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Multidimensional genome scans identify the combinations of genetic loci linked to diabetes-related phenotypes in mice

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Most quantitative trait loci (QTL) studies have focused on detecting the genetic effects of individual QTLs. This study thoroughly dissected the genetic components of type 2 diabetic mice, including a search for epistatic interactions and multi-locus additive effects that result in variation in diabetes-related phenotypes. F2 population was generated from BKS.Cg-*Lepr*^{db}+/+ *m* and DBA/2 intercross and separated into six subpopulations by sex and the *db*-dependent diabetes severity. Single-locus and pairwise genome scans first identified the QTLs in these F2 subpopulations, and next covariate-dependent scans confirmed their sex-, *db*- and sex-by-*db*-specific effects in the combined populations. Single-locus genome scans detected four QTLs (*QBIS1*, *QBIS2*, *QBIS3* and *QBIS4*) that presented their genetic effects beyond sex, but most QTLs showed their effects specifically in limited conditions. This highly conditional feature of the QTLs was accentuated in the pairwise analysis. The pairwise genome scans uncovered a total of 27 significantly interacting or additively acting pairs of loci, showing a better fit to explain the total phenotypic variation of the traits. These significant pairs affected the traits under constantly varying combinations of loci in a time series or in both sexes. In addition, pairwise analysis indicated the appropriate genetic background in constructing congenic strains to obtain the maximum power in the replication of phenotypes. Our study showed high degree of complexity in the genetics of type 2 diabetes in mice, and it suggested that a comprehensive understanding of the multi-locus effects was essential to disentangle the complex genetics of diabetes and obesity in humans.

INTRODUCTION

In complex diseases, such as diabetes and obesity, many genes of unknown function, combined with environmental influences, are hypothesized to control trait variation. To dissect genetic determinants in complex diseases, genetic analysis in rodents has been more effective than that in humans because of the minimized environmental variation and genetic heterogeneity in rodents. However, most studies in rodents have focused on detecting the genetic effects of individual QTLs, ignoring the possibility that these effects might be influenced by genetic background, either by individual loci or by the combination of other loci (1). This strategy has been successful for detecting the QTLs with large effects on quantitative traits and, in several instances, causal mutations for the QTLs have been identified in the coding as well as the regulatory regions of genes (2). However, in light of evident polygenic bases for diabetes and obesity, any analysis limited to treat each locus independently would be insufficient. It is important to establish whether and how the multiple loci contribute positively to the phenotype of interest. Similar opinion was expressed in the recent reviews (1,3), leading to the

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speculation that epistasis could be a factor that contributes to the failure to replicate the results of many human association studies and could be one cause of QTL effects that diminish or disappear if they are isolated on fixed genetic backgrounds in experimental organisms. Recently, simultaneous multi-locus genome scans have been proposed to better understand the complex genetics underlying common diseases (4–6). These approaches used a genetic model, including interaction terms, and allowed us to identify the significant locus pairs affecting the traits in two different modes, i.e. one interacting epistatically to affect the trait and the other affecting the trait in a multi-locus additive manner.

In this study, F2 population was generated from BKS.Cg-*Lepr*^{db}+/+*m* (BKS-*db*/−) and DBA/2 (D2) intercross and separated into six subpopulations by sex and the genotype of *Diabetes* (*db*) mutation. The *db* mutation is a result of a point mutation in the leptin receptor gene, *Lepr*, and the ligand, *leptin*, is a key weight control hormone. Therefore, the homozygous (*db*/*db*) progeny showed severe diabetes and obesity (7), whereas the heterozygous (*db*/−) and wild-type (−/−) progeny were nearly normal. Nevertheless, the severity of diabetes caused by the *db* mutation was markedly dependent on the genetic background (8), and wider distributions of phenotypic values were also found in our F2 *db*/*db* and *db*/− subpopulations. To dissect the diversity of the QTLs along with the *db*-dependent diabetes severity or sex, we further divided the F2 *db*/*db* and *db*/− subpopulations by sex and applied them to a simultaneous pairwise genome scan in addition to a conventional interval mapping (single-locus genome scan): the male *db*/*db* population ($n = 151$), the female *db*/*db* population ($n = 132$), the male *db*/− population ($n = 172$) and the female *db*/− population ($n = 179$) were analyzed individually and compared each other. However, to clarify the sex- or *db*-specific effects of the QTLs, it is not sufficient to analyze two sexes or *db* genotypes separately and interpret the differences. Because the smaller sample size reduces the power to detect the effects, it cannot be denied that the differences among subpopulations may be due to chance fluctuations in a setting where power to detect QTLs is moderate or low. We corrected the problem by incorporating sex and *db* genotype into genome scans as additive and interactive covariates. For these covariate-dependent scans, we used three combined populations: the combined population of *db*/*db* mice with sex ($n = 283$) and the combined population of *db*/− mice with sex ($n = 351$) were analyzed to determine the sex-specific effects of the QTLs. The whole combined population of F2 mice with sex and *db* genotype (without wild-types) ($n = 634$) was also analyzed to consider not only sex-specific effects but also *db*- and sex-by-*db*-specific effects of the QTLs. The process for detecting the QTLs and verifying their covariate-specific effect was illustrated in Supplementary Material, Fig. S1.

RESULTS

Characterization of F2 mice

To evaluate the distribution of diabetes-related phenotypes of F2 progeny generated from BKS-*db*/− and D2 intercross, a total of 634 F2 *db*/*db* and *db*/− mice were selected and partitioned by sex (Fig. 1).

Figure 2 illustrates the distribution of body weight, fat pad weight and blood glucose levels in the isogenic background strains and heterogeneous F2 mice. Compared with the wild-type [D2-−/− and BKS-−/− in Fig. 2 (white)] and the heterozygotes for *db* [BKS-*db*/− or F2-*db*/− in Fig. 2 (light gray)], the homozygotes for *db* [BKS-*db/db* or F2-*db/db* in Fig. 2 (dark gray)] exhibited much higher body weight, fat pad weight and fasting blood glucose levels (at 8 weeks of age and at 60 min in the glucose tolerance test, GTT). Again, compared with the background strains (BKS-*db*/− and BKS-*db/db*), F2 mice (F2-*db*/− and F2-*db/db*) showed an increase in body weight and fat pad weight, with a wide distribution of phenotypic values (Fig. 2A, B, E and F). This tendency was found in fasting blood glucose levels (at 8 weeks of age and at 60 min in GTT) in homozygotes for *db* (BKS-*db/db* or F2-*db/db*) (Fig. 2C, D, G and H). In heterozygotes for *db* (BKS-*db*/− and F2- *db*/−), there was little difference in the mean values of the blood glucose levels examined, although a wider distribution of phenotypic values was found in the F2 mice (Fig. 2C, D, G and H). Sex dimorphism of diabetes-related traits has been observed in many rodent models (9–11) and was observed throughout our study as well. In the F2-*db/db* population, females had a much higher fat pad weight than males ($P < 1 \times 10^{-10}$, 2788.4 ± 52.7 versus 1849.5 ± 27.3 mg) (Fig. 2B and F). In the F2-*db*/− population, males had a higher body weight ($P < 1 \times 10^{-10}$, 27.7 ± 0.2 versus 22.4 ± 0.2 g) (Fig. 2A and E) and blood glucose levels at 60 min in GTT ($P < 1 \times 10^{-10}$, 277.8 ± 5.7 versus 228.4 ± 3.7 mg/dl) (Fig. 2D and H) than females.

These results indicate that the severity of the diabetes-related phenotypes was strongly dependent on the genetic background: taken together, the mixture of the D2 and BKS background intensified the severity of the diabetes and obesity compared with the isogenic background strains. These effects also depended largely on the sex and *db* genotype. This finding allowed us to map the QTLs responsible for these conditional effects and analyze the diversity of the QTLs playing under the various conditions.

Single-locus genome scans

In our initial analysis of QTLs, single-locus genome scans were implemented with the 'scanone' function in R/qtl software (4,5), as an add-on package for the freely available statistical language R (http://www.r-project.org/) (12). Genome-wide significance thresholds ($\alpha = 0.05$) were generated by permutation tests for each subpopulation (number of permutations = 10 000) in R/qtl. The whole combined population, including F2-*db/db* and *db*/− mice, were first analyzed for *db* (Fig. 1A). In this QTL analysis, the location of the *db* locus near the marker *D4Mit186* on chromosome (Chr) 4 was clearly detected as a highly significant QTL responsible for body weight at 9 weeks of age with LOD score = 101 (Fig. 1B).

Next, to map the QTLs responsible for the conditional effects, we divided the whole combined population into four subpopulations defined by sex and *db* genotype. The

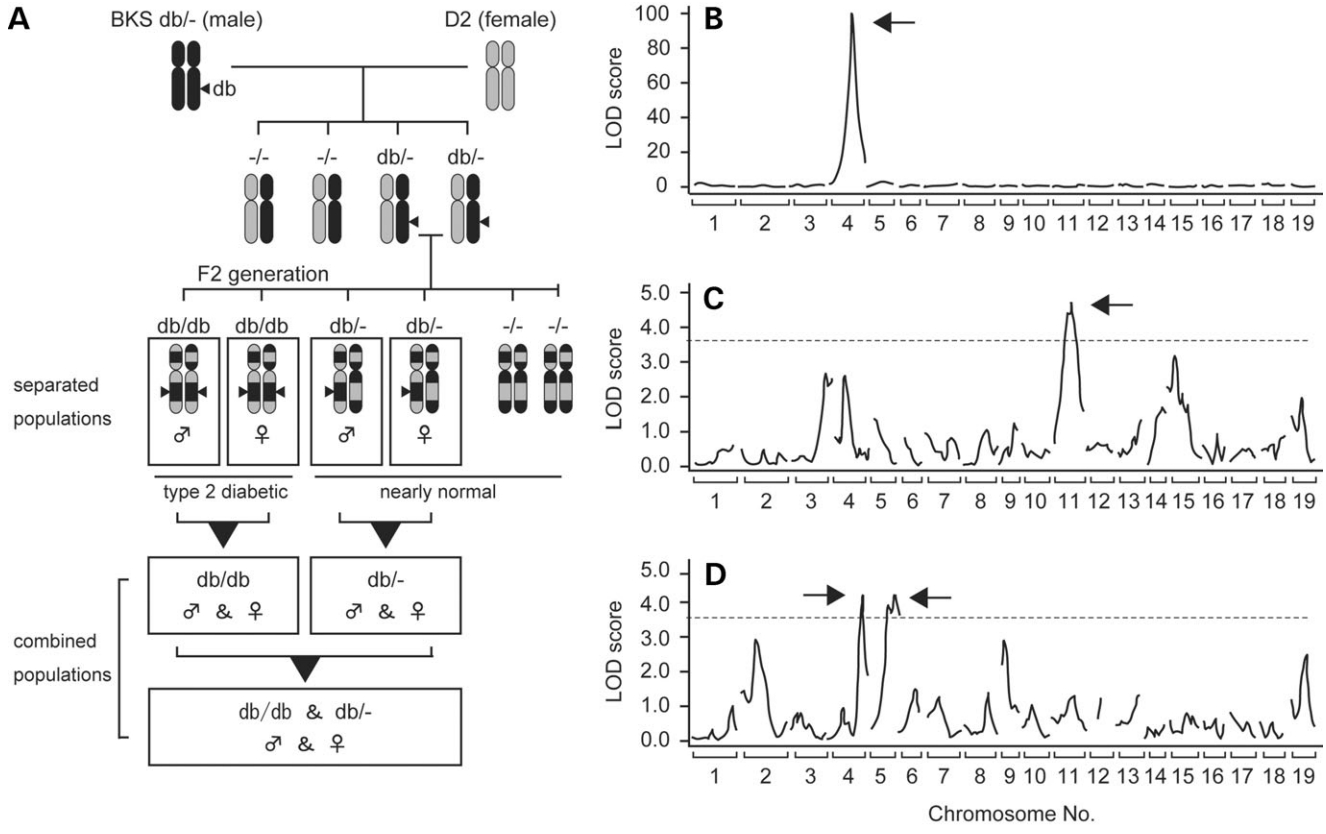

Figure 1. Breeding schema for generating F2 populations and single-locus genome scans in various conditions. (**A**) Breeding schema for generating F2 populations. After initially mating female D2 with male BKS- *db*/−, F1 progeny heterozygous for *db* were intercrossed to produce a F2 generation. F2 progeny homozygous and heterozygous for *db* were selected and partitioned by sex for the separated populations. For the combined populations, both sexes homozygous or heterozygous for *db* were individually combined. For whole combined population, all F2 progeny homozygous and heterozygous for *db* were combined. (**B**–**D**) Results of the single-locus genome scans on body weight at 9 weeks of age in the whole combined population (B), in the male *db*/− subpopulation (C) and in the female *db*/*db* subpopulation (D). The arrowheads indicate the location of the significant QTLs. Dashed lines (C and D) indicate genome-wide significance thresholds ($\alpha = 0.05$) generated by permutation tests.

genome scan in the male F2-*db*/− subpopulation revealed the significant QTLs for body weight at 9 weeks of age near *D11Mit179* on Chr 11 (LOD score = 4.75) (Fig. 1C). In the female F2-*db/db* subpopulation, three QTLs near *D4Mit54* on Chr 4 and near *D5Mit40* and *D5Mit372* on Chr 5 were also identified as the significant loci responsible for body weight at 9 weeks of age (LOD score = 4.14, 3.86 and 4.16, respectively) (Fig. 1D).

Topographical maps in Figure 3 show time-series variation of LOD scores for body weight in Chr 5 and 11 in every subpopulation analyzed. The QTLs identified near *D5Mit40* and *D5Mit372* on Chr 5 in the female F2-*db/db* population were also detected near *D5Mit40*, but not detected near *D5Mit372* in the male F2-*db/db* population (Figs 1D and 3A), and no QTLs were found on Chr 5 in the F2- *db*/− subpopulations (Fig. 3B). In contrast, the QTL identified near *D11Mit179* on Chr 11 in the male F2-*db*/− population was also identified in the female F2- *db*/− population (Figs 1C and 3D), but this QTL had little or no effect in the male and female F2-*db/db* subpopulations (Fig. 3C), suggesting that they acted specifically in limited conditions.

Table 1 summarizes the significant QTLs from the single-locus genome scans for every subpopulation and the LOD scores from covariate-dependent analysis in the combined populations of *db/db* or *db*/− mice with sex.

In the single-locus genome scan of male *db/db* subpopulation, five significant QTLs were identified on Chr 4, 5, 7 and 10. QTLs detected for different traits were considered the same QTL if their locations were estimated in the same marker bracket. The QTL between *D5Mit356* and *D5Mit40* on Chr 5, which had a broader peak of LOD curve, showed a continuous effect for body weight from 6 to 9 weeks of age (Table 1 and Fig. 3A male). This QTL showed an effect for fat pad weight as well. In the female *db/db* subpopulation, five significant QTLs were identified on Chr 4, 5 and 9. On Chr 4, two independent QTLs, which had a sharp peak of LOD curve, were closely located. One affected blood glucose levels near *D4Mit203* and the other affected body weight and fat pad weight near *D4Mit54*. In addition, two QTLs responsible for body weight and fat pad weight were independently found near *D5Mit372* or *D5Mit40* on Chr 5 (Table 1 and Fig. 3A female). In the male *db*/− subpopulation, six significant QTLs were mapped on Chr 4, 8, 11, 15 and 16. The QTL near *D8Mit155* on Chr 8 was responsible for blood glucose levels at 11 weeks of age and at 60 min in GTT (at 10 weeks of age). The QTL near

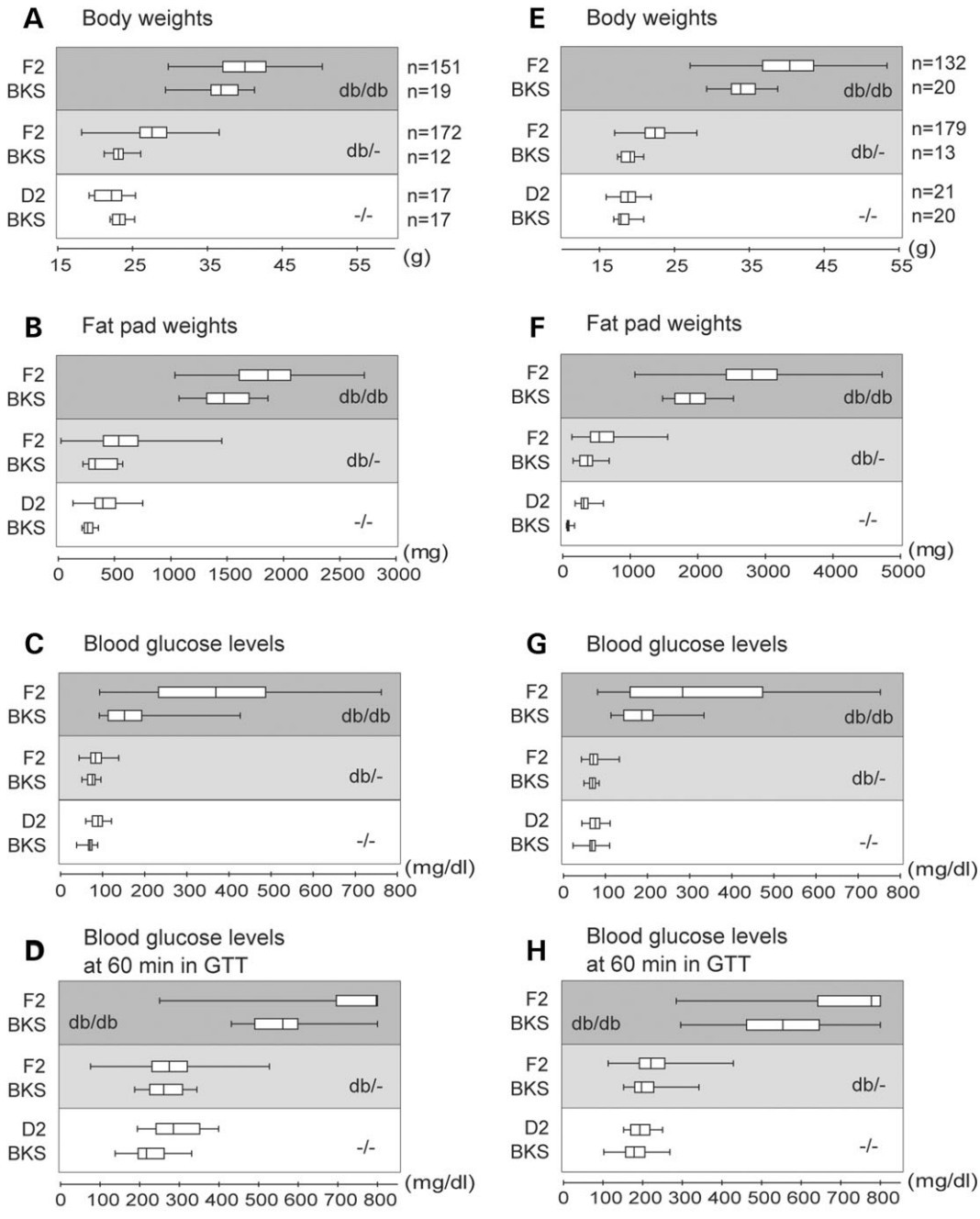

Figure 2. Effect of genetic background on phenotypic characteristics in the isogenic background strains and heterogeneous F2 mice. Box plots are displayed by sex [male: (**A**–**D**) and female: (**E**–**H**)] and *db* genotype [*db*/*db*: top (dark gray), *db*/−: middle (light gray) and −/−: bottom (white)]. (A and E) Body weight at 8 weeks of age. (B and F) Fat pad weight at 9 (*db*/*db*) or 11 (*db*/−, −/−) weeks of age. (C and G) Fasting blood glucose levels at 8 weeks of age. (D and H) Blood glucose levels in GTT (60 min) at 8 (*db*/*db*) or 10 (*db*/−, −/−) weeks of age.

D11Mit36 on Chr 11 showed the maximum effect for body weight at 5 weeks of age and this effect waned with age (Table 1 and Fig. 3D male). In the female *db*/− population, four significant QTLs were mapped on Chr 3, 11 and 15. Like the male *db*/− population, the QTL near *D11Mit179* (close to *D11Mit36*) showed a continuous effect for body weight, but this effect became larger at the later weeks of age in the female *db*/− population (Table 1 and Fig. 3D female). Also, Chr 15 had two independent QTLs responsible for the same trait of body weight near *D15Mit111* and *D15Mit239*.

For evaluating genotype effects of the QTLs, animals in each subpopulation were separated according to the genotype of the nearest marker of the QTLs (DD, D2 homozygotes; DB, D2/BKS heterozygotes; BB, BKS homozygotes), and mean values of each trait were calculated for each genotype group. The genotype effects of the QTLs are summarized in Table 1. In more than two-thirds of the QTLs, the DD

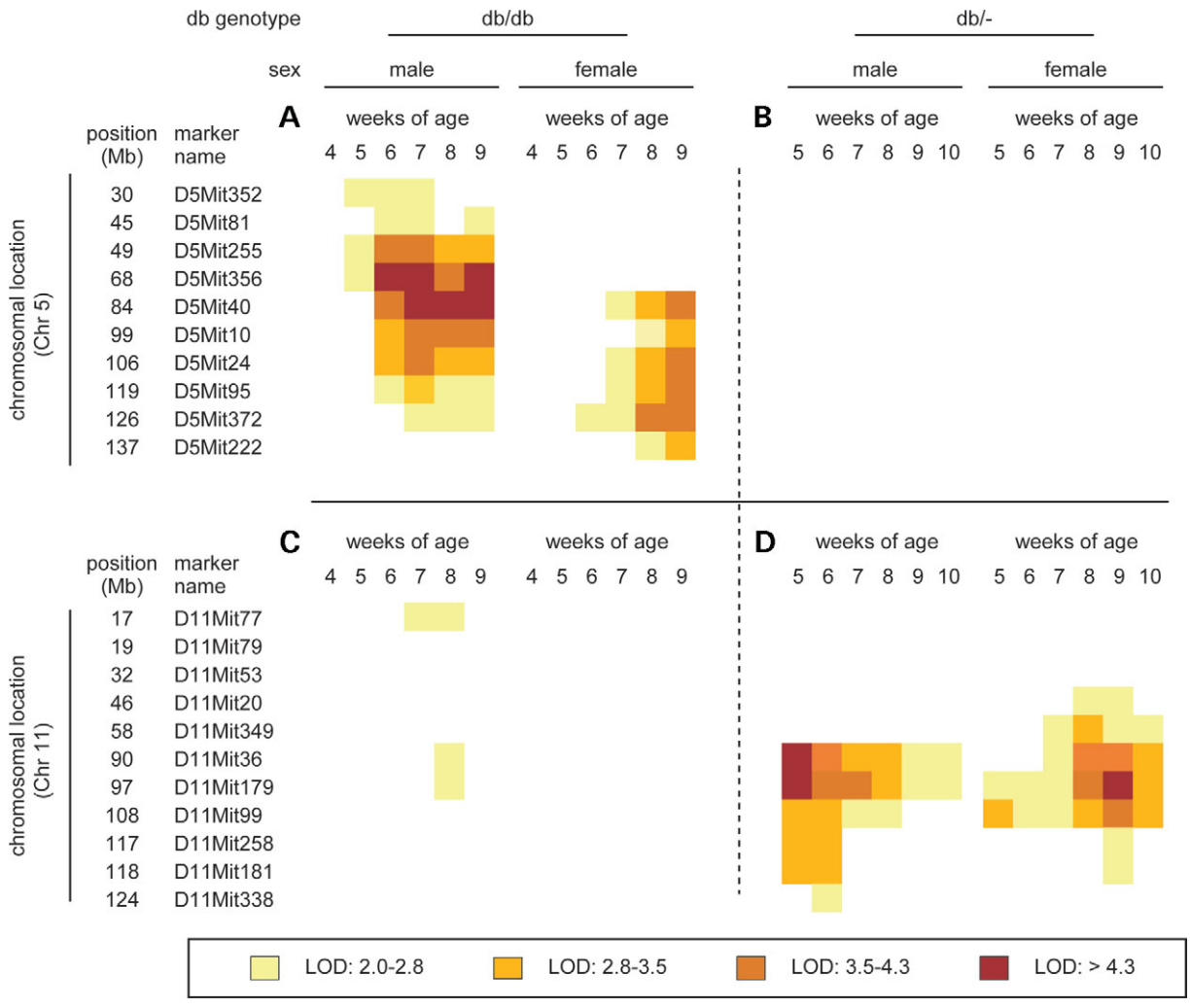

Figure 3. Topographical maps of LOD scores for body weight in Chr 5 and 11 from the single-locus genome scans. (**A** and **B**) Results in Chr 5. (**C** and **D**) Results in Chr 11. (A and C) Results in male and female *db*/*db* subpopulations. (B and D) Results in male and female *db*/− subpopulations.The *x*-axes of each figure show the weeks of age when the body weight was evaluated. The *y*-axes show the genetic markers in Chr 5 (A and B) and 11 (C and D) and their physical positions according to the Celera Mouse Genome Database. The color scale indicates the LOD scores.

genotype exhibited higher values in body weight, fat pad weight or blood glucose levels than the BB genotype.

The sex-specific effects of the QTLs that were first detected in the single-locus genome scans for every subpopulation were next analyzed in the covariate-dependent scans. The LOD scores taking sex as an additive or interactive covariate were compared in the right columns in Table 1. Most of the QTLs showed higher LOD scores in the scan model with sex as an interactive covariate than that with no interactive covariates, suggesting the sex-specific effects of them. However, the QTL for body weight and fat pad weight near *D5Mit40* that was co-identified in both male and female *db/db* subpopulations showed little change in LOD scores between two scan models, suggesting the sex-independent effect. The QTL near *D10Mit213* in male *db/db* population also showed little change between two scan models, but this QTL did not reach the significant threshold in the single-locus genome scan in female *db/db* subpopulation. Although the QTL near *D4Mit54* in the *db/db* subpopulations and the QTLs near *D11Mit36* (*D11Mit179* in female) or *D15Mit239* in the *db*/− subpopulations were detected in both sexes for body weight, the LOD scores were different between two scan models. It seemed to be caused by the difference between two sexes in the time points of QTL detected.

Table S1 in Supplementary Material included the results of the covariate-dependent scans for whole combined population of F2 mice with sex and *db* genotype. This population did not contain all phenotype data (see Materials and Methods). All of the QTLs analyzed using this population showed higher LOD scores in the scans with any interactive covariates than those with no interactive covariates, except for the QTLs near *D5Mit40* and *D10Mit213*. These results allowed us to consider not only sex-specific effects of the QTLs but also *db*- and sex-by-*db*-specific effects of them. The effects of QTLs near *D5Mit40* and *D10Mit213* were not sex-dependent in this scan but dependent on *db*.

Out of a total of 20 significant QTLs from the single-locus genome scans for all subpopulations, we selected four QTLs

Table 1. Significant QTLs detected by the single-locus analysis

Genotype of db	Sex	Locus[a]			Results of singe-locus genome scans (covariate-independent)					Results of covariate-dependent scans[b]	
		Chr	Nearest marker	Mb	Separated populations of F2 mice by sex and db genotype					Combined populations of db/db or db/- mice with sex	
					Traits	Weeks of age at evaluation (time points in GTT)[c]	Maximum LOD	% Variance explained	Genotype effect	LOD (no)[d]	LOD (sex)[e]
db/db	Male	4	D4Mit171	19.2	Blood glucose level in GTT	8 (60 min)	5.44	11	BB > DD	1.95	4.49
		4	**D4Mit54**	133.1	Body weight	4fe	3.98	11	BB > DD	3.67	5.18
		5	**D5Mit356-D5Mit40**	67.5–84.1	Body weight	6fe / **7fe** / 8fe / 9fe / 9	4.69	13	DD > BB	7.42	7.50
		5	**D5Mit356-D5Mit40**	67.5–84.1	Fat pad weight	9	3.86	11	DD > BB	7.36	7.70
		7	D7Mit310	28.0	Body weight	7fe	3.64	10	BB > DD	4.16	4.67
		10	D10Mit213	17.3	Body weight	8fe / **9fe** / 9	4.65	13	DD > BB	4.80	5.04
	Female	4	D4Mit203	124.9	Blood glucose level	9	4.87	16	DD > BB	4.02	5.73
		4	**D4Mit54**	133.1	Body weight	8fe / 9fe / **9**	4.20	13	BB > DD	4.13	4.89
		4	**D4Mit54**	133.1	Fat pad weight	9	5.18	15	BB > DD	5.89	8.02
		5	**D5Mit40**	84.1	Body weight	9fe / **9**	3.87	13	DD > BB	8.24	8.40
		5	**D5Mit40**	84.1	Fat pad weight	9	4.01	13	DD > BB	7.36	7.70
		5	D5Mit372	126.1	Body weight	8fe / 9fe / **9**	4.40	14	DD > BB	6.54	6.97
		5	D5Mit372	126.1	Fat pad weight	9	5.16	16	DD > BB	7.36	9.38
		9	D9Mit206	34.7	Blood glucose level in GTT	8 (120 min)	3.66	12	DD > BB	2.30	5.58
db/ −	Male	4	D4Mit42	150.9	Blood glucose level in GTT	10 (30 min)	5.08	13	BB > DD	5.20	7.62
		8	D8Mit155	2.1	Blood glucose level	11	3.70	9	DD > BB	3.78	4.84
		8	D8Mit155	2.1	Blood glucose level in GTT	10 (60 min)	4.67	12	DD > BB	5.18	7.70
		8	D8Mit4	30.0	Blood glucose level in GTT	10 (30 min)	4.10	10	DD > BB	5.19	6.31
		11	**D11Mit36**	90.4	Body weight	**5fe** / 6fe / 7fe	4.75	12	DD > BB	6.73	7.43
		15	**D15Mit239**	78.2	Body weight	8fe	4.49	10	DD > BB	5.30	7.30
		16	D16Mit103	29.5	Blood glucose level in GTT	10 (30 min)	3.76	10	DD > BB	4.59	5.73
	Female	3	D3Mit19	161.5	Body weight	7fe / **8fe**	4.41	11	DD > BB	3.15	4.44
		11	**D11Mit179**	96.5	Body weight	8fe / **9fe** / 10fe	4.99	11	DD > BB	5.60	6.80
		15	D15Mit111	29.6	Body weight	8fe / **10fe**	4.21	10	DD > BB	3.29	4.36
		15	**D15Mit239**	78.2	Body weight	10fe	3.65	9	DD > BB	6.18	7.01

QTLs with LOD scores that exceeded the significance thresholds ($\alpha = 0.05$) in both covariate-independent and -dependent analyses were included. The nearest marker denoted the marker closest to the position that showed the maximum LOD score for each trait. Physical position (Mb) was based on the Celera mouse genome assembly.

[a]QBIS regions exhibiting their genetic effects beyond sex are shown in underscore boldface.

[b]A covariate-dependent analysis was implemented for the phenotype data from weeks of age at which the LOD score was highest in the initial single-locus scans.

[c]Weeks of age with maximum LOD score are shown in boldface. Each phenotype was measured for the fasted conditions, unless specified as 'fe' denoting the fed condition.

[d]Results in the scans with sex as an additive covariate (no interactive covariates).

[e]Results in the scans with sex as an additive and interactive covariates.

that presented their genetic effects beyond sex. The QTLs near *D4Mit54* and *D5Mit40* in the *db/db* subpopulations and the QTLs near *D11Mit36* (*D11Mit179* in female) and *D15Mit239* in *db*/− subpopulations were co-identified in both sexes for body weight, but they did not affect body weight at the same time points between two sexes. We named them *QBIS1* (**Q**TL for **b**ody weight **i**ndependent of **s**ex **1**), *QBIS2*, *QBIS3* and *QBIS4*, respectively. They denoted the same directionality of their genotypic effects in both male and female subpopulations; DD mice had a higher body weight than BB mice, except for *QBIS1*.

Pairwise genome scans

In quantitative genetics, epistasis relates to the improvement in predicting phenotypic variation from simultaneously considering multi-locus genotypes, relative to predicting it from the sum of single-locus genotypes. In the single-locus genome scans, 20 individual genomic loci were identified, but each of them explained the relatively small proportions of variance ranging from 9 to 16%, with a mean of 12% in Table 1. Therefore, as a method to detect and characterize the epistatic and additive effects of loci, the pairwise genome scan was implemented with the 'scantwo' function in R/qtl except for Chr 4. The loci on Chr 4 were removed from the pairwise analysis because of the large bias in sample sizes (see Materials and Methods). This pairwise genome scan allowed the fitting of a two way ANOVA model to identify the significant locus pairs affecting the traits in two different modes, i.e. one interacting epistatically to affect the trait and the other affecting the trait in a two-locus additive manner. As well as single-locus scans, we first carried out the pairwise genome scans in all subpopulations and next incorporated covariates into pairwise scans to confirm the covariate-specific effects of the significant loci.

In the pairwise genome scans, both 'joint' and 'interaction' LOD scores were calculated for all pairwise combinations of loci. The former LOD provided an estimation of the whole two-locus effect for the trait and the latter LOD provided an estimation of the two-locus interaction (epistatic) effect for the trait. Genome-wide significance thresholds for the joint and interaction LOD scores were individually established through permutation tests in R/qtl. In this report, we considered that the locus pair interacted epistatically to affect the trait, when both joint and interaction LOD scores exceeded the significance threshold ($\alpha = 0.1$). When the interaction LOD score was not significant with the joint LOD score exceeding the threshold ($\alpha = 0.1$), two conditional LOD scores (LODq1 and LODq2) were assessed to distinguish 'the true effect from two loci affecting the trait in an additive manner' from 'the alleged coat-tail effect' (see Materials and Methods). Only when both LODq1 and LODq2 exceeded the threshold (nominal $P < 0.005$) in addition to the significance of the joint LOD score ($\alpha = 0.1$), the locus pair was considered as acting additively to affect the trait.

Table 2 summarizes the significant interacting pairs detected by the pairwise genome scans in every subpopulation and the LOD scores in covariate-dependent pairwise scans in the combined populations of *db/db* or *db*/− mice with sex. Five novel significant pairs were found in the female *db/db*, male *db*/− and female *db*/− subpopulations. No significant pairs were found in the male *db/db* subpopulation. All interacting pairs could have been identified only through the pairwise genome scans, showing little individual locus effects (LOD < 1.5) in the single-locus analysis. The *D1Mit206* and *D8Mit42* pair explained 47% of the variations of blood glucose levels at 6 weeks of age in the female *db/db* population. Three out of four interacting pairs affected blood glucose levels. This might suggest a higher correlation of blood glucose levels with multi-locus interactions than body weight or fat pad weight. In the covariate-dependent analysis for the combined populations of *db/db* or *db*/− mice with sex, the higher LOD scores in the scan model with an interactive covariate suggested their sex-specific interaction. However, the covariate-dependent dissection in the whole combined population provided another perspective to the *D1Mit206* and *D8Mit42* pair: it was found to be not only sex-specific but also more strongly *db*-by-sex-specific (in the right columns in Supplementary Material, Table S2).

Table 3 summarizes the significant additively acting pairs detected by the pairwise genome scans in every subpopulation and the LOD scores in the covariate-dependent pairwise scans in the combined populations of *db/db* or *db*/− mice with sex. A total of 22 significant pairs were found in all subpopulations: seven pairs in male *db/db*, five pairs in female *db/db*, five pairs in male *db*/− and five pairs in female *db*/− subpopulations. The significant pairs found for the different traits were considered the same pair. In the male *db/db* subpopulation, the position between *D5Mit40* and *D5Mit356* was considered as a single locus because of the large peak area on the LOD surface illustrated by the pairwise genome scan (as described later in Fig. 4A, B and D). Unlike the interacting pairs, most of the additively acting pairs affected body weight and fat pad weight. Several loci identified in the single-locus genome scans were not detected in the pairwise genome scans. They showed relatively large effects in isolation but they did not affect the traits additively as a pair. This meant that the conditional LOD score (LODq1 or LODq2) of their partner locus failed to achieve the threshold (nominal $P < 0.005$). In contrast, the pairwise genome scans revealed several loci that showed relatively small effects in isolation but in combination had a large effect on the traits. The half of loci in the additively acting pairs was detected only by the pairwise genome scans. For example, the pairwise genome scans have demonstrated that *D7Mit310* was an important locus as a partner of the pair affecting fat pad weight beyond sex in the *db/db* subpopulations (Table 3), but *D7Mit310* could be detected only in males in the single-locus genome scan [LOD = 2.81 ($\alpha = 0.2$) in females]. In addition, several complicated cases were found in which one locus paired with multiple loci at once, playing a key role in affecting the traits: *QBIS2* between *D5Mit40* and *D5Mit356* paired for affecting body weight with *D7Mit310* and *D19Mit89* at 7 weeks of age or *D9Mit182*, *D10Mit194* and *D11Mit36* at 9 weeks of age in the male *db/db* subpopulation. *D5Mit372* in the female *db/db* subpopulation paired with *D2Mit323*, *D7Mit310* and *D11Mit349* simultaneously for affecting fat pad weight at 9 weeks of age. In the male *db*/− subpopulation, *D8Mit155* affected blood glucose levels at 60 min in GTT with *D11Mit99* and *D15Mit239*. In the

Table 2. Significant interacting pairs detected by the pairwise analysis

Genotype of db	Sex	Locus 1			Locus 2			Results of pairwise genome scans (covariate-independent)				Results of covariate-dependent scans[a]	
		Chr	Nearest marker	Mb	Chr	Nearest marker	Mb	Separated populations of F2 mice by sex and db genotype				Combined populations of db/db or db/- mice with sex	
								Traits	Weeks of age at evaluation (time points in GTT)[b]	Interaction LOD	% Variance explained	Interaction LOD (no)[c]	Interaction LOD (sex)[d]
db/db	Female	1	D1Mit206	173	8	D8Mit42	128	Blood glucose level	6	9.75	47	2.98	10.22
db/-	Male	2	D2Mit525	130	8	D8Mit47	108	Blood glucose level in GTT	10 (0 min)	7.53	25	4.82	8.93
		7	D7Mit82	46	19	D19Mit35	55	Blood glucose level in GTT	10 (0 min)	8.15	14	3.92	10.58
	Female	2	D2Mit365	25	5	D5Mit372	126	Blood glucose level	10fe	6.72	21	4.14	11.39
		3	D3Mit89	160	16	D16Mit171	54	Body weight	11fe	7.67	42	2.11	12.58

Where the joint and interaction LOD scores exceeded the threshold ($\alpha = 0.1$) in the pairwise scans, we considered that the locus pair interacted epistatically to affect the trait. The nearest marker denotes the marker closest to the position that showed the maximum LOD score for each trait. Physical position (Mb) was based on the Celera mouse genome assembly. Some of significant interacting pairs identified in the initial pairwise scans were excluded, because they did not exceed the threshold ($\alpha = 0.1$) in the covariate-dependent scans.

[a]A covariate-dependent analysis was implemented for the phenotype data from weeks of age at which the LOD score was highest in the initial pairwise scans.

[b]Each phenotype was measured for the fasted conditions, unless specified as 'fe' denoting the fed condition.

[c]Interaction LOD scores in the scans with sex as an additive covariate (no interactive covariates).

[d]Interaction LOD scores in the scans with sex as an additive and interactive covariates.

female *db*/− subpopulation, *D3Mit89* paired with three loci (*D11Mit179*, *D14Mit94* and *D15Mit111*) and *D14Mit94* also paired with *D15Mit18* to affect body weight at 8 weeks of age.

In the covariate-dependent analysis of additively acting pairs in combined populations of *db/db* or *db*/− mice with sex, the higher LOD scores were obtained when analyzed using sex as an interactive covariate (Table 3). In addition, in the covariate-dependent dissection in the whole combined population, almost all of the pairs showed higher LOD scores in the scans with any interactive covariates than those with no interactive covariates (Supplementary Material, Table S3). These results indicated all of the additively acting pairs also affected the traits with the sex-, *db*- and sex-by-*db*-specificity.

In these pairwise genome scans, the proportions of variance explained by quantitative trait loci ranged from 14 to 47% with a mean of 23%, as seen in the column in Tables 2 and 3. Given that the proportions of variance explained from the single-locus genome scans ranged from 9 to 16% with a mean of 12% (Table 1), it was not surprising that the pairwise model was a better fit to explain the total phenotypic variations of complex traits than the single-locus model.

An interesting finding of our pairwise genome scan was the difference of the partner locus in the two sexes, even though one locus was shared between both sexes. In Table 3, *QBIS2* between *D5Mit40* and *D5Mit356* paired with the other five loci in Chr 7, 9, 10, 11 and 19 (*D19Mit89* at 40 Mb chromosomal location on the Celera mouse genome assembly) in the male *db/db* subpopulation, but it could not be detected as a pair in the female *db/db* subpopulation. When the relaxed threshold ($\alpha = 0.2$) was utilized in the pairwise genome scan, *QBIS2* in the female *db/db* subpopulation paired with *D19Mit30* (at 30 Mb chromosomal location) in Chr 19 for affecting body weight at 9 weeks of age (data not shown). *QBIS3* near *D11Mit179* was co-identified as a pair in the male and female *db*/− subpopulations, but the partner was not the same: *D5Mit352* in males and *D3Mit89* and *D15Mit29* (*QBIS4*) in females. The *QBIS* regions presented their genetic effects beyond sex in the single-locus genome scans, but the pairwise genome scans have revealed that their partners coordinately affecting the traits varied widely in males and females. Sex dimorphism of the diabetes-related traits observed in many rodent models might be linked with the different combinations of the multiple loci acting additively to affect the traits.

Time-series shift of the combination of loci

Graphical examples of the pairwise genome scans are displayed as the additively acting pairs for body weight and fat pad weight in the male *db/db* subpopulation (Fig. 4) and as the interacting pairs for blood glucose levels in GTT in the male *db*/− subpopulation (Fig. 5). Whereas a single-locus scan resulted in a statistical support curve (LOD curve) for the individual effects of a QTL at each tested location in the genome, a simultaneous pairwise scan resulted in a statistical support surface of the LOD score for all potential combinations of QTL locations in the genome. This statistical support surface indicated the test statistic for the joint LOD score below the main diagonal and the test statistic for the interaction LOD score above the diagonal.

Table 3. Significant additively acting pairs detected by the pairwise analysis

Genotype of db	Sex	Locus 1[a]			Locus 2[a]			Results of pairwise genome scans (covariate-independent)				Results of covariate-dependent scans[b]	
		Chr	Nearest marker	Mb	Chr	Nearest marker	Mb	Separated populations of F2 mice by sex and db genotype				Combined populations of db/db or db/- mice with sex	
								Traits	Weeks of age at evaluation (time points in GTT)[c]	Joint LOD	% Variance explained	Joint LOD (no)[d]	Joint LOD (sex)[e]
db/db	Male	5	**D5Mit356**	68	10	D10Mit194	45	Body weight	**9fe** / 9	8.44	21	13.27	14.16
		5	**D5Mit356**	68	11	**D11Mit36**	90	Body weight	8fe / 9fe / **9**	11.84	30	12.83	15.82
		5	**D5Mit40**	84	7	***D7Mit310***	28	Fat pad weight	9	10.20	24	18.43	20.68
		5	**D5Mit40**	84	7	***D7Mit310***	28	Body weight	**7fe** / 8fe	12.56	26	13.66	15.66
		5	**D5Mit40**	84	9	D9Mit182	98	Body weight	9fe / **9**	8.68	22	11.71	13.82
		5	**D5Mit40**	84	19	D19Mit89	40	Body weight	7fe	8.62	21	11.62	14.52
		5	D5Mit10	99	7	D7Mit84	54	Body weight	6fe / **9**	11.30	27	14.54	17.08
		5	D5Mit10	99	17	D17Mit96	92	Body weight	8fe / 9fe / **9**	8.96	25	12.07	14.04
	Female	2	D2Mit323	54	5	D5Mit372	126	Body weight	9fe / **9**	8.54	23	11.12	13.55
		2	D2Mit323	54	5	D5Mit372	126	Fat pad weight	9	9.12	22	13.49	16.47
		2	D2Mit323	54	9	D9Mit93	41	Fat pad weight	9	8.35	23	8.18	14.24
		2	D2Mit92	70	17	D17Mit139	53	Blood glucose level	8fe	8.62	27	5.25	11.27
		5	D5Mit372	126	7	***D7Mit310***	28	Fat pad weight	9	10.13	26	11.38	16.42
		5	D5Mit372	126	11	D11Mit349	58	Fat pad weight	9	9.05	24	11.18	16.27
db/-	Male	1	D1Mit87	114	9	D8Mit112[c]	34	Body weight	4fe	8.67	22	6.27	11.24
		5	D5Mit352	30	11	**D11Mit179**	97	Body weight	5fe	8.75	20	8.73	14.25
		8	D8Mit155	2	11	D11Mit99	108	Blood glucose level in GTT	10 (60 min)	8.48	19	8.47	13.44
		8	D8Mit155	2	15	**D15Mit239**	78	Blood glucose level in GTT	10 (60 min)	9.31	18	11.35	14.48
		9	D9Mit90	27	11	D11Mit338	124	Body weight	5fe	8.96	26	11.21	13.29
	Female	3	D3Mit89	160	11	**D11Mit179**	97	Body weight	8fe	9.02	18	9.72	14.22
		3	D3Mit89	160	14	D14Mit94	100	Body weight	8fe	8.61	15	7.89	11.27
		3	D3Mit89	160	15	D15Mit111	30	Body weight	8fe	8.96	16	7.27	11.57
		11	**D11Mit179**	97	15	**D15Mit29**	73	Body weight	10fe	8.72	17	13.47	15.09
		14	D14Mit94	100	15	D15Mit18	26	Body weight	8fe	8.82	16	10.44	13.64

When both LODq1 and LODq2 exceeded the threshold (nominal $P < 0.005$) with the significant joint LOD score ($\alpha = 0.1$), we considered that the locus pair acted additively to affect the trait in the pairwise genome scans. Some of significant pairs identified in the initial pairwise genome scans were excluded, because they did not exceed the threshold ($\alpha = 0.1$) in the covariate-dependent scans.

[a]QBIS regions exhibiting their genetic effects beyond sex are shown in underscore boldface and common markers identified across populations are shown in boldface italic.

[b]A covariate-dependent analysis was implemented for the phenotype data from weeks of age at which the LOD score was highest in the initial pairwise scans.

[c]Weeks of age with maximum LOD score are shown in boldface. Each phenotype was measured for the fasted conditions, unless specified as 'fe' denoting the fed condition.

[d]Joint LOD scores in the scans with sex as an additive covariate (no interactive covariates).

[e]Joint LOD scores in the scans with sex as an additive and interactive covariates.

[f]D8Mit112 was mapped on Chr 9.

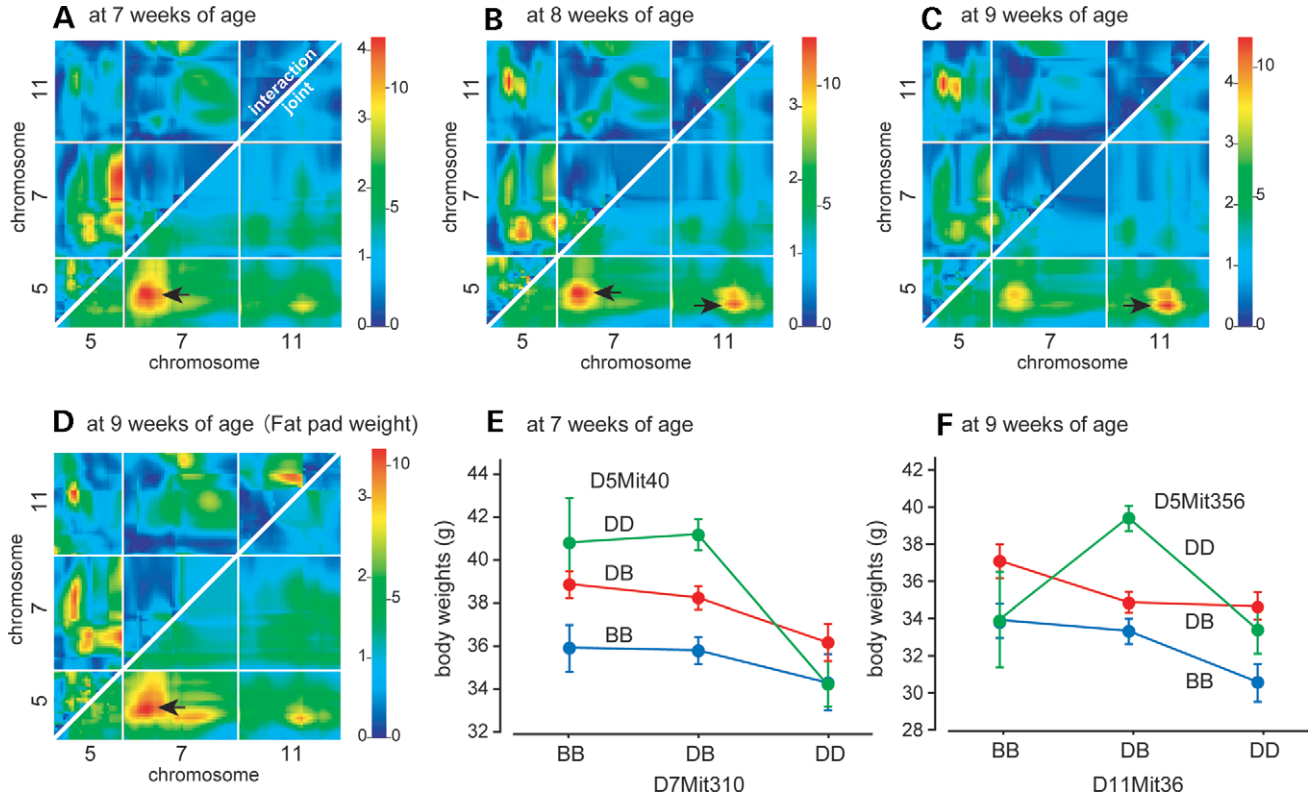

Figure 4. The results in the pairwise genome scans for body weight and fat pad weight in the male F2-*db/db* subpopulation. (**A**–**C**) Close-up of the pairwise genome scans on Chr 5, 7 and 11 for body weight at 7 (A), 8 (B) and 9 (C) weeks of age. (**D**) Close-up of the pairwise genome scans on Chr 5, 7 and 11 for fat pad weight at 9 weeks of age. The *x*- and *y*-axes of the figures show genetic positions on Chr 5, 7 and 11. The axis starts with Chr 5 (left of *x*-axis, bottom of y-axis) and ends with Chr 11 (right of *x*-axis, top of *y*-axis). The primary test statistic for joint LOD is shown below the main diagonal and the test statistic for interaction LOD is shown above the diagonal. The color scale indicates LOD scores; note that the scale is different for joint LOD (as shown on the right-hand side of the color bar) and the interaction LOD (as shown on the left-hand side of the color bar). Though the results in the pairwise genome scans for the loci on the same chromosome were displayed in the figures, they were not analyzed in this study (see Materials and Methods). (**E** and **F**) Graph showing the genotype versus the mean value of body weight at an additively acting pair; the pair of *D5Mit40* and *D7Mit310* showed a joint LOD score of 12.56 ($\alpha = 0.001$) for body weight at 7 weeks of age (A and E) and the pair of *D5Mit356* and *D11Mit36* showed a joint LOD score of 11.84 ($\alpha = 0.001$) for body weight at 9 weeks of age (C and F). The *y*-axes are the body weight. The *x*-axes are the genotypes at *D7Mit310* (E) and *D11Mit36* (F). The blue line is BB, the red line is DB and the green line is DD at *D5Mit40* (E) and *D5Mit356* (F).

Figure 4 (A–C) showed the close-up of the pairwise scans on Chr 5, 7 and 11 for body weight at 7, 8 and 9 weeks of age as the most representative additively acting pairs. *QBIS2* between *D5Mit356* and *D5Mit40* in Chr 5 was one of the major QTLs for body weight and fat pad weight in our single-locus genome scans. In the pairwise scans, from 7 to 8 weeks of age, *QBIS2* formed the relatively large spot of joint LOD score [joint LOD = 12.56 ($\alpha = 0.001$)] with the combination of *D7Mit310* in Chr 7, exhibiting the additive effects for body weight [LODq1 = 5.49 ($P < 1 \times 10^{-5}$) and LODq2 = 5.17 ($P < 1 \times 10^{-4}$)] (Fig. 4A and B, left-pointing arrowheads). Their partnership dissolved at 9 weeks of age for body weight (Fig. 4C), though they maintained cooperative ties for affecting fat pad weight (Fig. 4D). In place of *D7Mit310*, from 8 to 9 weeks of age, *QBIS2* worked together with *D11Mit36* in Chr 11 as a new partner that additively affected body weight [joint LOD = 11.84 ($\alpha = 0.001$), LODq1 = 5.48 ($P < 1 \times 10^{-5}$) and LODq2 = 3.06 ($P < 1 \times 10^{-3}$)] (Fig. 4B and C, right-pointing arrowheads). At 7 weeks of age, the combination of D2 homozygotes at *D5Mit40* and heterozygotes at *D7Mit310* exhibited a 7 g higher body weight (41.21 $\pm$ 0.75 g) compared with double D2 homozygotes at the *D5Mit40* and *D7Mit310* locus pair (34.24 $\pm$ 1.07 g) (Fig. 4E). At 9 weeks of age, the combination of D2 homozygotes at *D5Mit356* and heterozygotes at *D11Mit36* exhibited a 9 g higher body weight (39.45 $\pm$ 0.70 g) than that of the combination of BKS homozygotes at *D5Mit356* and D2 homozygotes at *D11Mit36* (30.57 $\pm$ 1.02 g) (Fig. 4F). As for pairwise interaction effects, weak evidence was found among these loci (maximum interaction LOD = 3.65 for the pair of *D5Mit40* and *D7Mit310* at 7 weeks of age and LOD = 4.77 for *D5Mit356* and *D11Mit36* at 9 weeks of age).

Figure 5 (A–C) shows the close-up of the pairwise scans on Chr 2, 8 and 15 for blood glucose levels in GTT in the male *db*/− population as one of the representative interacting pairs. We also attached Supplementary Material, Figure S2 that included all of the interacting pairs at 0 min in GTT in the male *db*/− subpopulation. As well as a large time-series analysis for body weight (Fig. 4), the combination of loci varied with a much shorter time period in GTT. The arrowheads above the diagonal in Figure 5A indicated that the combination of *D2Mit525* in Chr 2 and *D8Mit47* in Chr 8 exhibited the interaction effects for blood glucose levels at 0 min in GTT [joint

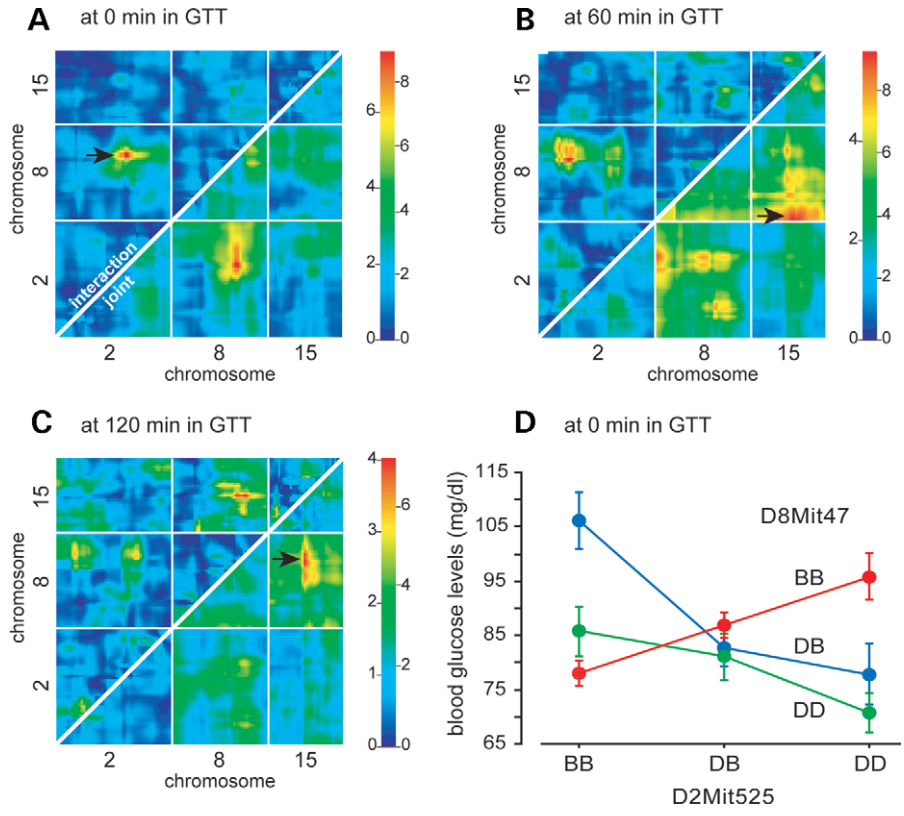

Figure 5. The results in the pairwise genome scans for blood glucose levels in GTT in the male F2- *db*/− subpopulation. (**A**–**C**) Close-up of the pairwise genome scans on Chr 2, 8 and 15 for blood glucose levels at 0 min (A), 60 min (B) and 120 min (C) in GTT. The *x*- and *y*-axes of the figures show the genetic positions on Chr 2, 8 and 15. The axis starts with Chr 2 (left of *x*-axis, bottom of *y*-axis) and ends with Chr 15 (right of *x*-axis, top of *y*-axis). The primary test statistic for joint LOD is shown below the main diagonal and the test statistic for interaction LOD is shown above the diagonal. Though the results in the pairwise genome scans for the loci on the same chromosome were displayed in the figures, they were not analyzed in this study (see Materials and Methods). (**D**) The graph showing the genotype versus the mean value of blood glucose levels at an interacting pair; the pair of *D2Mit525* and *D8Mit47* had an interaction LOD score of 7.53 ($\alpha = 0.05$) for blood glucose levels at 0 min in GTT (A and D). The *y*-axis is blood glucose levels at 0 min in GTT. The *x*-axis shows the genotypes at *D2Mit525*. The blue line is BB, the red line is DB and the green line is DD at *D8Mit47*.

LOD = 9.00 ($\alpha = 0.05$) and interaction LOD = 7.53 ($\alpha = 0.05$)]. The genotypes homozygous for alleles of the two loci that originated from the same mouse line (i.e. BB with BB or DD with DD) lowered the blood glucose levels (Fig. 5D). However, their interacting effect disappeared almost entirely at 60 min in GTT (Fig. 5B, above the diagonal), and a newly found pair of *D8Mit155* and *D15Mit239* acted additively for blood glucose levels at 60 min in GTT (Fig. 5B, arrowhead below the diagonal) [joint LOD = 9.31 ($\alpha = 0.05$), LODq1 = 4.17 ($P < 1 \times 10^{-4}$) and LODq2 = 2.40 ($P < 0.5 \times 10^{-}$)]. At 120 min in GTT, *D8Mit47* reappeared and worked not interactively but additively with *D15Mit105* on Chr 15 as a new partner (Fig. 5C, arrowhead below the diagonal), exhibiting moderate significant effects for blood glucose levels [joint LOD = 8.17 ($\alpha = 0.2$)].

Just for reference, we made the combined data including blood glucose levels at 0–120 min in GTT and applied it to covariate-dependent analysis using the time points in GTT as a covariate. This analysis had a problem because of the repeated measurements of the same animal. Although a repeated measures analysis was beyond the current capabilities of R/qtl, it could be implemented. The results of this analysis showed the time-specific effect of the interacting pair of *D2Mit525* and *D8Mit47* at 0 min in GTT (LOD with no interactive covariates = 7.16 and LOD with time as an interactive covariate = 13.91). In the same sense, we implemented covariate-dependent analysis with the weeks of age as a covariate. The results also showed the week-specific effect of the additively acting pair of *QBIS2* and *D7Mit310* (LOD with no interactive covariates = 26.37 and LOD with weeks of age as an interactive covariate = 43.13) or *QBIS2* and *D11Mit36* (LOD with no interactive covariates = 36.51 and LOD with weeks of age as an interactive covariate = 50.58).

Our multidimensional genome scans in all subpopulations and covariate-dependent scans in the combined population led us to know the different loci or the different combinations of loci affected the traits specifically in each condition. In addition, our pairwise genome scans suggested that the combinations of loci varied dramatically from week to week for body weight and from time to time for blood glucose levels in GTT, even within the same population. These results described here were observed throughout our populations as well, when the relaxed threshold ($\alpha = 0.2$) was utilized to select pairs of loci in the pairwise genome scans.

DISCUSSION

In this report, a multidimensional genome scan was conducted for all F2 subpopulations. The diversity of QTLs varied with the db-dependent diabetes severity and the difference in sex. The single-locus genome scans have led to the identification of four QTLs that presented their genetic effects beyond sex: *QBIS1*, *QBIS2*, *QBIS3* and *QBIS4*. Nevertheless, most QTLs showed their effects specifically in the limited conditions, i.e. in the presence or absence of *db/db*-induced diabetes and in the male or female population, even though all genetic backgrounds were composed of either the D2 or the BKS strain. The highly conditional feature of the QTLs was accentuated in the pairwise genome scans. A total of 27 significant pairs of loci were detected in the pairwise genome scans: five were interacting pairs and 22 were additively acting pairs. Nonetheless, no pair of loci could be found commonly among all subpopulations. All pairs of loci affected the traits under varying combinations in both sexes, even though one locus was shared between sexes. Instead, even within the same subpopulation, the pairwise analysis for body weight or blood glucose levels in GTT showed that the pairs of loci affected the traits under constantly varying combinations in the time series. Incorporating covariates into genome scans assessed these highly conditional effects of the QTLs or the combinations of loci. Although some QTLs and the combinations of loci were significant in the covariate-independent scans, they were excluded from our results because they did not reach the threshold in the covariate-dependent scans. They might be the artifacts of the analysis where power to detect QTLs was low.

Our multidimensional genome scans uncovered high degree of complexity in the genetics of type 2 diabetes. These characteristics of the loci could not be discovered solely by the analysis of the one-to-one relationship between a marker locus and the quantitative trait. It has inferred that we would never be able to fully understand the nature of complex traits, unless an elaborate investigation was undertaken, including the simultaneous multi-locus analysis at different time points under various conditions. Toward this end, a larger sample size and higher recombination will be required to obtain the full benefits of the multi-locus analysis, and the development of new approaches that allow us to analyze a pair of loci on the same chromosome will also be needed.

From another point of view, the information from pairwise genome scans is expected to help us select donor and recipient strains in the construction of congenic strains. In the analysis for complex traits, congenic strains are powerful tools because they allow the characterization of a single locus of interest in the absence of genetic variation throughout the remainder of the genome. Congenic strains are usually produced by repeated backcrossing to introduce a particular chromosomal (QTL) region from donor strain to recipient (background) strain. However, at any time of the first backcrossing, it becomes an inevitable subject of discussion which strain is better for the background of a congenic strain. Although either of the two inbred strains used in the initial QTL study can be backcrossed to another strain, we needed to select an appropriate genetic background that allowed the expression of the phenotype in the progeny. We found the pairwise analysis could guide us to the appropriate genetic background to obtain the maximum power to detect single-locus effects of the QTL. For example, *QBIS2* between *D5Mit356* and *D5Mit40* additively affected body weight with the combination of *D7Mit310* or *D11Mit36* in the male F2-*db/db* population (Fig. 4). To evaluate a single-locus effect of *QBIS2*, if we construct a congenic strain that has the D2-derived *QBIS2* region with a BKS-derived background (that is to say, the DD genotype at *D5Mit40* and the BB genotype at *D7Mit310* in Fig. 4E), we will obtain the maximum power for the detection of the phenotypic difference of body weight at 7 weeks of age from the control strain that has the BKS-derived *QBIS2* region with the BKS-derived background (the BB genotype at both *D5Mit40* and *D7Mit310*). However, at 9 weeks of age, this congenic strain will be unable to show the additively acting effect with *D11Mit36* for body weight when compared with the control strain (DD at *D5Mit356* with BB at *D11Mit36* versus BB at *D5Mit356* with BB at *D11Mit36* in Fig. 4F). In contrast, if the reciprocal congenic strains that have the BKS-derived *QBIS2* region with the D2-derived background (BB at *D5Mit40* and DD at *D7Mit310* in Fig. 4E) are constructed, they can no longer be expected to have any impact on body weight at 7 weeks of age from the control strain (DD at both *D5Mit40* and *D7Mit310*), while this combination has the potential to make moderate differences in body weight at 9 weeks of age from the control strain (BB at *D5Mit356* and DD at *D11Mit36* versus DD at both *D5Mit356* and *D11Mit36* in Fig. 4F). Recently, another effective approach using the recombinant congenic strains (RCSs) was proposed for understanding the complex susceptibilities to cancer, type 1 diabetes and type 2 diabetes. Leiter and Reifsnyder (13,14) have used the RCSs strategy successfully to dissect the multi-locus interaction in type 2 diabetes without the development of monocongenic stocks and to produce new mouse models that developed a more moderate obesity formed by the interaction of numerous genes with small effects.

As described in Single-locus genome scans section, *QBIS2* was identified between *D5Mit356* and *D5Mit40* in Chr 5 with a broader peak of LOD curve. This locus lies close to the previously published QTL, *Nob1*, which affects body weight in a backcross population of New Zealand obese with Swiss/Jim-Lambert mice (15). Interestingly, whereas the effect of *QBIS2* was dependent on the severity of diabetes and obesity triggered by the *db*/*db*-induced hyperphagia, *Nob1* was also dependent on the high fat diet-induced obesity and it showed no significant LOD score with standard chow. Further investigation is required to clarify whether *QBIS2* is *Nob1*.

Several studies of epistasis among major genes or candidate genes have found epistasis in the expression of complex traits of medical importance in humans, including type 1 diabetes and type 2 diabetes (16,17). A better understanding of such multi-locus effects will be essential to fully understand the complex nature of diabetes and obesity in humans. Presently, however, the obstacles to increase the power in the multi-locus approach still remain high in human natural populations due to sample heterogeneity and the limitations of sample sizes. The similarity between the human and mouse genomes and the availability of genomic sequences of several mouse strains make the mouse an excellent model for elucidating complex traits. We hope that the genetic dissection of complex traits

in mice could help to unravel the complexity of common diseases in humans and provide a rationale for the design of new drugs. To that end, it will be also necessary to evaluate all combinations of loci in mice using other post-mapping analyses to further explore whether the multi-locus effects can be explained by biological gene interactions. Effective use of the recently established genome-wide panel of congenic strains may help us to directly investigate the multi-locus effects in the absence of background variation (18). Here, we described multidimensional QTL analysis in the absence or presence of covariates as a starting point for a more thorough understanding of the genetic networks of complex traits. Our pairwise genome scans have uncovered a total of 27 significantly interacting or additively acting pairs of loci, showing a better fit to explain the total phenotypic variation of the traits. These significant pairs affected the traits under constantly varying combinations of loci in the time series or in the two sexes and told us the appropriate genetic background in constructing congenic strains to obtain the maximum power to replicate the phenotypes. Now, we should routinely explore the multi-locus effects in the complex trait studies and look at multiple loci comprehensively to disentangle the complex genetics. We believe this study and future multi-locus investigations in mice will continue to provide valuable insights into the genetics of common diseases and add light to the clarification of entire biological systems in other mammals, including humans.

MATERIALS AND METHODS

Animals and populations for genome scans

The following inbred male and female mice were purchased from CLEA Japan (Tokyo, Japan) for phenotypic comparisons with F2 populations (Fig. 2): BKS.Cg-*Lepr*^{db}+/+*Lepr*^{db} (BKS-*db*/*db*), BKS.Cg-*Lepr*^{db}+/+*m* (BKS- *db*/−), BKS.Cg-*m*+/+*m* (BKS- −/−) and DBA/2 (D2). The recessive misty (*m*) mutation causes a mild dilution of coat color. As both male and female BKS-*db*/*db* are sterile, *m* has been incorporated into the stocks for maintenance of the *db* mutation.

All mice were maintained under specific pathogen-free conditions in a temperature and humidity controlled environment with a 12 h light and dark cycle. After weaning at 4 weeks of age (29 ± 1 days), mice were group housed by sex (2–5 mice/cage) with free access to standard rodent chow (MF, Oriental Yeast Co., Tokyo, Japan) and water at all times, except for the 16 h fasting periods prior to blood sampling. All protocols for animal use were reviewed and approved by the Animal Care Committee of Tokushima University.

To generate F2 populations, F1 progeny heterozygous for *db* were intercrossed after an initial mating of female D2 with male BKS-*db*/−. We obtained a total of 634 F2 homozygous (*db*/*db*) and heterozygous (*db*/−) mice. The wild-types were not analyzed in this study. In our initial analysis of linkage, F2 *db*/*db* and *db*/− progeny were separated by sex and *db* genotype as the independent subpopulations to map the QTLs responsible for the conditional effects: the male *db*/*db* population ($n = 151$), the female *db*/*db* population ($n = 132$), the male *db*/− population ($n = 172$) and the female *db*/− population ($n = 179$) were analyzed individually. In the next covariate-dependent QTL analysis, we used three combined populations: the combined population of *db*/*db* mice with sex ($n = 283$) and the combined population of *db*/− mice with sex ($n = 351$) were analyzed to infer the sex-specific effects of the QTLs. The whole combined population of F2 *db*/*db* and *db*/− mice ($n = 634$) was also analyzed to consider sex-, *db*- and sex-by-*db*-specific effects of QTLs.

DNA isolation and genotyping

Genomic DNA was prepared from the tail tips of all F2 and their parental (F1) mice using a DNeasy 96 Tissue Kit for mouse tails (QIAGEN, Valencia, CA, USA).

The genotype at the *db* locus in F2 and F1 mice was determined by the TaqMan-PCR method (Applied Biosystems; ABI; Foster City, CA, USA). The minor groove binder (MGB) TaqMan probe was created using a G/T point mutation of the leptin receptor (7). The sense primer was 5′-CAAC TTCCCAACAGTCCATACAATATTA-3′ and the antisense primer was 5′-AAACTGAACTACATCAAACCTACATT GTG-3′. The TaqMan MGB probe for the wild-type was 5′-FAM-TGGAGGGAAACAAA-MGB-3′. The TaqMan MGB probe for the mutation was 5′-VIC-TGGAGGTAAAC AAAC-MGB-3′. The PCR assay mixture contained 20 ng of genomic DNA, 900 nM each of primer, 200 nM of each TaqMan MGB probe and Platinum qPCR Supermix UDG (Invitrogen Corporation, Carlsbad, CA, USA).

A set of 227 well-amplified microsatellite loci distributed across all autosomes was genotyped in all F2 populations. PCR was performed following the recommendations of the suppliers using Platinum Taq DNA Polymerase (Invitrogen Corporation) with the fluorescently labeled primers. Amplicons were electrophoretically separated on a 3700 capillary sequencer (ABI) and alleles were scored by Genotyper 3.7 software (ABI). The X chromosome was not genotyped because of the absence of polymorphisms between the BKS and D2 strains.

Measurements of phenotypic traits

All mice were weaned at 4 weeks of age (29 ± 1 days), and homozygotes for *db* were sacrificed at 9 weeks of age (63 ± 1 days), and the heterozygotes and wild-types for *db* were sacrificed at 11 weeks of age (77 ± 2 days) after overnight fasting (16 h).

Body weight (under fed conditions) was serially measured every week from weaning until sacrifice. At sacrifice, body weight was also measured for both the fasted and fed conditions, and abdominal fat pad weight was determined by collecting epididymal (in males) or parametrial (in females) adipose tissues.

Fasting blood glucose levels were monitored by the glucose oxidase method (ANTSENSE II Bayer Sankyo Inc., Japan) once every week and at sacrifice. The GTT was performed at 8 weeks of age (56 ± 1 days) for homozygotes for *db* and at 10 weeks of age (72 ± 2 days) for heterozygotes and wild-types for *db*. Glucose tolerance was assessed in overnight-fasted mice by injecting glucose (2 g/kg body weight in physiological saline) intraperitoneally and collecting blood from a tail vein at 0, 30, 60 and 120 min after injection. Non-fasting blood

glucose levels were also measured on the day before GTT, and serum triglycerides (TG) levels at sacrifice were assayed using a COBAS MIRA autoanalyzer (Roche Diagnostics, Japan) with the TG GPO Unimate 5 Kit (Roche Diagnostics).

In all genome scans reported here, all traits were analyzed after taking logarithmic transforms of the data to reduce skewness in the distributions.

Statistical analysis of phenotypic traits

Phenotypic comparison between parental and F2 populations was analyzed using the Statview 5.0 statistical software package (SAS Institute Inc., Cary, NC, USA) and displayed using a box plot summary. The plot's vertical line represents the median; the box encompasses the 25–75th percentiles; and error bars encompass the 10–90th percentiles. All values above the 90th percentile and below the 10th percentile are not plotted. Comparisons were evaluated by the unpaired Student's *t*-test.

For evaluating genotype effects of the QTLs, F2 mice in each subpopulation were separated according to the genotype of the nearest marker of the QTLs (DD, D2 homozygotes; DB, D2/BKS heterozygotes; BB, BKS homozygotes), and all group comparisons of the phenotypic traits were evaluated by the Kruskal–Wallis test with Scheffe's *post hoc* analysis. All quantitative values were expressed as mean $\pm$ SEM. A value of $P < 0.05$ was considered statistically significant.

Single-locus genome scans

We carried out single-locus genome scans separately in subpopulations defined by sex or *db* genotype. Single-locus genome scans were implemented by using the 'scanone' function of R/qtl (http://www.biostat.jhsph.edu/~kbroman/qtl/) with Harley–Knott regression (19). Haley–Knott regression required that multipoint genotype probabilities were first calculated using the 'calc.genoprob' function in R/qtl. The genotype probabilities were calculated at 2 cM intervals for the maximum distance between positions.

In each subpopulation, significance thresholds of single-locus genome scans ($\alpha = 0.05$) were individually generated through permutation tests by specifying the 'n.perm' command in the 'scanone' function of R/qtl (10 000 permutations) as described by Churchill and Doerge (5,20). These thresholds were more stringent than or almost equal to the multiplicity adjusted thresholds estimated by the false discovery rate (FDR) procedure (21) at FDR = 0.1 as recommended by Mosig *et al*. (22).

To calculate the proportion of variance explained by the QTLs, we analyzed each locus by fitting one-way ANOVA model on the Statview 5.0 statistical software. The microsatellite marker with the highest LOD score for a QTL was localized on Celera's physical map from the Celera mouse genome assembly R13.

Pairwise genome scans

Several procedures were followed step-wise to identify the significant locus pairs affecting the traits in two different modes, i.e. one interacting epistatically to affect the trait and the other affecting the trait in a two-locus additive manner. The pairwise genome scans were implemented using the 'scantwo' function of R/qtl with Harley–Knott regression to assess the simultaneous effects of the loci for the traits. The genotype probabilities were calculated at 5 cM intervals for the maximum distance between positions. The 'scantwo' function searched through all pairs of loci by fitting a two-way ANOVA model in the following two-step procedure.

First, the likelihood under the full regression model with interaction $(y = m + b[q1] + b[q2] + b[q1q2] + Ag + Zd[q1] + Zd[q2] + Zd[q1q2] + e)$ was compared to that under the null model $(y = m + Ag + e)$ with no genetic effects, where $q1$ and $q2$ are the unknown QTL genotypes at two locations, A is a matrix of covariates and Z is a matrix of covariates that interact with QTL genotypes. The columns of Z are forced to be contained in the matrix A (http://www.biostat.jhsph.edu/~kbroman/qtl/html/scantwo.html). This comparison generated the LOD scores for every pair of loci. This LOD score, which is named 'joint LOD' in R/qtl, provided an estimation of the whole two-locus effect for the trait. Next, the likelihood under the full regression model was compared to that under the following additive model $(y = m + b[q1] + b[q2] + Ag + Zd[q1] + Zd[q2] + e)$ with two main effects but no interaction. This comparison generated the LOD scores, which is named 'interaction LOD' in R/qtl, estimating the two-locus interaction (epistatic) effect for the trait.

Where the joint and interaction LOD scores for a locus pair exceeded the significance threshold ($\alpha = 0.1$), we concluded the locus pair interacted epistatically to affect the trait. If the interaction LOD score for a locus pair was not significant even though the joint LOD score exceeded the threshold ($\alpha = 0.1$), we assessed two conditional LOD scores (LODq1 and LODq2) to distinguish 'the true effect from two loci affecting the trait in an additive manner' from 'the alleged coat-tail effect (the significance of one of the loci could carry along another locus to make a significant pair)' (23,24). The conditional LOD score for locus q1, LODq1, was estimated from the 'scantwo' function in R/qtl as the $\log_{10}$ likelihood ratio comparing the model with q1 and q2 acting additively to the model with q2 alone, along with single-point *P*-values calculated by the chi-square approximation. In this study, only when both LODq1 and LODq2 exceeded the threshold ($\alpha = 0.005$) in addition to the significant joint LOD score ($\alpha = 0.1$), we concluded the locus pair acted additively to affect the trait. If one of the loci failed to achieve the threshold, they were dropped from the significant additive pairs. Each test for the conditional LOD score was carried out using nominal *P*-values. By applying the threshold of $\alpha = 0.005$ that was arbitrary but stringent, we could obtain unmistakably additive pairs and drop the subtle pairs.

Genome-wide significance thresholds for the joint and interaction LOD scores were individually established through permutation tests by specifying the 'n.perm' command in the 'scantwo' function of R/qtl (2000 permutations) (5,20). The proportion of variance explained by a locus pair was defined as $r2 = (s02 - s12)/s2$, where $s02$ and $s12$ are the residual variance under the null and alternative hypotheses, respectively, and $s2$ is the trait variance (6). To calculate the proportion of variance explained, we also analyzed each pair

of loci by fitting a two-way ANOVA model on the Statview 5.0 statistical software.

Our populations analyzed in this study had a large bias of the genotype on Chr 4, especially near the *db* locus, because they were originally divided by sex and genotype at *db* locus in Chr 4. If, in the pairwise genome scans, we again divided the populations by the genotype combination of the locus in Chr 4 and the other locus, the numbers of mice in each genotype group leaned to one side enormously. Therefore, we could not consider our sample sizes to be large enough to reliably cover Chr 4 in pairwise genome scans and removed the loci in Chr 4 from our pairwise analysis. For a similar numerical reason, the pairwise analysis of loci on the same chromosome could not be performed.

Covariate-dependent genome scans

The covariate-dependent genome scan has previously been described in details (25,26). A simple genome scan compares two linear models written as

$$y_i = \beta_0 + \varepsilon_i \quad [1]$$

$$y_i = \beta_0 + \beta_1 Q_i + \varepsilon_i \quad [2]$$

where y_i are the phenotypes, β_0 and β_1 are regression coefficients, Q represents the QTL genotype and ε_i are normal errors. The index i runs through all individuals in the cross. The LOD score is the difference in $\log_{10}$ likelihood values between models [1] and [2], where the individual model likelihoods are maximized with respect to the regression coefficients.

If a phenotype differs on average between the two sexes but the QTL has the same effect on both males and females, this can be modeled by including sex as an additive covariate in the genome scan. The genome scan with additive covariates provides LOD scores contrasting the models

$$y_i = \beta_0 + \beta_1 X_i + \varepsilon_i \quad [3]$$

$$y_i = \beta_0 + \beta_1 X_i + \beta_2 Q_i + \varepsilon_i \quad [4]$$

where X represents the covariate. Alternatively, if a QTL has an effect in only one of the sexes, we can allow for covariate-dependent QTL effects by using a model that includes a QTL-by-covariate interaction term:

$$y_i = \beta_0 + \beta_1 X_i + \beta_2 Q_i + \beta_3 Q_i X_i + \varepsilon_i \quad [5]$$

To make inferences about covariate-dependent QTL effects, one must consider all three models [3], [4] and [5].

In this study, the traits that showed the highest LOD score in the single-locus or pairwise genome scans in subpopulations, were re-analyzed by using sex as an additive covariate [model [4] − model [3]]. For this analysis, the subpopulations of F2 mice were combined with sex but separated by *db* genotype. They were also re-examined by using sex as an interactive covariate [model [5] − model [3]]. The change in likelihood between models [4] and [5] at the peak position helped us to identify sex-specific effects of the QTLs. These models could be computed in R/qtl by specifying the 'addcovar' or 'intcovar' command in the 'scanone' and 'scantwo' functions. Significance thresholds of covariate-dependent genome scans ($\alpha = 0.05$ for single-locus and $\alpha = 0.1$ for pairwise scans) were also generated in R/qtl by permutation tests (200 permutations) (5,20).

As for our populations, *db*/*db* and *db*/− progeny were not phenotyped exactly the same way, i.e. GTT was performed at 8 weeks of age for *db*/*db* and at 10 weeks of age for *db*/−, and *db*/*db* were sacrificed at 9 weeks of age, whereas *db*/− were phenotyped at 10 and 11 weeks of age. Because blood glucose levels of *db*/*db* populations increased steeply with weeks of age, and GTT must be performed until 8 weeks of age to avoid exceeding an upper measuring limit of blood glucose. In contrast, the phenotypes of *db*/− populations were nearly normal, and their phenotypic values were not widely distributed at earlier weeks of age. However, most phenotype data were collected at the same time points between *db*/*db* and *db*/− populations, and we could merge them as the data of the whole combined population. For this whole combined data, we ran additional genome scans with sex, *db* genotype and sex-by-*db* genotype, respectively, as additive and interactive covariates. These scans allowed us to consider not only sex-specific effects but also *db*- and sex-by-*db*-specific effects of the QTLs.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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