et al., 2011), and the development of an algorithm that corrects for population structure in association analysis (Kang et al., 2008), we recently

developed a systems genetics resource in the mouse capable of high resolution genome-wide association mapping (Bennett et al., 2010). This resource, termed the Hybrid Mouse Diversity Panel (HMDP) is composed of more than 100 commercially available mouse strains and is ideal for systems-level analyses of gene-by-environment interactions. Association-based mapping approaches in rodents have recently been reviewed (Flint and Eskin, 2012).

Employing a systems genetics approach in the mouse, we integrated physical, molecular traits, and gut microbiota composition data in response to an energy-rich diet. Using GWAS rather than quantitative trait locus (QTL) analyses, we obtained biologically meaningful genetic mapping, such that several of the genetic loci identified contained between 1-3 genes, comparable to human GWAS. These genes were prioritized using expression quantitative trait locus (eQTL) analysis and we were able to show a significant overlap between mouse and human GWAS loci. We measured the change in fat dynamically, at five different points following high-fat/high-sucrose (HF/HS) feeding, providing strong evidence for a genetically controlled body fat set-point. Our use of inbred mice strains also enabled detailed analysis of the relationship between gut microbiota composition, obesity traits, and diet. Overall, gene-by-diet interactions were highly reproducible and pervasive, providing a partial explanation for the failure of human studies to explain a larger fraction of the genetic basis of obesity. Our results indicate that mouse GWAS and systems genetics analyses provide a powerful method to complement human studies and to address factors, such as gene-by-diet interactions, that would be difficult to study directly in humans.

RESULTS

Robust Variation in Gene-by-Diet Interactions

To assess gene-by-diet interactions common to obesity, male mice were fed *ad libitum* a HF/HS diet that represents a typical fast food diet, in terms of fat and refined carbohydrates (32% kcal from fat and 25% kcal from sucrose). Mice were maintained on a chow diet (6% kcal from fat) until 8 weeks of age and subsequently placed on a HF/HS diet for 8 weeks. Body fat percentage was assessed by magnetic resonance imaging (MRI) every two weeks and food intake was monitored for a period of one week at the middle of the study timeline (study schematic shown in Figure 1A). Altogether, about 100 inbred strains of male mice were studied, with an average of 6 mice of each strain (Table S1).

A wide distribution in body fat percentage was observed in male mice before HF/HS feeding (Figures 1B). Dietary responses, as assessed by the body fat percentage increase during HF/HS feeding, varied widely among the strains (Figures 1C and S1). Although many strains exhibited a significant increase in body fat percentage throughout the study timeline, their individual responses differed significantly, from no change to a 200% increase in body fat percentage within the first two weeks (Figures 1C). Most strains responded during the first 4 weeks of HF/HS feeding and did not accumulate additional fat during the remainder of the study, suggesting an upper set-point whereby continued body fat percentage growth is resisted (Figure 1C) (Speakman et al., 2011). The large effect of the HF/HS feeding on fat accumulation was confirmed with age-matched (16 weeks old) male mice (Table S2) fed a chow diet, which displayed similar body fat percentage to male mice before the HF/HS diet intervention (Figure S1A). Additionally, comparison of individual male strains maintained on a chow diet or fed a HF/HS diet for 8 weeks showed an average increase in body fat percentage from 0 to more than 600% (Figure S1B).

We observed high heritability of about 80% for body fat percentage across the study timeline (Table 1). Changes in body fat percentage after HF/HS feeding were also highly heritable (>70%), suggesting that dietary responses are strongly controlled by genetics. Our

results are consistent with the heritability estimates for body mass index (BMI) and obesity in humans (Barsh et al., 2000; Stunkard et al., 1986) and emphasize the importance of genetics in controlling obesity traits, such as gene-by-diet interactions.

Other Factors Contributing to Dietary Responsiveness

Over-consumption of high-calorie, energy-rich foods is a key environmental factor contributing to the global obesity epidemic (McCaffery et al., 2012). To understand the relationship between food intake and obesity, food intake was monitored and found to range from 2-5 grams per mouse per day. Total food intake per day was significantly correlated with body weight and lean mass (Figures 1D and E). In contrast, body fat percentage and body fat percentage change after 4 weeks of HF/HS feeding showed little to no correlation with food intake (Figures 1F and G). This suggests that factors outside of food intake largely underlie the variation of obesity and fat mass gain between the strains in response to HF/HS feeding.

To further define the contribution of energy consumption to the differences in fat accumulation, we performed *in vivo* metabolic chamber analyses of five inbred strains that are the progenitors of the recombinant inbred strains (and therefore contribute importantly to the overall genetic component) on chow diet. Significant strain differences were observed in total food intake, activity, heat production, and utilization of different energy substrates, as indicated by respiratory exchange ratio (RER) (Figures S2A, B, C, and D), all of which can influence dietary responses and subsequent fat accumulation.

GWAS and Systems Genetics Analysis

Association analysis was performed using about 100,000 informative SNPs, spaced throughout the genome, with efficient mixed model association (EMMA) adjusting for population structure (Kang et al., 2008). The threshold for genome-wide significance was based on simulation and permutations, as previously described (Farber et al., 2011). This approach has been validated using transgenic analyses and by comparison with linkage analysis (Bennett et al., 2010). Altogether, 11 genome-wide significant loci were found to be associated with obesity traits (Table 2). Loci averaged 500 kb to 2 Mb in size and in most cases contained 1 to 20 genes within a linkage disequilibrium (LD) block, an improvement of more than an order of magnitude as compared to traditional linkage analysis in mice which has a resolution of 10 to 20 Mb (Flint et al., 2005).

In order to help identify candidate genes at loci, global expression analyses of epididymal adipose tissue in male mice (16 weeks old) fed a chow diet were carried out to determine genetic regulation and correlation between gene expression and body fat percentage. The loci controlling transcript levels in adipose tissue were mapped using EMMA and are referred to as expression Quantitative Trait Loci (eQTL). Loci are termed "*cis*" if the locus maps within 1 Mb of the gene encoding the transcript and "*trans*" if the locus is outside 1 Mb. Overall, 3,960 *cis* and 4,496 *trans* eQTL were identified to have a genome-wide significance threshold (*cis* threshold: $p < 1.4 \times 10^{-3}$ and *trans* threshold: $p < 6.1 \times 10^{-6}$) (Figure S3A). *Cis* regulation indicates a potential functional genomic variation within or near a gene that significantly influences gene expression of a given gene. For example, *Fto*, the most widely replicated gene in human GWAS for obesity shows a strong *cis* eQTL in the adipose tissue of mice (Figure S5D), indicating genetic variation of this gene.

Global gene expression in epididymal adipose tissue was correlated with body fat percentage in chow fed mice (top 50 genes shown in Table S3). Many genes known to play a vital role in adipose biology showed significant correlations with body fat percentage. Leptin is a key adipose-derived hormone correlating with adipose tissue mass (Ioffe et al.,

1998) and is strongly correlated ($r = 0.75$; $p < 2.2 \times 10^{-16}$) with body fat percentage (Figure 2A). Other important adipose tissue genes, such as *Sfrp5* (Ouchi et al., 2010) ($r = 0.76$; $p < 2.2 \times 10^{-16}$), *Chrebp* (Herman et al., 2012) ($r = -0.57$; $p = 2.28 \times 10^{-9}$) and *Tmem160* ($r = 0.71$; $p = 4.21 \times 10^{-16}$), a recently identified gene from a human GWAS for body mass index (BMI) (Speliotes et al., 2010), were also found to be highly correlated with body fat percentage (Figure 2B, S3B and S3C).

Genetic Control of Obesity and Dietary Responsiveness

Most strains in the study showed a striking increase in body fat percentage within the first two weeks of HF/HS feeding (Figure 1C). Association analysis with body fat percent increase after 2 weeks of HF/HS feeding identified genome-wide significant loci on Chromosomes 2 and 6 (Table 2 and Figure S4A). The chromosome 2 locus (rs13476804; $p = 2.95 \times 10^{-6}$) contains one gene within the LD block, *Sptlc3*, which has been implicated in biogenesis of sphingolipids (Demirkan et al., 2012; Hornemann et al., 2009). The locus on chromosome 6 contains 11 genes within LD and the peak single nucleotide polymorphism (SNP), (rs13478690; $p = 2.8 \times 10^{-7}$) is 33.5kb upstream of *Klf14*, a primary candidate causal gene at this locus (Table 2 and Figure S4B). *Klf14* has previously been identified in human GWAS for type 2 diabetes (Voight et al., 2010) and has recently been shown to be a master regulator of gene expression in adipose tissue (Small et al., 2011). Our results support a role of *Klf14* in regulating changes in adipose tissue and indicate that *Klf14* may also regulate dietary interactions.

Eight genome-wide significant loci were associated with body fat percentage growth after 8 weeks of HF/HS feeding (Table 2 and Figure 2C). The most significant signal (rs31849980; $p = 1.4 \times 10^{-8}$) maps to chromosome 1 and has genome-wide significant SNPs spanning a 5 Mb region with 60 genes within LD (Table 2). A primary candidate gene within this locus is *Degs1*, a fatty acid desaturase involved in the metabolism of important bioactive sphingolipids (Ternes et al., 2002). *Degs1* expression in adipose tissue of chow fed male mice is strongly correlated (within top 10 genes) with body fat percentage ($r = 0.7$; $p = 1.3 \times 10^{-15}$) (Figure 3E). Previous linkage studies in mice have identified distal chromosome 1 as contributing importantly to obesity (Chen et al., 2008) and our results greatly refine this region and suggest *Degs1* as a high-confidence candidate gene in the locus, although given the size of the locus multiple genes may be contributing to the signal.

Of the eight loci associated with body fat percent growth after 8 weeks of HF/HS feeding both loci on chromosomes 16 and 18 contained genes with genome-wide *cis* eQTL and strong expression correlation with body fat percentage in epididymal adipose tissue (Figure 3C). The peak SNP at chromosome 18 (rs30078681; $p = 4.3 \times 10^{-8}$) contained 26 genes within LD and one gene, *Npc1* was previously identified in a human GWAS for obesity (Meyre et al., 2009). Gene expression analysis of *Npc1* indicated a strong negative correlation with body fat percentage ($r = -0.4$; $p = 1.5 \times 10^{-5}$) (Figure S3D) and the presence of a genome-wide significant *cis* eQTL (Figure S5A). Genomic sequence analysis indicated multiple non-synonymous coding variations within the *Npc1* gene (Keane et al., 2011). Furthermore, *Npc1* heterozygous knockout mice exhibit increased obesity on a high fat diet, but not on a chow diet (Jelinek et al., 2010), confirming the importance of *Npc1* in regulating dietary interactions. The chromosome 16 locus (rs3148854; $p = 9.0 \times 10^{-8}$) has 8 genes within LD of the genome-wide significant SNPs and one gene in this region, *Cbr1* (Figure 3D) is highly expressed in adipose tissue and is negatively correlated ($r = -0.5$; $p = 1.6 \times 10^{-7}$) with obesity (Figure 2F). Furthermore, *Cbr1* has a *cis* eQTL ($p = 1.1 \times 10^{-4}$) (Figure 5B) and contains multiple non-synonymous coding variants (Keane et al., 2011). *Cbr1* reduces carbonyl compounds via NADPH-dependent oxidoreductase activity and through its glutathione (GSH)-binding site *Cbr1* can act on GSH-conjugated molecules and

play a role in controlling oxidative stress (Bateman et al., 2008), an important component of obesity (Furukawa et al., 2004).

The loci for body fat growth following 8 weeks of HF/HS feeding also contained genes with known links to adipose biology and metabolism. The locus on chromosome 5 (rs13478388; $p = 1.5 \times 10^{-7}$) contains several C-X-C motif chemokine genes within LD. *Cxcl5* is one of the genes in this cluster (Figure S4C), and expression of *Cxcl5* is increased in adipose tissue of obese human subjects (Chavey et al., 2009). *Cxcl5* contains non-synonymous coding polymorphisms and an alternative splice site among multiple mouse strains (Keane et al., 2011), making *Cxcl5* a strong candidate at the chromosome 5 locus. Similarly, the significant SNP at chromosome 3 (rs29982345; $p = 9.9 \times 10^{-7}$) is within 1 Mb of three amylase genes, *Amy2b, Amy2a5*, and *Amy1* (Figure S4D). Amylases play a critical role in the breakdown of polysaccharides to sugars and targeting amylases has been suggested as a potential method for treating obesity (Barrett and Udani, 2011). The significant peak on chromosome 11 (rs29417268; $p = 2.8 \times 10^{-7}$) has 11 genes within LD and one gene, *Glp2r*, plays an important role in intestinal homeostasis and feeding behavior (Guan et al., 2012). We also observed genome-wide significant loci for dietary response at two positions on chromosome 18 (rs13483184; $p = 2.6 \times 10^{-07}$ and rs29628302; $p = 2.6 \times 10^{-7}$) with no obvious candidate genes (Table 2).

In addition to loci influencing dietary response, there was one genome-wide significant locus at chromosome 7 associated with body fat percentage after 8 weeks of HF/HS feeding. This locus was gene-rich and contained 113 genes within LD of the peak SNP (rs13479513; $p = 6.7 \times 10^{-7}$). Two genes at this locus, *Atp2a1* and *Apob48r* have previously been identified in human GWAS for BMI (Speliotes et al., 2010; Thorleifsson et al., 2009). Furthermore, this locus contains *Sephs2*, a strong candidate gene with both a significant *cis* eQTL ($p = 1.4 \times 10^{-4}$) and an exceptionally strong negative correlation with body fat percentage ($r = 0.68$; $p = 2.0 \times 10^{-14}$) (Figures S3E and S5C). *Sephs2* is an evolutionarily conserved protein that is essential for production of selenocysteine (Xu et al., 2007), a key amino acid found in a diverse group of proteins with roles in the pathology of metabolic syndrome and diabetes (Rayman, 2012).

Conservation of Human and Mouse Metabolic Loci

A comparison of the genes/loci identified in our study with GWAS genes for human obesity and BMI revealed significant overlap ($p = 4.0 \times 10^{-4}$) (described in Experimental Procedures). In addition to genome-wide significant loci, we analyzed significant human GWAS genes in our association results and identified suggestive associations near *Opcml* and *Sorl1* (Table 3). Both *Opcml* and *Sorl1* have been found in large human GWAS for visceral adipose/subcutaneous adipose ratio and waist circumference, respectively (Fox et al., 2012; Smith et al., 2010). Our results demonstrate that genes associated with obesity and BMI in humans show functional variation in mice and are associated with dietary interactions and obesity.

Contributions of Gut Microbiota to Obesity

A number of recent studies have implicated gut microbiota in metabolic and cardiovascular diseases (Turnbaugh et al., 2006; Vijay-Kumar et al., 2010; Wang et al., 2011). In order to investigate the genetic contributions to the gut microbiome and their relationship to obesity and dietary responses we examined the gut microbiota composition and distribution in strains after chow and HF/HS feeding. Since the microbiome is highly affected by environmental factors, all mice in the study were bred in the same facility and each strain was maintained in separate cages. We observed significant phylum-level shifts in the gut microbiota composition after HF/HS feeding (Figure 3A). The shifts between the two

dominant phyla *Bacteroidetes* and *Firmicutes* after HF/HS feeding (Figure 3A and Table S4), are consistent with previous reports (Turnbaugh et al., 2009b). Principal Coordinates Analysis (PCoA) of unweighted UniFrac (similar results for weighted UniFrac shown in Figure S6B) (Lozupone and Knight, 2005) distances detected two clear clusters corresponding to the two dietary conditions (Figure 3B), suggesting that HF/HS feeding dramatically alters gut microbial communities across a variety of genetic backgrounds. Compared to mice on a chow diet the HF/HS fed mice had greater abundance of several genera classified to order *Clostridales* in Phyla *Firmicutes* and lower abundances of *Bacteroidetes*, classified to family *Porphyromonadaceae* (41% chow vs 18% HF/HS, $p=4.36\times10^{-11}$). In total we identified 17 genera whose abundance was significantly changed by diet ($p<0.01$) (Figure 3C, and Table S4). Interestingly, the abundance of *Akkermansia* genera of the *Verrucomicrobia* phylum explains much of the unweighted UniFrac distance clusters on PCoA plots in HF/HS diet (Figures S6C and S6D).

Robust effects of diet on the gut microbiota are well documented and recent evidence indicates that the gut microbiota constitute a complex polygenic trait under genetic regulation (Benson et al., 2010; McKnite et al., 2012; Qin et al., 2012). Consistent with the idea that the gut microbiota is influenced by genetic factors, we observed a strong effect of genetic background on the composition and plasticity of the gut microbiota after HF/HS feeding. Some strains showed large shifts in all major phyla, while other strains had little fluctuation in their gut microbiota after HF/HS feeding (Figure 4A). Additionally, Procrustes analysis was used to compare the relative orientation of matched strains after HF/HS and chow feeding and confirmed that the plasticity of gut microbial community is highly individualized (Figure 4B). Taken together, these results demonstrate a profound effect of HF/HS feeding on gut microbiota and show the strong influences of host genetics on influencing the plasticity of the gut microbiota in response to altered dietary compositions.

To test the relationship between obesity, dietary responsiveness, and gut microbiota composition we analyzed all 11 genome-wide significant loci with the abundances of specific gut phylotypes. Only the chromosome 3 locus (rs29982345), which is associated with body fat growth after 8 weeks of HF/HS feeding showed a significant enrichment ($p = 0.0033$) of the genera *Enterobacteriaceae* (phylum *Proteobacteria*). This region of chromosome 3, which contains three amylase genes, may therefore contribute to both dietary responsiveness and gut microbiota composition. We also examined the relationship between specific gut phylotypes and obesity traits and observed a modest but statistically significant negative correlation with the abundance of the genera *Akkermansia* (phylum *Verrucomicrobia*) and body fat percentage growth after 8 weeks of HF/HS feeding (Figure 4C). Furthermore, body fat percentage growth after HF/HS feeding was positively correlated with the relative abundances of *Lactococcus* from phylum *Firmicutes* (Figure 4D) and with the genera *Allobaculum* (phylum *Bacteroidetes*) (Figure 4E). Additional studies are warranted to validate the connections of these specific gut microbiota and dietary interactions.

DISCUSSION

Our results have led to several findings about the genetic control of responses to energy-rich diets consumed by an increasing fraction of the world's population. Although biochemical and genetic studies have revealed important regulators of body fat, including feedback pathways that modulate food intake and energy expenditure, these studies have not revealed how common genetic variation controls these processes. Human GWAS studies have been successful at identifying many loci controlling measures such as body mass index, but they have been unable to examine gene-by-environment interactions. Classic linkage studies in mice, on the other hand, have been able to partially address issues such as dietary

responsiveness (Ehrich et al., 2005b), but have been hindered by poor mapping resolution, and, thus, only a handful of genes have been identified (Buchner et al., 2012; Dokmanovic-Chouinard et al., 2008; Ehrich et al., 2005a; York et al., 1999; Zhang et al., 1994). Our results add to this body of knowledge in several important ways. First, the improved mapping resolution enabled us to identify several candidate genes for gene-by-environment interactions in obesity. Second, because we examined obesity traits in a dynamic way, studying the mice following dietary challenge at multiple time points, we were able to provide strong evidence for the concept of a biologic set-point. Third, we have integrated the obesity traits with global adipose transcript levels and gut microbiota composition. Fourth, our results emphasize the importance of gene-by-environment interactions, with important implications for an understanding of the overall genetic architecture of obesity. Finally, our results provide the basis of a systems genetics resource for obesity traits that can be expanded to include multiple biologic scales using metabalomics, proteomics, and epigenetics. In particular it will be of interest to examine behavioral and neurological differences among the strains as they relate to obesity traits.

Using association rather than linkage, we were able to obtain biologically significant mapping data, as several of the loci identified for the response to a HF/HS diet contained between 1-3 genes, comparable to human GWAS results. Altogether, we examined only about 800 mice, much fewer than the tens of thousands examined in human GWAS. It is likely that power is greatly increased in mice, as compared to humans, because of the ability to accurately monitor the phenotypes (body fat, food intake) and control the environment. Also, for the loci containing larger numbers of candidates, examination of transcript levels in adipose tissue and access to complete DNA sequence information, allowed us to prioritize candidates. Indeed, several of the genes at such loci (*Npc1* and *Glp2r*) have already been studied using transgenic approaches with results consistent with our findings (Guan et al., 2012; Jelinek et al., 2010).

Several different models have been developed to understand how genes and environment combine to regulate body fat. However, only limited studies have addressed the genetic basis of gene-by-environment interactions in obesity. The dual intervention point model postulates an upper and lower limit where genetic factors become dominant, and between which there is only weak genetic regulation (Speakman et al., 2011). Our results strongly support this concept, because after 4 weeks of HF/HS feeding most mouse strains reach an upper limit where there is no further increase in body fat (Figure 1). Thus, there must be strong genetic factors that resist continued fat accumulation beyond a certain point. Clearly, this set-point varies widely in the population; some strains have already achieved the set-point level on a chow diet whereas a few strains continue to accumulate fat throughout the 8 week feeding period, suggesting that the set-point mechanism is disrupted. It is noteworthy that obesity prevalence appears to be leveling off in developed countries, which is also consistent with the idea of a set-point (Flegal et al., 2012). Based on our data (Table 1) and a monozygotic twin study (Bouchard et al., 1990), acute body fat changes in response to an obesogenic environment are under strong genetic regulation, although this may be part of the same pathway that determines the set-point. Our data also show that food intake is only modestly correlated with obesity and dietary responsiveness, and indicates energy expenditure may be a likely mechanism contributing to the obesity set-point. Further metabolic studies of strains at the extremes will be required to confirm the effect of energy expenditure on obesity set-points.

The experimental design of our study, involving inbred strains of mice, allowed us to study the gut microbiota composition of the mice both on a chow and HF/HS diet. The HF/HS diet clearly influenced gut microbiota composition in all of the 52 strains examined on both chow and the HF/HS diet (Figure 3). Shifts in gut microbiota composition and diversity in

response to dietary changes are similar to those previously noted in both human (Ley et al., 2006; Turnbaugh et al., 2009b) and mouse studies (Ley et al., 2005; Turnbaugh et al., 2006). Our data indicate that host genetic factors influence gut microbiota plasticity in response to diet; however, since the gut microbiota can vary significantly even in well-controlled cohorts we cannot exclude the possibility that other factors could partly explain the observed differences. We observed significant relationships between gut microbiota and metabolic traits (Figure 4C, 4D, 4E). The results are consistent with the concept that gut microbiota contribute to systemic functions and common diseases (Clemente et al., 2012). Future studies in the HMDP have the potential to test the roles of specific gut microbiota using transplantation and cross-fostering to systematically examine the relationship between genetic background, diet, and gut microbiota composition.

Recent successes in human GWAS for common diseases have yielded many genetic loci, but in most instances they have revealed only a very small fraction of the estimated heritability (Lander, 2011). While it is likely that some of the "missing heritability" will be found in rare variants in the population, our results, and those from other studies in animal models (Burrage et al., 2010; Lawson et al., 2011; Shao et al., 2008) suggest that genetic interactions are likely to have important contributions. Clearly, human studies have limited ability to identify gene-by-environment or gene-by-gene interactions (Zuk et al., 2012), and our results suggest that GWAS in mice will provide a powerful complement to human studies. A key feature of our experimental design is that genetically identical mice can be studied under multiple environmental conditions. Also, the greatly improved resolution of association as compared to classical linkage studies in mice greatly facilitates gene identification and mechanistic understanding. The substantial overlap of our study with human GWAS for obesity traits suggests that at least for metabolic disorders, the mouse is highly relevant and that natural variations of the two species affect shared pathways.

Because the inbred strains of the HMDP are permanent, data obtained about the strains is cumulative, allowing integration with previous results. The data compiled here provides a basis for further genetic studies of obesity-related traits in mice. It will be of interest, for example, to examine additional intermediate phenotypes, such as protein and metabolite levels, in the same set of strains, and to relate these to the obesity traits using association mapping, correlation, and modeling. In this study, we examined transcript levels in adipose tissue, and the composition of gut microbiota and in the future it would be valuable to examine transcript levels in other relevant tissues, such as the hypothalamus, liver, and muscle, as well as protein, metabolite levels, and epigenetic markers. Moreover, traits such as epigenetic variation and gut microbiota composition can be tested for effects on obesity-related traits in this set of strains.

EXPERIMENTAL PROCEDURES

Animals

All mice were obtained from The Jackson Laboratory and were bred at University of California, Los Angeles to generate mice used in this study. Mice were maintained on a chow diet (Ralston Purina Company) until 8 weeks of age when they were given a high-fat, high-sucrose diet (Research Diets-D12266B) with the following composition, 16.8 % kcal protein, 51.4 % kcal carbohydrate, 31.8 % kcal fat. Age-matched control male mice were fed a chow diet (18% kcal fat) for 16 weeks. A complete list of the strains included in our study is included in Supplemental Tables 1 and 2. The animal protocol for the study was approved by the Institutional Care and Use Committee (IACUC) at University of California, Los Angeles.

Association Analysis and Heritability Calculations

We performed the association testing of each SNP using a linear mixed model, which accounts for the population structure among the n animals using the following model (Kang et al., 2008):

$$y = 1_n\mu + x\beta + u + e$$

,where μ is the mean, β is the allele effect of the SNP, x is the (nx1) vector of observed genotypes of the SNP, u is the random effects due to genetic relatedness with $var(u) = \sigma_u^2 K$ and e is the random noise with $var(e) = \sigma_e^2 I$. K denotes the identity-by-state (IBS) kinship matrix estimated from all the SNPs, I denotes the (nxn) identity matrix and 1_n is the (nx1) vector of ones. We estimated σ_u^2 and σ_e^2 using restricted maximum likelihood (REML), and computed p-values using the standard F-test to test the null hypothesis $\beta = 0$. Genome-wide significance threshold and genome-wide association mapping are determined as the family-wise error rate as the probability of observing one or more false positives across all SNPs for phenotype. We ran 100 different sets of permutation tests and parametric boot strapping of size 1,000, and observed that the mean and standard error of the genome-wide significance threshold at the family-wise error rate of 0.05 were $3.9 \times 10^{-6} \pm 0.3 \times 10^{-6}$, and $4.0 \times 10^{-6} \pm 0.3 \times 10^{-6}$, respectively. A detailed explanation of the analyses is provided in Bennett *et. al.* (Bennett et al., 2010). Linkage disequilibrium (LD) was determined by calculated pairwise r^2 SNP correlations for each chromosome. Approximate LD boundaries were determined by visualizing $r^2 > 0.8$ correlations in MATLAB (MathWorks)

Heritability (in the narrow-sense) is defined as the phenotypic variance explained by additive genetic effects and is computed using the following model (Yang et al., 2010):

$$y = 1_n\mu + u + e$$

We estimated σ_u^2 and σ_u^2 using REML and calculated the heritability (h^2) for each trait as follows:

$$h^2 = \frac{\text{var}(u)}{\text{var}(u) + \text{var}(e)} = \frac{\sigma_u^2 tr(PK)}{\sigma_u^2 tr(PK) + \sigma_e^2}$$

where tr(.) is the matrix trace and $P = \left(I - \frac{1}{n} 1_n 1_n^T\right)$.

Body Composition Analysis

Animals were measured for total body fat mass and lean mass by magnetic resonance imaging (MRI) using Bruker Minispec with software from Eco Medical Systems, Houston, TX (Taicher et al., 2003). All animals in the study were measured at 0, 2, 4, 6, and 8 weeks of HF/HS feeding.

Assessment of food intake

After 4 weeks of HF/HS feeding, food intake was monitored for 5 days. Ninety to 100 grams of food was weighed and put in the food hopper, after 24 hours food was weighed, and then weighed again after 24 hours. The procedure was repeated again with fresh food, and a total of 4 × 24 hour period total food weight consumed was calculated by subtracting food

weights; grams per mouse was then calculated by dividing average total food weight consumed in 4 × 24 hour periods by number of mice in cage.

In vivo metabolism assessment

Metabolic rate, activity, food and water consumption were assessed using a Columbus Instruments Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH) equipped with sub-systems for open circuit indirect calorimetry, and monitoring of activity, feeding, and drinking. Detailed methods for in vivo metabolism assessment provided in Supplementary Experimental Procedures.

Gut Microbiota Analysis

Microbial community composition was assessed by pyrosequencing 16S rRNA genes derived from the cecal samples of chow diet and HF/HS diet animals. All mice used in the study were bred at UCLA to ensure uniform environmental conditions. Furthermore, strains were individually housed within the same vivarium throughout the duration of the study. To ensure comprehensive analysis we sequenced biological replicates on all mice included in the study. Biological replicates were carried out on all mice included in the study. For mice fed a HF/HS diet we sequenced a range of 2 to 5 mice per strain, with an average of 3 mice per strain (n=339 male mice from 110 strains). Chow diet mice were sequenced by pooling 2 to 4 mice per strain, with an average of 2 per strain (n=108 male mice from 52 strains). We also sequenced technical replicates on 8 mouse strains (5 strains on chow diet and 3 on HF/HS diet) and observed high reproducibility of UniFrac analysis between technical replicates (Figure S6A). Detailed methods for gut microbiota analysis provided in Supplementary Experimental Procedures.

Adipose RNA isolation and global gene expression analysis

Flash frozen epididymal adipose samples from male mice (16 weeks old) maintained on a chow diet were weighed and homogenized in Qiazol (Qiagen) and RNA isolated according to the manufacturer's protocol using RNeasy columns (Qiagen). Isolated RNA (2 mice per strain as indicated in Supplementary Table 2) was analyzed for global gene expression using Affymetrix HT_MG430A arrays and was filtered as described (Bennett et al., 2010).

Expression eQTL analysis

GWAS for gene expression was performed using EMMA and defined as *cis* if peak SNP mapped within 1 Mb of gene position and *trans* if mapped outside (*cis* threshold: $p < 1.4 \times 10^{-3}$ and *trans* threshold: $p < 6.1 \times 10^{-6}$).

Statistics

Correlations were calculated using the biweight midcorrelation, which is robust to outliers (Wilcox, 2005). Overlap p-value between mouse and human GWAS genes was calculating using hypergeometric cumulative distribution function in MATLAB (MathWorks) using pval = 1 - hygecdf(*x,M,K,N*), where x = total number of genes in genome, M = total number of genes identified in mouse and humans, K = number of genes identified in obesity and BMI human GWAS, N = number of genes identified in mouse obesity traits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Detailed analysis of diet-induced obesity in more than 100 inbred mouse strains
- Identification of 11 genetic loci associated with obesity and dietary responsiveness
- Significant overlap between mouse loci with human GWAS loci for obesity
- Strain-specific shifts in gut microbiota composition in response to dietary intervention

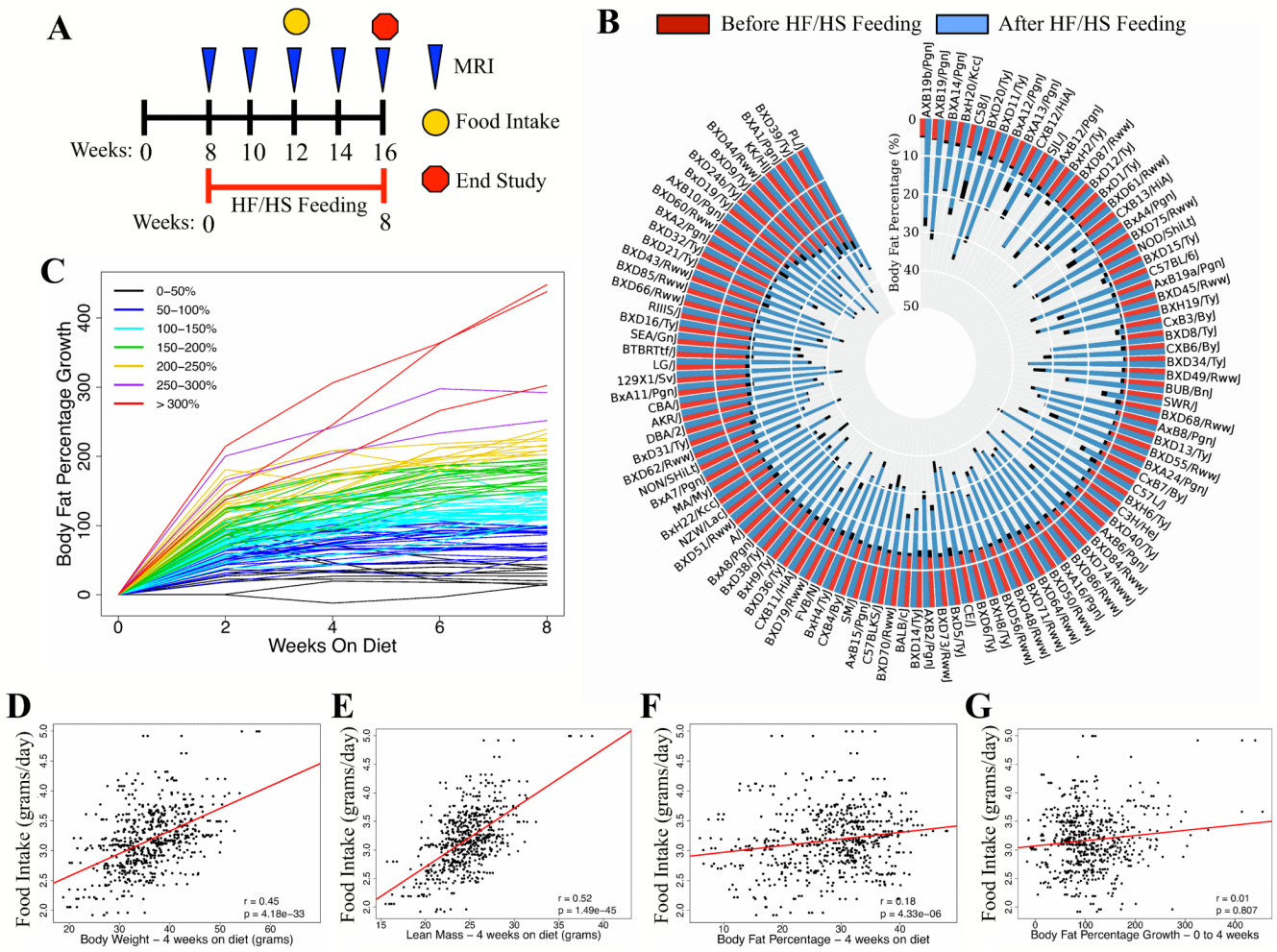

Figure 1. Natural Variation in Gene-by-Diet Interactions

(A) Schematic of study design with indicated time points for HF/HS feeding (red), MRI (blue), food intake monitoring (yellow), and end of study (red). MRI, magnetic resonance imaging.

(B) Body fat percentage in male mice (108 strains) before (red) and after (blue) 8 weeks of HF/HS feeding. Error bars (black) represent standard error of the mean (SEM)

(C) Bi-weekly percent body fat percentage increase in male mice with indicated body fat percentage increase after 8 weeks of HF/HS feeding.

(D-G) Correlation of food intake (grams/day/mouse) with body weight (D), lean mass (E), body fat percentage – 4 weeks on HF/HS diet (F), and body fat percentage growth – 0 to 4 weeks (G), regression line (red). r, biweight midcorrelation, p, p-value.

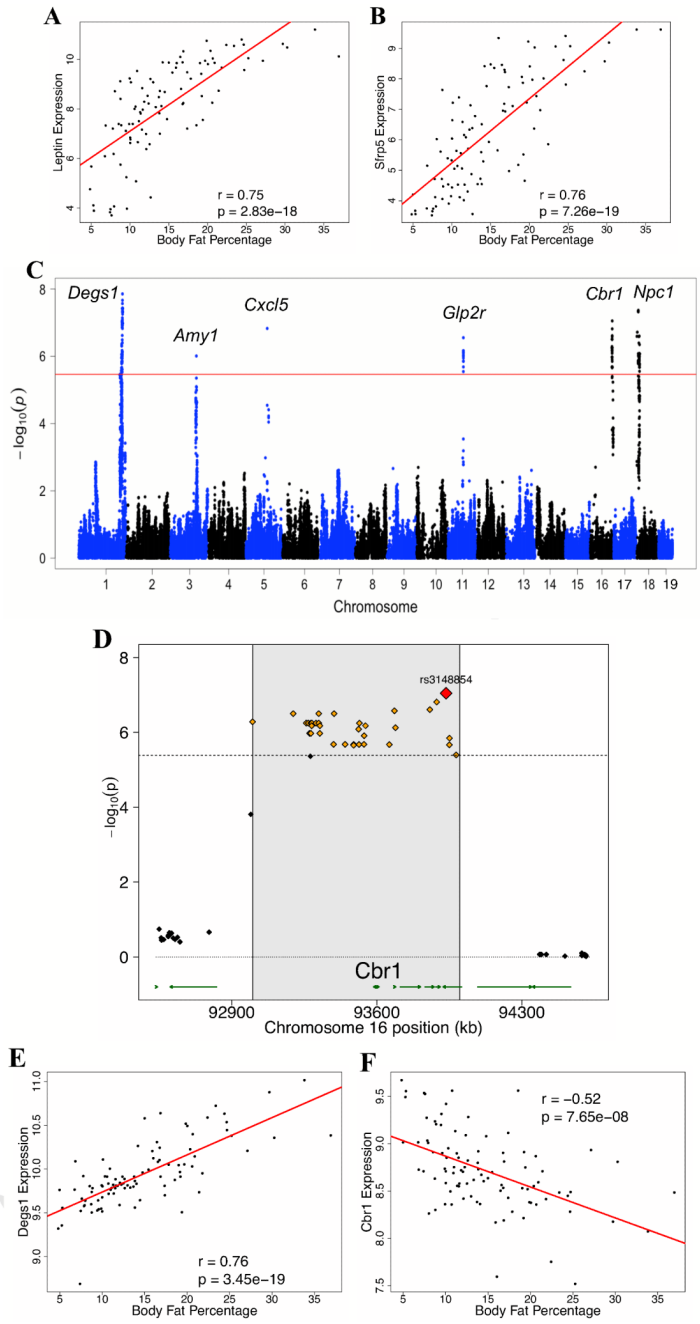

Figure 2. Genetic Control of Dietary Responses to HF/HS Feeding

(A-B) Correlation of epididymal adipose gene expression of Leptin (*Lep*) (A) and *Sfrp5* (B) with body fat percentage in male HMDP mice fed a chow diet, regression line (red). r, biweight midcorrelation, p, p-value.

(C) Manhattan plot showing the significance ($-\log_{10}$ of p) of all SNPs and percent body fat percentage increase after 8 weeks of HF/HS feeding in male HMDP mice. Candidate genes for genome-wide significant loci are indicated above genome-wide significant loci. Genome-wide significant threshold (red) of $p=4.1 \times 10^{-06}$ is indicated.

(D) Locus plot for genome-wide significant association at chromosome 16qC4 with approximate LD block (shaded in grey) and genome-wide significant SNPs (yellow) with the peak SNP (red).
(E-F) Correlation of epididymal adipose gene expression of *Degs1* (E) and *Cbr1* (F) with body fat percentage in male HMDP mice fed a chow diet, regression line (red). r, biweight midcorrelation, p, p-value.

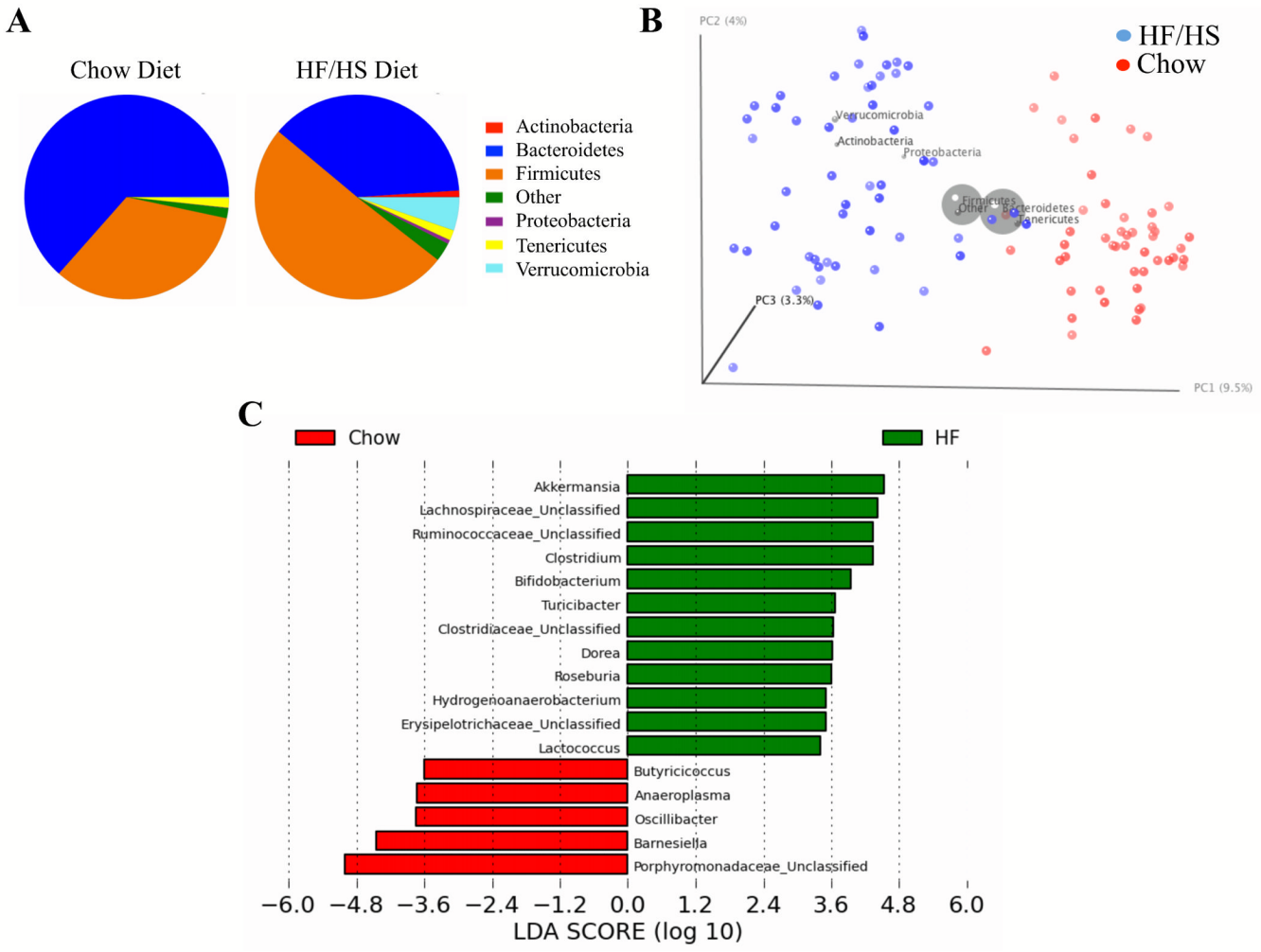

Figure 3. Robust Shifts in Gut Microbiota Composition After HF/HS Feeding

(A) Relative abundances of the different phyla after chow diet and HF/HS feeding (average among 52 matched strains).

(B) Principal Coordinates Analysis (PCoA) plot of the unweighted UniFrac distances. Each circle representing a different mice strain is colored according to the dietary conditions. PC1, PC2 and PC3 values for each mouse sample are plotted; percent variation explained by each PC is shown in parentheses.

(C) Linear Discriminant Analysis (LDA) coupled with effect size measurements identifies the most differentially abundant taxons between chow and HF/HS diets. HF/HS diet enriched taxa are indicated with a positive LDA score (green), and taxa enriched in normal chow diet have a negative score (red). Only taxa meeting an LDA significant threshold >2 are shown.

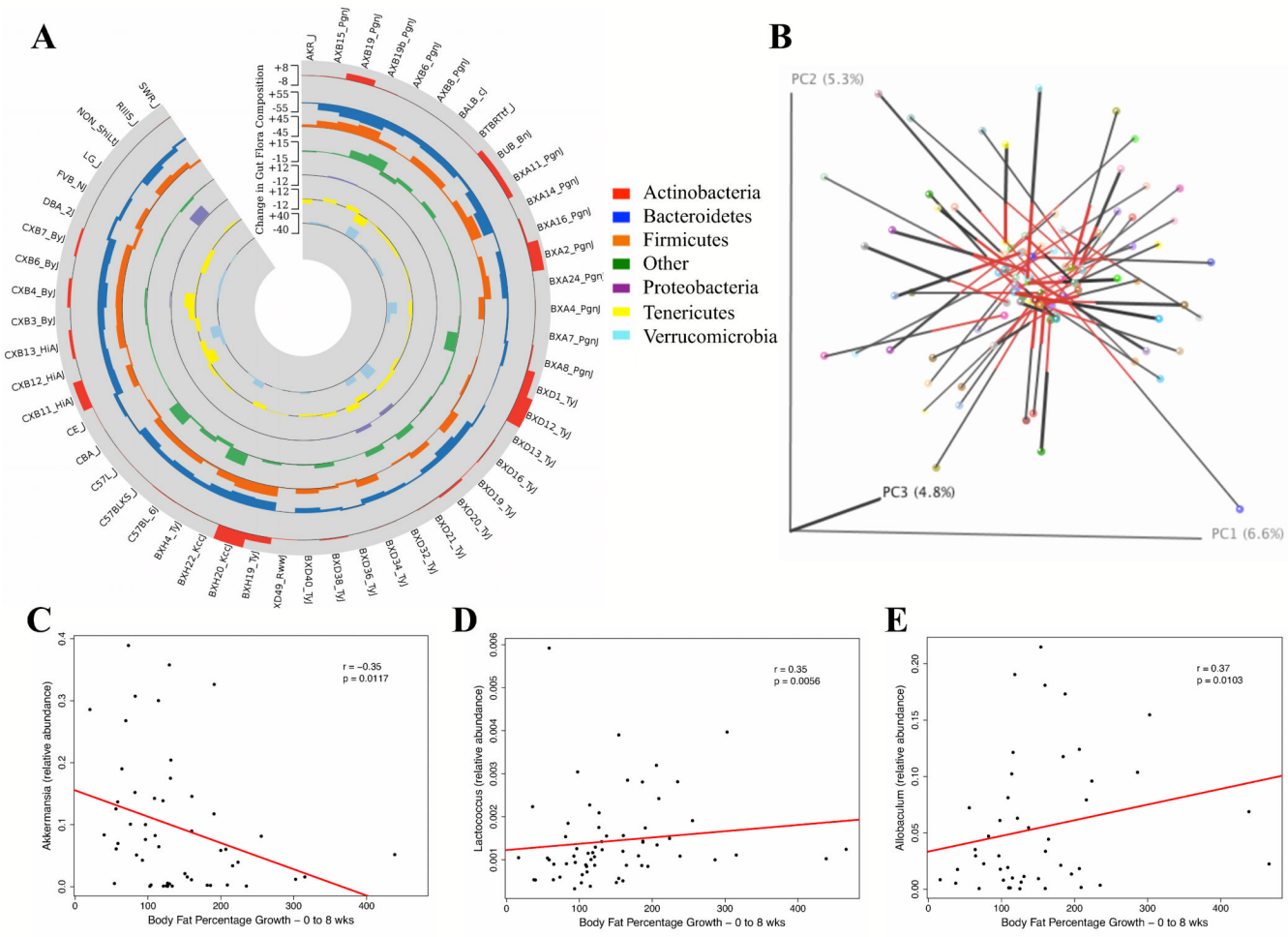

Figure 4. Plasticity of Gut Microbiota is Strain-Specific

(A) Gut Microbiota phyla shift in 52 strains after HF/HS feeding for 8 weeks indicated by shift in percent composition of indicated phylum.

(B) Procrustes analysis of the same strains on chow diet and the HF/HS diet are linked with a bar. The unweighted UniFrac distances between the diets varies across strains, but are in general long, highlighting the shifts in microbial composition in response to diet.

(C-E) Correlation of relative abundance of Akkermansia (A), Lactococcus (B), and Allobaculum (C) with body fat percentage increase from 0 to 8 weeks in male mice fed a HF/HS diet, regression line (red), r, biweight midcorrelation, p, p-value.

Table 1
Heritability Estimates for Obesity and Dietary Responsiveness

Trait	Heritability (%)
Body Fat Percentage – 0 weeks on HF/HS diet	80
Body Fat Percentage – 2 weeks on HF/HS diet	82
Body Fat Percentage – 4 weeks on HF/HS diet	83
Body Fat Percentage – 6 weeks on HF/HS diet	83
Body Fat Percentage – 8 weeks on HF/HS diet	85
Body Fat Percentage growth - 0 to 2 weeks	
Body Fat Percentage growth - 0 to 4 weeks	63
Body Fat Percentage growth - 0 to 6 weeks	67
Body Fat Percentage growth - 0 to 8 weeks	73

Body fat percentage growth calculated by quantifying the percentage increase of body fat after beginning HF/HS diet. Heritability calculated as described in experimental procedures.

Table 2

Genome-wide Significant Loci For Obesity and Dietary Responsiveness

Trait	Chromosome	Peak SNP	Position (Mb)	P-value	MAF	LD (Mb)	No. of Genes
Body Fat % increase – 0 to 8 wks	1	rs31849980	183730026	1.4E-08	15	178.3-184.5	60
Body Fat % increase – 0 to 8 wks	18	rs30078681	9731125	4.3E-08	8	8.5-12.5	26
Body Fat % increase – 0 to 8 wks	16	rs3148854	93933923	9.0E-08	40	93.0-94.0	8
Body Fat % increase – 0 to 8 wks	5	rs13478388	91288973	1.5E-07	37	90.9-92.2	17
Body Fat % increase – 0 to 8 wks	18	rs29628302	5395236	1.9E-07	11	4.6-5.4	3
Body Fat % increase – 0 to 8 wks	18	rs13483184	3796540	2.6E-07	13	3.8-4.6	3
Body Fat % increase – 0 to 8 wks	11	rs29417268	67079767	2.8E-07	12	65.8-67.4	14
Body Fat % increase – 0 to 2 wks	6	rs13478690	30872499	2.8E-07	7	30.5-31.5	11
Body Fat % increase – 0 to 8 wks	3	rs29982345	111983084	9.9E-07	14	110.2-113.4	7
Body Fat % - 8 wks	7	rs13479513	134251677	6.7E-07	21	133.0-136.0	113
Body Fat % increase – 0 to 2 wks	2	rs13476804	139322068	2.95E-06	10	138.9-139.4	1

MAF, Minor Allele Frequency; LD, Linkage Disequilibrium

Indicated genome-wide significant loci for obesity traits with indicated location, position, P-value, MAF, LD, and number of genes with in LD block (described in experimental procedures)

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Table 3

Significant Overlap of Mouse and Human GWAS

Mouse Trait	Chromosome	Peak SNP	P-value	Candidate Gene	Corresponding Human GWAS Trait
Body Fat % increase – 0 to 8 weeks on HF/HS diet	18	rs30078681	4.33E-08	*Npc1*	Obesity
Body Fat % increase – 0 to 2 weeks on HF/HS diet	6	rs13478690	2.75E-07	*Klf14*	Type 2 Diabetes, HDL Cholesterol
Body Fat % - 8 weeks on HF/HS diet	7	rs13479513	6.7E-07	*Atp2a1*	BMI, Weight
Body Fat % - 8 weeks on HF/HS diet	7	rs13479513	6.7E-07	*Apob48r*	BMI
Body Fat % increase – 0 to 2 weeks on HF/HS diet	9	rs13459107	1.01E-05	*Opcml*	Visceral Adipose/Subcutaneous Adipose Ratio
Body Fat % - 0 weeks on HF/HS diet	9	rs30236502	4.90E-05	*Sorll*	Waist Circumference

Identified mouse genetic loci containing genes previously identified in human GWAS studies for obesity and related traits. The corresponding mouse trait, location, P-value, candidate gene, and human GWAS Trait is indicated.