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ASSOCIATION STUDIES ARTICLE

A collective diabetes cross in combination with a computational framework to dissect the genetics of human obesity and Type 2 diabetes

Heike Vogel[1,2], Anne Kamitz[1,2], Nicole Hallahan[1,2], Sandra Lebek[2,3], Tanja Schallschmidt[2,3], Wenke Jonas[1,2], Markus Jähnert[1,2], Pascal Gottmann[1,2], Lisa Zellner[1,2], Timo Kanzleiter[1,2], Mareike Damen[2,3], Delsi Altenhofen[2,3], Ralph Burkhardt[4], Simone Renner[2,5,6], Maik Dahlhoff[2,5,6], Eckhard Wolf[2,5,6], Timo D. Müller[2,7,8], Matthias Blüher[9], Hans-Georg Joost[1,2], Alexandra Chadt[2,3], Hadi Al-Hasani[2,3] and Annette Schürmann[1,2,10,*]

[1]Department of Experimental Diabetology, German Institute of Human Nutrition Potsdam-Rehbrücke, Nuthetal D-14558, Germany, [2]German Center for Diabetes Research (DZD), München-Neuherberg D-85764, Germany, [3]Medical Faculty, Institute for Clinical Biochemistry and Pathobiochemistry, German Diabetes Center (DDZ), Heinrich Heine University, Düsseldorf D-40225, Germany, [4]Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, University Hospital Leipzig, Leipzig D-04303, Germany, [5]Chair for Molecular Animal Breeding and Biotechnology, Gene Center and [6]Department of Veterinary Sciences, Center for Innovative Medical Models (CiMM), LMU Munich, D-81377 Munich, Germany, [7]Institute for Diabetes and Obesity, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg D-85764, Germany, [8]Division of Metabolic Diseases, Department of Medicine, Technische Universität München, Munich D-80333, Germany, [9]Department of Medicine, University of Leipzig, Leipzig D-04103, Germany and [10]Institute of Nutritional Science, University of Potsdam, Nuthetal D-14558, Germany

*To whom correspondence should be addressed. Tel: +49 33200882368; Fax: +49 33200882334; Email: schuermann@dife.de

Abstract

To explore the genetic determinants of obesity and Type 2 diabetes (T2D), the German Center for Diabetes Research (DZD) conducted crossbreedings of the obese and diabetes-prone New Zealand Obese mouse strain with four different lean strains (B6, DBA, C3H, 129P2) that vary in their susceptibility to develop T2D. Genome-wide linkage analyses localized more than 290 quantitative trait loci (QTL) for obesity, 190 QTL for diabetes-related traits and 100 QTL for plasma metabolites in the outcross populations. A computational framework was developed that allowed to refine critical regions and to nominate a small number of candidate genes by integrating reciprocal haplotype mapping and transcriptome data. The efficiency of the complex procedure was demonstrated for one obesity QTL. The genomic interval of 35 Mb with 502 annotated candidate genes

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was narrowed down to six candidates. Accordingly, congenic mice retained the obesity phenotype owing to an interval that contains three of the six candidate genes. Among these the phospholipase *PLA2G4A* exhibited an elevated expression in adipose tissue of obese human subjects and is therefore a critical regulator of the obesity locus. Together, our broad and complex approach demonstrates that combined- and comparative-cross analysis exhibits improved mapping resolution and represents a valid tool for the identification of disease genes.

Introduction

The obesity epidemic is associated with adverse health consequences—Type 2 diabetes (T2D), cardiovascular disease, hypertension, hyperlipidemia and increased cancer risk and involves a complex interplay of genetic environmental and behavioral factors (1,2). Despite following multiple strategies such as genome-wide association studies (GWAS) in humans, the genetic basis of this complex disease still remains incompletely defined. Novel approaches combining classical familial linkage analysis with whole-genome sequencing are currently emerging as an important and powerful method, especially since rare variants, which are not well interrogated by GWAS, could be responsible for a substantial proportion of complex human diseases (3,4).

Animal models offer an additional, complementary method for identifying candidate genes (5). However, linkage studies using inbred mouse strains are time-consuming, and the identified quantitative trait loci (QTL) display a complex genetic architecture still containing a high number of genes. To accelerate the process of gene discovery, it would be beneficial to integrate and combine different crossbreeding approaches with well-defined genome and phenome data (6) in combination with available sequence resources of various inbred strains of mice (7–9). Therefore, we initiated the Collective Diabetes Cross project within the DZD (German Center for Diabetes Research), whereby four lean inbred strains of mice (B6, DBA, C3H, 129P2), each with varying susceptibility to develop T2D, were crossed with obese and diabetes susceptible New Zealand Obese (NZO) mice. The NZO mouse strain was the common breeding partner in all crosses as it represents an excellent model for polygenic obesity and T2D (10,11). On the basis of this mouse strain we previously identified the adipogenic and diabetogenic genes *Tbc1d1* (12), *Ifi202b* (13,14) and *Zfp69* (15,16) by positional cloning of which the human orthologues also appear to be involved in the progression of human obesity and T2D (13,15,17).

In the present study, the purpose was to combine the collective cross with a computational framework to dissect the genetics of obesity and T2D. In detail, (i) we identified QTL, (ii) dissected QTL intervals, (iii) established allelic states for haplotype analysis and (iv) included expression profiles finally allowing the discovery of single gene variant(s) linked to obesity and/or T2D.

Results

Phenotypic heterogeneity and genetic diversity of parental strains

Within the German Center for Diabetes Research (DZD), we initiated a cross project using four lean inbred mouse strains with varying T2D susceptibility (diabetes-resistant: B6, 129P2, C3H; diabetes-prone: DBA), which were crossed with the obese and diabetes-prone NZO mouse strain. As expected, NZO mice gained more body weight in comparison to all other strains and NZO males were the only group that developed overt diabetes (>16.6 mM), reaching severe hyperglycemia with body weight loss by the endpoint of 16 weeks (Supplementary Material, Figs S1 and S2).

In addition to the phenotypic diversity between the different inbred strains of mice we analyzed their genetic structure. The residual patterns of heterozygosity within the five breeding partners were evaluated with the Mouse Phylogeny Viewer (18) for regions that are identical by descent (Supplementary Material, Fig. S3). Segregating regions, varying in size, are present on each chromosome and clearly confirmed the evolutionary distance within the mouse family tree as each strain could be assigned to another evolutionary subgroup of mouse inbred strains.

The heterogeneity of the different metabolic traits and the genetic diversity further provided the basis for the subsequent investigation of QTL and the identification of genes by crossing the different inbred strains with the obese and diabetes-prone NZO mice.

QTL mapping of different NZO backcross populations

To improve the mapping resolution of QTL and to identify disease genes, we developed a computational framework which integrates linkage data of the outcross populations, strain-specific haplotype information, and genome-wide expression data (Fig. 1A). First, ~600 mice (~300/sex) of each backcross population (N2) were characterized on an HFD according to a standardized protocol (Supplementary Material, Fig. S4). The various phenotypical recordings were identical between the different NZO crosses. Genotypes were determined according to distinctive SNP markers positioned in 10–20 Mb intervals and linkage analysis was performed for individual traits and separately for each cross and gender. The genome-wide linkage analysis for traits related to obesity revealed QTL for male mice on Chromosomes 2, 3, 4, 6, 7 and 13 and QTL for female mice on Chromosomes 1, 2, 4, 5, 6, 10, 13, 14 and 17 (Fig. 1B) with LOD-score values exceeding the genome-wide significance threshold of 5%. Commonly occurring loci, i.e. QTL hotspots associated with obesity traits were identified on Chromosomes 1 and 4. Interestingly, several QTL (Chromosomes 1, 4, 7, 10) exhibited sex-specificity and no common QTL for all crosses was detected.

As expected, male N2 cohorts were more prone to T2D and thus had a higher rate of association with diabetes-related traits (Fig. 1C). The most prominent consensus region with linkage to diabetes traits was Chromosome 4 with a strong correlation for increased blood glucose values in the NZO cross with DBA, C3H and 129P2 as breeding partners. Additionally, QTL for altered blood glucose, plasma insulin and/or pancreatic insulin were identified on Chromosomes 2, 4, 7, 8, 9, 11, 13 and 15 in male and Chromosomes 1, 9 and 18 in female N2 mice (Fig. 1C). Linkage analysis for different metabolites was performed with blood samples from each animal of the outcross populations and overlapping regions with QTL for obesity and blood glucose parameters were identified on Chromosomes 4, 6, 10 and 15 for

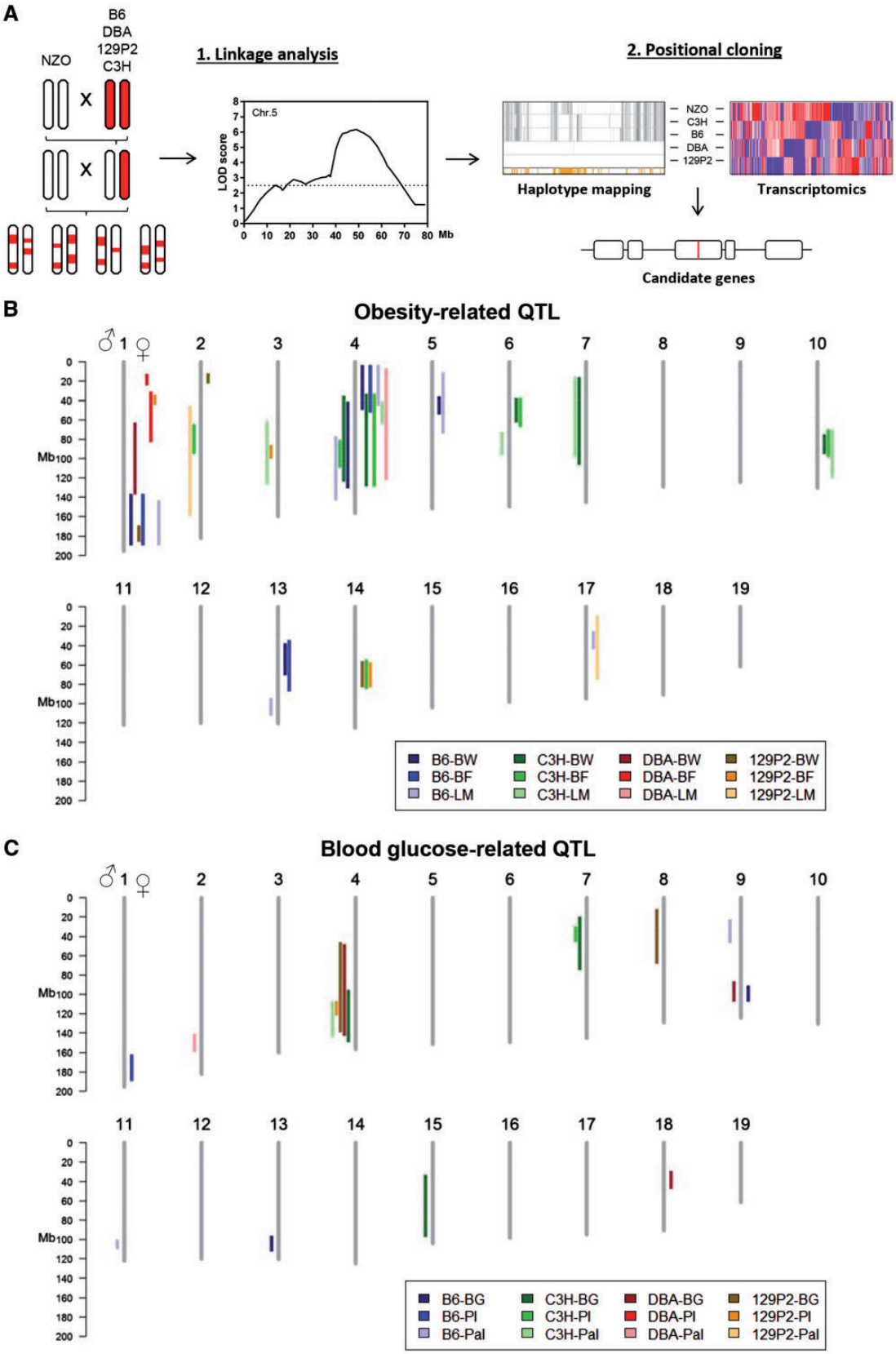

Figure 1. Physical map of QTL identified by genome-wide linkage analysis of NZO backcross populations. (**A**) Establishment of a computational framework integrating linkage data of all four outcross populations, strain-specific haplotype information, and genome-wide expression data to improve the mapping resolution of quantitative trait loci (QTL) and to finally identify disease genes. Genome-wide linkage maps for QTL relating to (**B**) obesity and (**C**) glucose homeostasis at the age of 16 (males, left side of indicated chromosome) and 22 (females, right side of indicated chromosome) weeks. Genome-wide linkage analysis of N2 mice including genetic map, genotyping errors, and single QTL scans for individual traits were assessed with R/qtl and QTL intervals exceeding a genome-wide 5% significance threshold are shown. NMR, nuclear magnetic resonance spectroscopy; BW, body weight; BF, body fat; LM, lean mass; BG, blood glucose; PI, plasma insulin; PaI, pancreatic insulin.

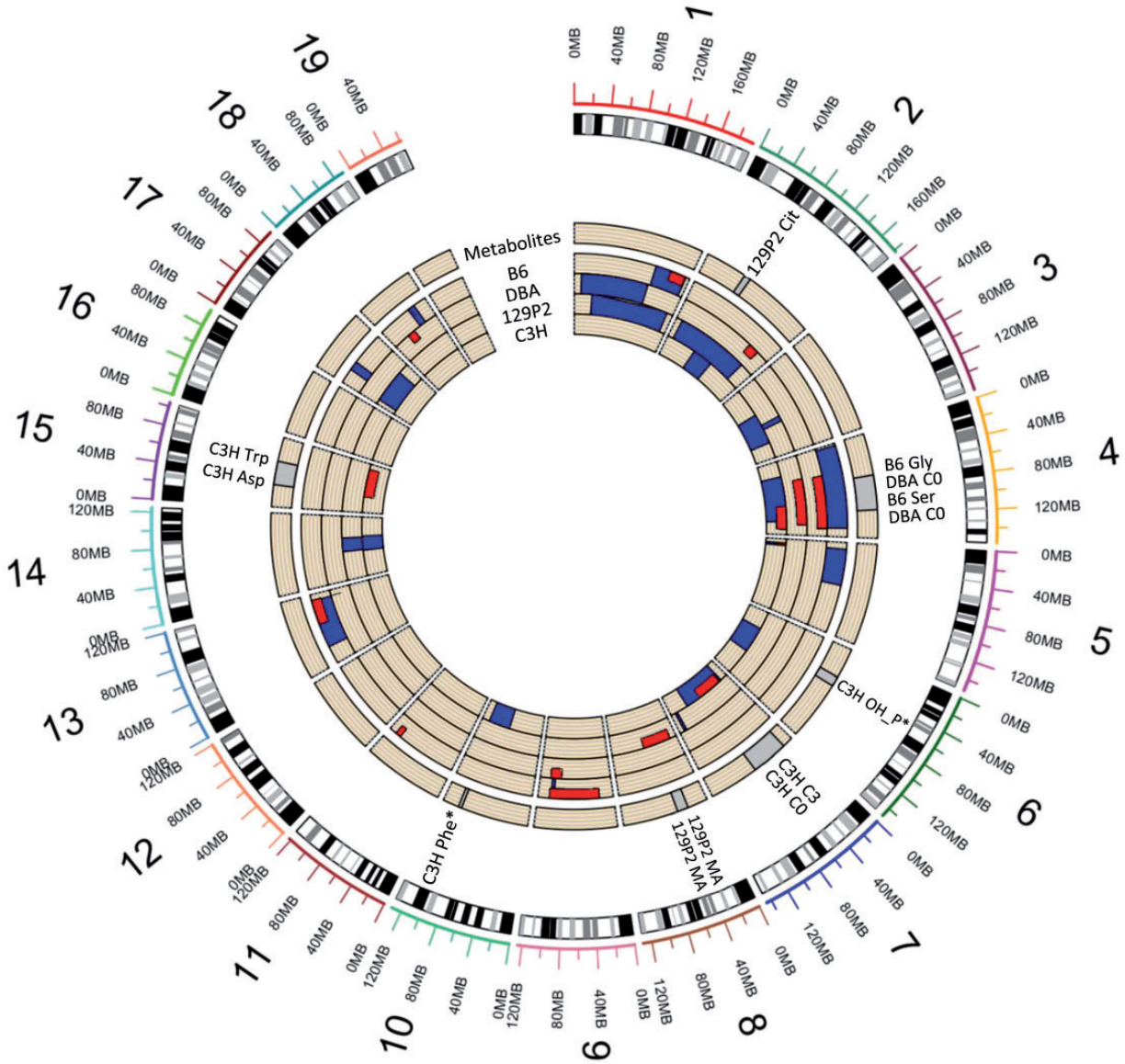

Figure 2. Linkage analysis for blood metabolites in NZO backcross mice. Circos plot showing the mouse chromosome ideogram with QTL for blood metabolites (grey, outer circle) in male or *female backcross mice with overlapping positions to QTL detected for obesity- (blue) and glucose-related (red) traits. Each circle represents one NZO backcross population starting outside with NZOxB6, followed by NZOxDBA, NZOx129P2 and NZOxC3H. The plot was generated with the RCircos package (19,20). C0, free carnitine; C3, propionylcarnitine; MMA, methylmalonyl-carnitine.

blood amino acids and Chromosomes 4, 7 and 8 for blood acylcarnitines (Fig. 2; Supplementary Material, Fig. S5).

In conclusion, these data clearly document the complexity of diabetes development as multiple QTL, presumably including various disease genes, are crucial for determination of clinically relevant metabolic endpoints. In the following part we will focus on one obesity QTL, *Nob5*, and demonstrate that the collective cross strategy allows narrowing down critical regions by haplotype mapping and integration of gene expression analysis without the time-consuming procedure of breeding recombinant congenic mice.

Nob5, an obesity QTL on Chromosome 1

The obesity QTL *Nob5* was unique for the female N2 population of the NZOxB6 backcross and localized on Chromosome 1 with a peak at 153.2 Mb (rs3674280; LOD = 3.6) and a separate, distal peak at 189.8 Mb (rs3675669; LOD = 5.2) (Fig. 3A and B). A more proximal linkage peak (rs3691057; 123.4 Mb) was also identified in the NZOxDBA cross, but was not intersecting with QTL intervals of the B6 cross as the level of significance was below the 5% threshold ($P < 0.05$) at these positions.

The distal peak at 189.8 Mb overlapped with the position of *Nob3*, an obesity QTL that we have previously identified and characterized in an intercross population derived from NZO and B6 mice (13,14). The actual analysis focused on the identification of the gene variant responsible for an increased body weight and fat mass in animals carrying the homozygous NZO alleles for the QTL *Nob5* ($Nob5^{N/N}$) in comparison to heterozygous controls ($Nob5^{N/B}$) (Fig. 3C and D). As $Nob5^{N/B}$ mice showed a lower body weight than $Nob5^{N/N}$ mice and all other crosses did not show a QTL, it can be interpreted that the B6 allele represents

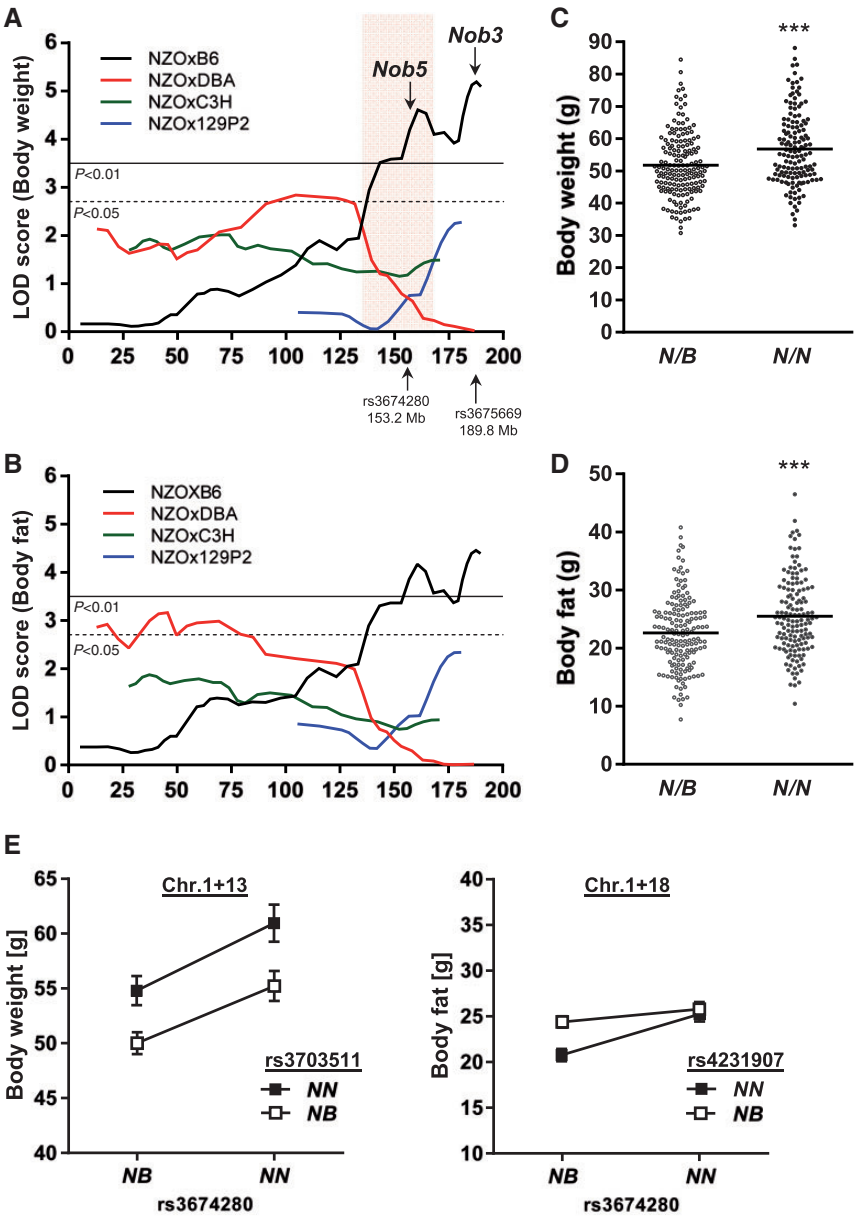

Figure 3. Identification of *Nob5*, a B6-specific obesity QTL on Chromosome 1. Genome-wide linkage analysis of the different DZD crosses in respect to (**A**) body weight at Week 18 and (**B**) body fat at Week 15. Introducing one NZO allele at the *Nob5* peak position (rs3674280, 153.2 Mb) is combined with an increased (**C**) body weight and (**D**) fat mass (*N/B*: $n = 167$; *N/N*: $n = 141$). (**E**) The effects of gene interactions determined by pair-wise genome scan of the *Nob5* peak marker (rs3674280) revealed the interaction with Chr.13 (left panel) at 64 Mb (rs3703511) for the trait body weight (Week 18) and with Chr.18 (right panel) at 65 Mb (rs4231907) for body fat (Week 15). Data represent mean±SEM. Differences between groups were calculated with Student's *t*-test between genotypes. $^{***}P < 0.001$. *N/N*, homozygous NZO; *N/B*, heterozygous NZO/B6.

an obesity suppressor gene. The proximal boundary of *Nob5* was defined by the first genotyping marker at 133.4 Mb (rs3022833) reaching genome-wide significance of 5% and the distal marker at 168.2 Mb (rs3714825), where LOD-score values started to drop again (Fig. 3A).

In addition, pairwise interaction was calculated to clarify whether the *Nob5* locus interacts with other genomic regions affecting obesity. Comparing mice carrying homozygous NZO alleles on Chromosomes 1 (*Nob5*) and 13 (64 Mb, rs3703511) with heterozygous animals lead to a significantly increased body weight. In contrast, introducing one B6 allele on Chromosome 18 (65 Mb, rs4231907) increased the body weight of mice with heterozygous genotypes for the Nob5, suggesting an adipogenic B6 component on Chromosome 18 (Fig. 3E).

In summary, a QTL hotspot associated with obesity-related traits was identified on Chromosome 1 caused by a unique genetic alteration within the B6 genome.

Comparative genomics and gene expression analysis to dissect the QTL *Nob5*

A combined-cross analysis was used to refine the critical region of the *Nob5* locus assuming that the genetic alteration of the

identified locus was owing to the inheritance of alleles from common progenitors.

The sequenced genomes of the parental mouse strains are available (8,9) and used to generate a haplotype map based on SNP information (Fig. 4A). As the *Nob5* QTL is unique for the NZOxB6 cross, all genes within the critical region (133.36–168.22 Mb) which do not share a common haplotype between NZO, DBA, 129P2, C3H and are different from B6, were excluded as being causal for the effect of the QTL (Fig. 4A). Thus, with block haplotyping, the number of genes within the critical region of *Nob5* was reduced from 502 to 188 annotated genes. To narrow down the list of candidates, the genomic sequences were filtered for coding changes, but none of these 188 genes contains any variations that were predicted to affect the protein-coding sequences.

In addition, whole-genome transcriptome data from gonadal white adipose tissue (gonWAT), brown adipose tissue (BAT), liver and quadriceps of the parental strains (6 weeks of age) were analyzed. We detected numerous genes within the *Nob5* locus which fulfil the criteria of a differential expression in B6 compared with all other strains and are located in the appropriate haplotype block. In BAT, four genes (*Timm17a*, *Ralgps2*, *Astn1*, *Mettl13*) exhibited differential expression, in liver five genes (*Timm17a*, *Ipo9*, *Colgalt2*, *Ralgps2*, *Gm10176*) and in skeletal muscle three genes (*Phlda3*, *Pappa2*, *Mrps14*) (Supplementary Material, Fig. S6). However, as fat mass was linked to *Nob5* we hypothesized that the white adipose tissue could be one driver for the effect; a conclusion that can be supported with recently published data from the distal peak on Chromosome 1 (*Nob3*), where the gonWAT was the main source for the obese phenotype (22). Therefore, we focused our analysis on the gonWAT as one possible target tissue. Out of the differently expressed genes in the gonWAT, only six genes (*Atp2b4*, *Lmod1*, *Pla2g4a*, *Cep350*, *Soat1*, *Mrps14*) were located within the B6-specific haplotype block suggesting that those genes may be causal for the identified QTL (Fig. 4B and C). The sequence information from the Welcome Trust Sanger Institute for the six candidate genes reported no obviously damaging alterations within the coding regions in any of the five parental strains, but a large number of SNPs in the putative promotor regions which might explain the differences in expression (Supplementary Material, Table S2).

Taken together, the implementation of combined-cross analysis including block haplotyping and gene expression analysis allowed the refinement of the obesity locus *Nob5* and the reduction of candidates from 502 to a total number of six genes.

Confirmation of the relevant fragment of *Nob5* by recombinant congenic mice

In order to demonstrate that our approach is capable to identify the most likely responsible genes within the QTL, we generated congenic mice carrying different fragments of the QTL on Chromosome 1. First, we confirmed the existence of two separate QTL (*Nob3* and *Nob5*), demonstrating the occurrence of at least two adipogenic alleles on Chromosome 1 (Fig. 5A). Congenic mice carrying 91 Mb ($Nob3.91^{N/B}$, 104–195 Mb) of the QTL interval had a significantly higher body weight than homozygous B6 mice ($Nob3.91^{B/B}$), and higher body weight than mice carrying heterozygous alleles for 38 Mb of the distal region ($Nob3.38^{N/B}$, 157–195 Mb) (Fig. 5B).

Additional congenic mice carrying different fragments of the obesity locus (Fig. 5C) revealed that the homozygous NZO allele carriers $Nob5.53^{N/N}$ (104.0–156.8 Mb) had a significantly higher body weight compared with congenic line $Nob5.40^{N/N}$ (104.0–143.8 Mb; D1Mit468-D1Mit423, Fig. 5D). This further confirmed the critical *Nob5* fragment and allowed a definitive refinement of the relevant interval with coordinates from 143.8 to 156.8 Mb (D1Mit423-D1Mit14). The procedure resulted in a final reduction to three candidate genes; *Pla2g4a*, *Cep350* and *Soat1* (Fig. 5C), which overlap with the genes identified with the bioinformatics approach. Expression analysis of these genes in gonWAT of congenic mice revealed a trend toward a higher expression of *Pla2g4a* in homozygous NZO mice carrying the critical *Nob5* fragment (Fig. 5E). The gene *Soat1* showed a significantly higher expression in *Nob5* allele carriers compared with controls, although the level of expression in adipose tissue was remarkable low.

Expression analysis in adipose tissue of pigs and humans

In order to translate our findings to other organisms, we first investigated the expression of the orthologues in subcutaneous (s.c.) and visceral adipose tissue of female lean and obese Göttingen minipigs. The expression of *SOAT1* revealed a positive correlation with percentage of fat mass in s.c. ($r = 0.511$, $P < 0.02$) as well as in visceral ($r = 0.441$, $P < 0.05$) adipose tissue (Fig. 6A and B). In visceral adipose tissue, also *PLA2G4A* showed a significantly higher expression in obese pigs compared with lean controls ($r = 0.716$, $P < 0.005$) (Fig. 6B).

To clarify if the identified genes are relevant for human obesity, we also analyzed the expression of all three candidate genes of the *Nob5* locus in adipose tissue of lean and overweight subjects (Fig. 7). The expression of *PLA2G4A* showed a positive correlation with BMI in s.c. fat ($r = 0.337$, $P < 0.02$); an effect that was even stronger in visceral fat ($r = 0.387$, $P < 0.005$), whereas no differences were detected for *CEP350* nor *SOAT1* (Fig. 7A). As the obesity locus *Nob5* was more pronounced in female mice we asked whether the effect of *PLA2G4A* is also stronger in adipose tissue of women. Indeed, the Pearson correlation of *PLA2G4A* expression with BMI ($r = 0.230$, $P =$ n.s.; $r = 0.400$, $P < 0.02$) and also percentage of body fat ($r = 0.307$, $P =$ n.s.; $r = 0.724$, $P < 0.0001$; Fig. 7B) revealed a stronger effect in female than in male subjects in visceral adipose tissue. Thus, according to their phenotype data we divided the human subjects into lean and overweight and observed a significantly higher expression in overweight compared with lean controls (Fig. 7C).

Together, the data from pig and human adipose tissue indicate an association with an increased expression of *PLA2G4A* in pigs and subjects with a higher body fat/body weight suggesting *Pla2g4a*/*PLA2G4A* as critical regulator of the identified locus. The data furthermore demonstrate that the complex collective cross approach is sufficient for the identification of disease genes in human.

Discussion

Previous linkage studies of single outcross populations of inbred mouse strains have led to the identification of numerous genetic loci that predispose for obesity and T2D, but subsequent positional cloning of candidates has mostly been unsuccessful (23–25). In our present study we took advantage of the genetic architecture of widely used inbred mouse strains that share common genetic ancestry (26). By establishing a computational framework combining linkage data from outcross populations

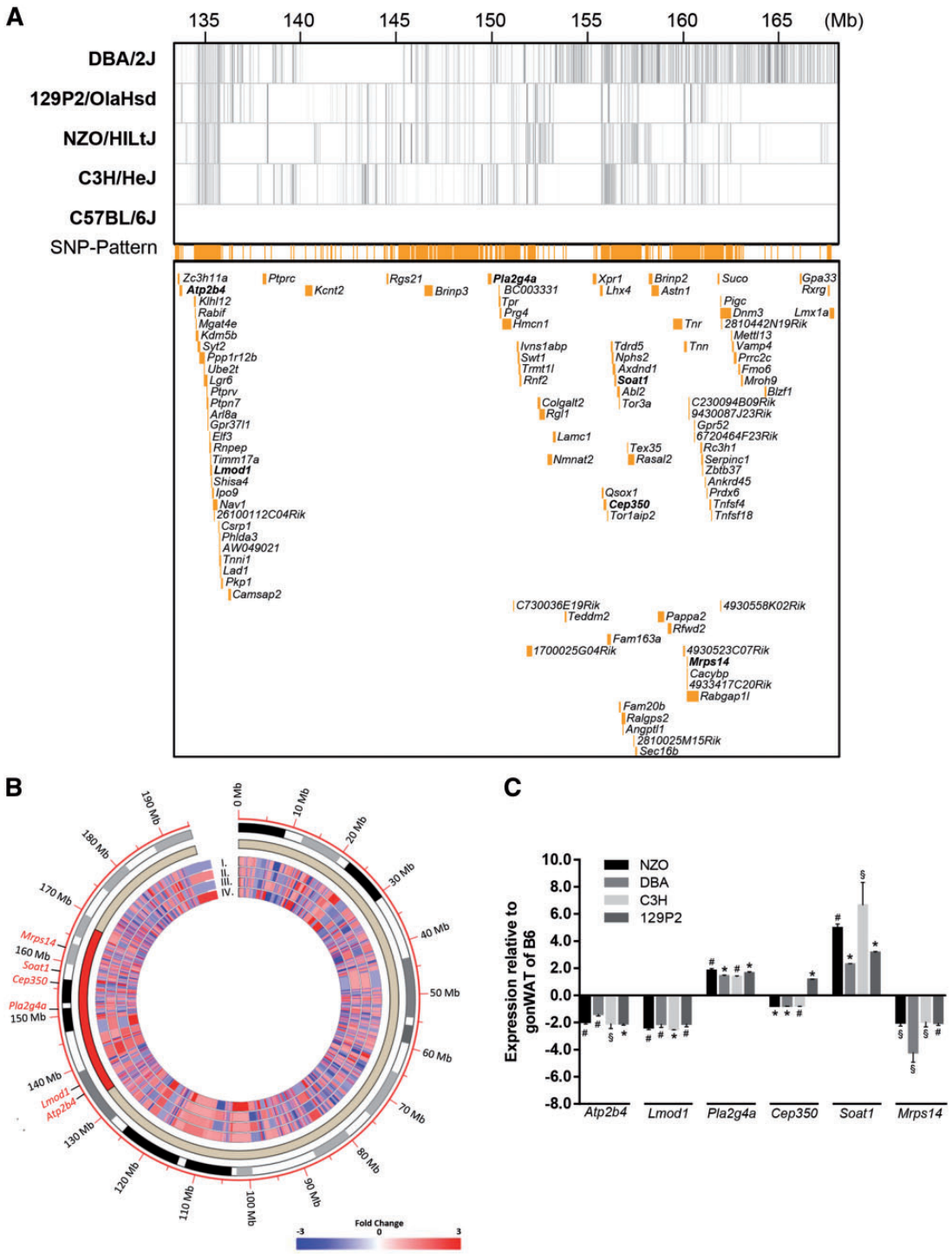

Figure 4. Integration of different NZO backcross populations for comparative cross analysis. (**A**) Single nucleotide polymorphisms (SNPs) within the critical region (133.6–168.2 Mb) between the different parental strains and C57BL/6J are presented in grey vertical lines. Identical SNPs between DBA/2J, 129P2/OlaHsd, NZO/HILtJ, C3H/HeJ that were different to C57BL/6J are highlighted in orange. Lower panel represents the corresponding genes located within the polymorphic haplotype block. Owing to clarity, gene models are not listed. Haplotype map was generated by using a script written by Gatti (21). It is based on SNPs published by the Sanger Wellcome Trust Institute (8,9). (**B**) Circos plot showing mouse Chromosome 1 with integration of the transcriptome data from gonWAT of the different parental strains (I. DBA; II. 129P2; III. NZO; IV. C3H) compared with B6. The red bar within the outer circle indicates the critical region of the *Nob5* locus and out of the differently expressed genes, six were located within the relevant haplotype block. Expression data are presented as fold change relative to B6 of three to four samples/strain. The plot was generated with the RCircos package (19,20). (**C**) Expression data in gonWAT of the six candidate genes located within the critical haplotype block of *Nob5*. Differences are presented as fold change of male DBA, 129P2, NZO and C3H mice in comparison to B6 at the age of 6 weeks ($n = 3$–4). Data represent mean±SEM. Differences between groups were calculated with one sample *t*-test. $^{\S}P < 0.05$; $^{\#}P < 0.01$; $^{*}P < 0.001$.

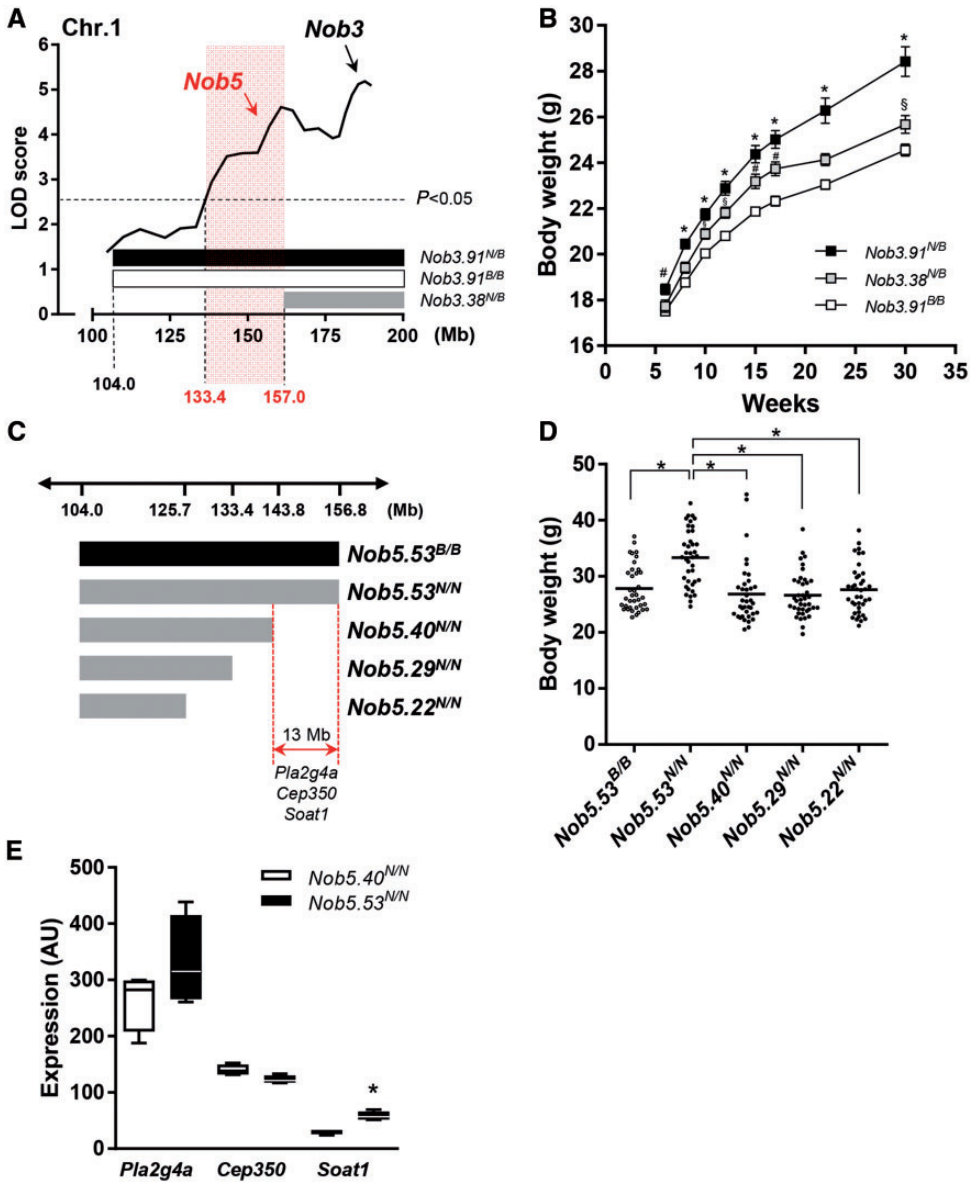

Figure 5. Confirmation of the obesity QTL *Nob5* by generation of congenic mice. (**A, B**) Definition of two adipogenic alleles on Chromosome 1 by generation of congenic mice (N4 generation) carrying 91 Mb (*Nob3.91*$^{N/B}$, $n = 32$) or 38 Mb (*Nob3.38*$^{N/B,}$ $n = 21$) of the critical region on Chromosome 1 in comparison to control mice with homozygous B6 alleles (*Nob3.91*$^{B/B}$, $n = 63$). (**C, D**) Body weight of congenic female mice (N10 generation) at the age of 22 weeks on a high-fat diet reflecting different fragments of the obesity locus *Nob5* and differentially expressed genes between B6 and NZO, DBA, C3H, 129P2 localized in the critical 13 Mb fragment. Differences between groups were calculated with one-way ANOVA followed by post hoc Bonferroni test. $^{\S}P < 0.05$; $^{\#}P < 0.01$; $^{*}P < 0.001$. $n = 47$–50. (**E**) Gene expression analysis in gonWAT of congenic mice (8 weeks of age) carrying 40 Mb (*Nob5.40*$^{N/N}$, $n = 4$) or 53 Mb (*Nob5.53*$^{N/N}$, $n = 4$) of the *Nob5* locus. Statistical differences between groups were calculated with Mann–Whitney test ($^{*}P < 0.05$). Data represent mean±SEM. B/B, homozygous B6; N/N, homozygous NZO; N/B, heterozygous NZO/B6.

derived from NZO and four lean, commonly used but genetically distinct inbred mouse strains (B6, DBA, 129P2, C3H) and by superimposing strain-specific haplotype information, genome wide-expression data we reduced the number of candidate genes within a critical interval linked to obesity-related traits on Chromosome 1 by two orders of magnitude. Breeding and phenotyping of recombinant congenic strains carrying B6-specific variants of three novel obesity candidate genes on a NZO background validates our unique approach, demonstrating the superior resolution of integrative genetics in comparison to classical analysis. Finally, translation of these results into humans revealed *Pla2g4a/PLA2G4A* as putative obesity gene of the *Nob5* locus.

The NZO strain has evolved as an established polygenic mouse model for T2D exhibiting early onset obesity, hyperglycemia and late onset loss of pancreatic beta cells (10,11). In contrast, the lean strains B6, DBA, 129P2 and C3H display generally low but varying susceptibility for obesity and T2D-related traits (23,27). Many studies have demonstrated that the penetrance of obesogenic and diabetogenic alleles strongly depends on the genetic background (28,29). Importantly, B6 mice were used for building the reference mouse genome, whereas 129 strains are the source of the most common embryonic stem cell lines used in generating knockout mice. The lean DBA/2 strain develops obesity-induced diabetes when fed a high-fat diet and is genetically distinct from most other strains

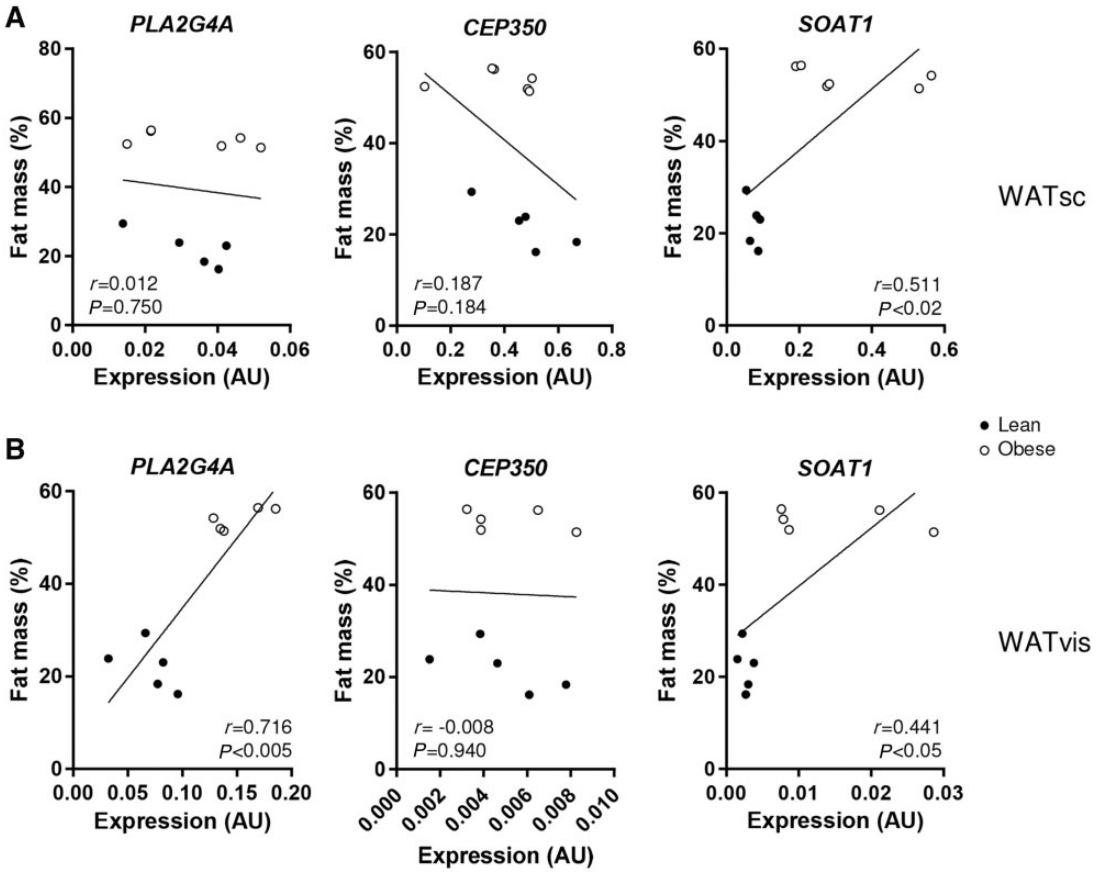

Figure 6. Expression analysis in adipose tissue of female lean and obese Göttingen minipigs. Correlation of the expression of the different *Nob5* candidate genes and fat mass (%) in s.c. (**A**) and visceral (**B**) adipose tissue of female Göttingen minipigs ($n = 5–6$). filled circles: lean pigs; open circles: obese pigs.

used in diabetes research (25,30,31), whereas the lean C3H mouse represents a diabetes-resistant model (23).

In the present study, we conducted linkage analysis of ~2400 N2 mice, both male and female offspring from four backcross populations. Several phenotypes measured in the four backcross populations depended on each other and therefore correlated. For the discovery of new obesity genes we measured body weight, fat mass and the weight of adipose tissue depots were analyzed. For the identification of genes involved in (dys)-regulation of glucose homeostasis, the most valuable and informative traits were the blood glucose concentrations, plasma insulin and total pancreatic insulin values. We catalogued more than 360 QTL in males and 230 QTL in females for various metabolic traits. The most prominent consensus regions with linkage to obesity traits were localized on Chromosomes 1 and 4, and a major locus affecting metabolic traits associated with diabetes-related traits was mapped to Chromosome 4. Some of these QTL, e.g. on Chromosome 4 for blood glucose and on Chromosome 14 for body fat, are shared between the different strains, assuming that they originate from the same allelic variation, whereas other QTL, e.g. on Chromosome 5 for body weight and on Chromosome 8 for blood glucose, are specific for one certain cross. This further demonstrates that the application of one common breeding partner in different backcross panels facilitates interpretation of multiple linkage signals in the genetics of complex diseases.

The direct approach for narrowing down a critical QTL region is the positional cloning by isolation of the QTL through the generation of congenic lines (32). However, this procedure is a time- and labor-intensive effort to identify the causal gene (33). The nomination of genes underlying QTL effects can also be facilitated by improving the mapping resolution and by utilizing genomic sequence and transcriptome information (34). Adding further genotyping markers to increase the mapping resolution is helpful but limited by the number of recombination events within a specific offspring population. Therefore, Li *et al.* (32) developed a method for combining data from multiple crosses to increase the power and mapping resolution. This has successfully been used to increase the resolution of shared QTL and to identify new QTL not recognized in individual crosses for traits such as lipid metabolism (35). We have further developed this method by applying computational biology to include and combine for the first time strain-specific information from expression profiling and sequence information.

Owing to the importance of B6 as reference genome and the absence of linkage signals from all other strains except B6, we selected the B6-specific QTL on Chromosome 1 to test and validate the entire procedure of combined-cross analysis for the identification of novel disease genes. Two distinct peaks at 153 Mb (*Nob5*) and 190 Mb (*Nob3*) were detected on Chromosome 1. The causal gene variant for the distal peak *Nob3* has already been identified and assigned to the transcriptional regulator *Ifi202b* (13). Owing to a large genomic deletion, the *Ifi202b* gene is not expressed in tissues of B6 mice but highly abundant in NZO mice (14). As the *Nob5* locus occurred exclusively in the NZOxB6 cross, haplotype information was assembled to define

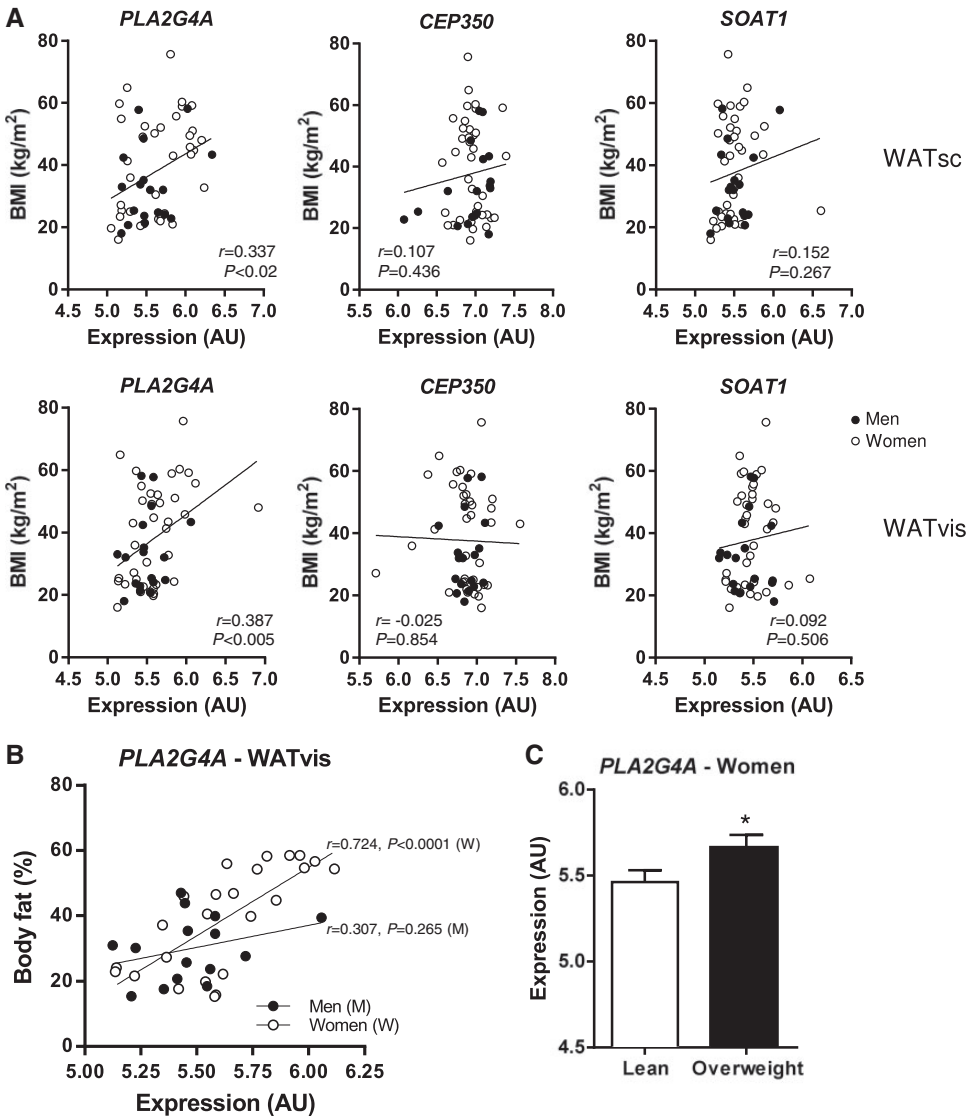

Figure 7. Expression analysis in human adipose tissue. (**A**) Correlation of the expression of the different *Nob5* candidate genes and BMI in s.c. (upper panel) and visceral (lower panel) adipose tissue ($n = 55$). (**B**) Correlation of *PLA2G4A* expression and body fat in visceral adipose tissue of male ($n = 18$) and female ($n = 37$) human subjects. (**C**) Expression of *PLA2G4A* in lean ($n = 11$) and overweight ($n = 26$) female subjects. Data represent mean±SEM. The Pearson correlation test was used to determine the relationship between *PLA2G4A* expression and different metabolic parameters. Differences between lean and overweight subjects were calculated with one-tailed Student's *t*-test. *$P < 0.05$. M, men; W, women.

regions within this locus that were unique for the B6 genome. In addition transcriptome data were integrated allowing to significantly minimize the total number of potential candidate genes from 502 genes within *Nob5* to six genes (*Atp2b4*, *Pla2g4a*, *Lmod1*, *Cep350*, *Soat1* and *Mrps14*) as most likely causal genes for the linkage signal.

In fact, *Pla2g4a*, *Cep350* and *Soat1* were validated as obesity candidate genes after phenotyping of interval-specific congenic lines, whereas *Soat1* and tendentially also *Pla2g4a* showed a differential expression in adipose tissue of *Nob5* congenic mice. By analyzing the expression of the corresponding orthologues in adipose tissue of pigs and human subjects only *PLA2G4A* revealed a significant difference between lean and overweight subjects. *PLA2G4A* expression in human adipose tissue positively correlates with BMI and percentage of body fat, a similar pattern that was also observed in pigs and mice. Interestingly, *Pla2g4a* (cytosolic group IVA phospholipase A2), also designated cPLA2-α, was described to be involved in body weight regulation. cPLA$_2$α is one of the most studied members of the PLA$_2$ superfamily of enzymes, whose common feature is to hydrolyze the fatty acid present in the *sn*-2-position of glycerophospholipids (36–38). Recently, cPLA$_2$α was identified as an early factor of adipocyte differentiation *in vitro*. High-fat diet fed animals deficient in cPLA$_2$α showed a reduced gain in body weight and fat mass (36). Moreover, genomic variants associated with resistance to high-fat diet induced obesity have been described in a primate model (39). Another PLA gene has previously been suggested to be connected to obesity in mice as the candidate genes *Ccna2* and *Trpc3* affected the serum levels of the metabolite PC aa C42: 1 via the enzymes CHKA and PLA2G1B (40).

However, within the critical interval of the *Nob5* locus two miRNAs and 24 long non-coding RNAs (lncRNAs) are listed in addition to the protein coding genes. Out of these 24 lncRNAs, 5 revealed a total number of 36 SNPs between the breeding

partners NZO and B6. Therefore, it cannot be excluded entirely that the sequence differences within one and/or more lncRNAs are causative for the obesity effect of the *Nob5* locus.

Linkage analysis for different blood metabolites was performed for the first time in a collective cross approach. Several chromosomal regions with a linkage to acylcarnitine species were localized and overlap to the QTL map of obesity- and blood glucose-related traits. Increased plasma concentration of different acylcarnitine species have already been observed under conditions of obesity and T2D and utilized as early biomarkers for insulin resistance (41–45). However, further investigations are necessary to examine the exact physiological role of the different blood acylcarnitines in relation to the metabolic traits and QTL detected in the current study. Plasma-free amino acids have also been highlighted in their association with the risk of developing T2D in individuals with differing degrees of obesity (46). Within the current study chromosomal regions associated with altered glycine (Gly) and phenylalanine (Phe) concentrations were detected on Chromosomes 4 and 10, respectively. Increased circulating levels of Phe have been reported to be associated with states of insulin resistance, T2D or cardiovascular disease (47–49). In studies using blood metabolites to predict T2D and determining correlations between metabolites and insulin sensitivity (43,49), Phe provided one of the strongest associations. The positive correlation between the amino acid and insulin secretion may be involved in pathways to compensate early stage of insulin resistance through stimulating insulin secretion (46).

Together, our approach provides evidence that combining multiple strain-specific linkage signals and expression data substantially improves the resolution of genetic mapping of disease genes and provides instant benefits by means of information on the allelic state of QTL that considerably facilitate gene identification. The method allowed the identification of three putative obesity genes which were also present in a smaller fragment of an interval-specific recombinant congenic strain displaying elevated body weight. The final translational approach allowed the identification of *PLA2G4A* as interesting human obesity gene. Moreover, by combined-cross analysis the generated data can also be used repeatedly for the identification of additional genetic variants on other chromosomes to obtain further insights into the genetic determinants of this complex disease.

Materials and Methods

Animals

Female NZO mice from our own breeding colonies (NZO/HIBomDife) (13) were mated with male DBA/2J (Jackson Lab, Maine, USA), C57BL/6JRI (B6; Janvier Laboratories, Le Genest St Isle, Fance), C3H/FeJ (C3H; Helmholtz Center, Munich, Germany) or 129P2/OlaHsd (129P2; German Institute of Human Nutrition, Nuthetal, Germany) mice to produce F1 hybrids. Male F1 mice from each cross were subsequently backcrossed to female NZO to produce N2 mice that were metabolically characterized. We used the N2 approach in order to increase the probability of diabetes in the offspring. Mice were housed in Type 2 or Type 3 macrolon cages with bedding made of soft wood shavings (spruce) in groups of three to six and kept under standard conditions (conventional germ status, 22°C with 12 h light/dark cycling). After weaning at 3 weeks of age animals were placed on a high-fat diet (HFD, D12451 Research Diets, Inc., NJ, USA), containing 45, 35 and 20% kcal from fat, carbohydrate and protein, respectively. Recombinant congenic mice were bred by repeated backcrossing of male mice selected for the *Nob5* locus (*N/N* or *B/B*) with NZO females and were characterized in the N10 generation. All experiments were approved by the ethics committee of the State Agency of Environment, Health, and Consumer Protection (State of Brandenburg, Germany) and Ethics Committee of the State Ministry of Agriculture, Nutrition and Forestry (State of North Rhine-Westphalia, Germany).

Body composition

Body composition was determined by non-invasive nuclear magnetic resonance spectroscopy (EchoMRI™-100 system, Echo Medical Systems, Houston, USA).

Blood glucose progression

Blood glucose was measured in the morning between 8 and 10 am with a CONTOUR® XT glucometer (Bayer Consumer Care AG, Leverkusen, Germany).

Plasma analysis

Insulin concentrations were determined by ELISA (ALPCO, Salem, USA). Triglyceride and free glycerol concentrations were determined with the Triglyceride/Glycerol Calorimetric Assay (Sigma, Hamburg, Germany). Cholesterol content was determined by Cholesterol liquicolour colorimetric assay (Human Gesellschaft für Biochemica und Diagnostica mbH, CHOD-PAP-Method, Wiesbaden, Germany). Non-esterified fatty acids were quantified with an assay purchased from Wako Chemicals (ACS-ACOD-Method, Neuss, Germany) All measurements were applied according to the manufacturer's recommendations.

Pancreatic insulin content

Whole pancreas was homogenized in ice-cold acidic ethanol (0.1 mol/l HCl in 70% ethanol) and incubated overnight at 4°C. After centrifugation (16 000*g*, 10 min) insulin content was measured in the supernatant fraction with the Mouse High Range Insulin ELISA (ALPCO).

Hepatic triglycerides

In order to determine triglyceride content in the liver, ∼20 mg of powered tissue was homogenized in 10 mmol/l sodium dihydrogen phosphate, 1 mmol/l EDTA and 1% (vol./vol.) polyoxyethylene-10-tridecyl ether. After homogenization with a TissueLyser (Qiagen, Hilden, Germany) for 5 min, enzymes were deactivated by incubating the samples at 70°C while shaking and then rested on ice for a minimum of 5 min before centrifugation at 13 000*g* and 4°C for 10 min. Liver triglyceride content in the supernatant was quantified using a commercial kit (RandoxTR-210, Crumlin, UK). Briefly, during a two-step enzyme-linked reaction an indicator dye is formed and the absorbance measured at 500 nm. The triglyceride content was determined using a standard curve and were normalized to protein concentration (μg TG/μg protein).

Metabolite profile in blood samples

At the age of 10 weeks whole blood was collected between 8 and 10 am on filter paper (Protein Saver™ 903™ Card, GE Healthcare Bio-Sciences Corp., Chalfont St Giles, UK). The determination of blood amino acid and acylcarnitine content was

performed using liquid chromatography-tandem mass spectrometry as previously described (50).

Genotyping

Genomic DNA was extracted from mouse tail-tips using the Invisorb Genomic DNA Kit II (STRATEC Molecular GmbH, Berlin, Germany), following the manufacturer's instructions. Competitive allele-specific PCR (KASP) genotyping of the different NZO backcross mice was performed by LGC genomics (LGC group, Teddington, UK). Recombinant congenic mice containing the *Nob5* locus were genotyped by PCR with oligonucleotide primers obtained from Sigma and the microsatellite length was determined by non-denaturing polyacrylamide gel electrophoresis.

RNA extraction and gene expression in mice

Total RNA was isolated and purified from murine tissue using TRIzol™ reagent (Invitrogen, Carlsbad, USA). From parental NZO and B6 mice (6 weeks of age) and congenic animals (8 weeks of age), 20–100 mg samples of gonWAT were aliquoted for RNA extraction. The procedure was carried out according to the manufacturer's instructions. RNA quality was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the manufacturer's instructions were followed to measure RNA integrity (RIN). Samples with RIN values >8 were subsequently selected for microarray analysis. Genome-wide mRNA gene expression was performed by Oaklabs GmbH (Henningsdorf, Germany) using a Mouse GE 8× 60k Microarray V2 (Agilent), and data were normalized by the method of ranked median quantiles (51).

Linkage analysis

Genome-wide scans of N2 mice [NZOxB6, $n = 311/308$ (males/females); NZOxDBA, $n = 288/299$; NZOxC3H, $n = 329/310$; NZOx129P2, $n = 290/307$] including the genetic map, genotyping errors and linkage between individual traits and genotypes were assessed with the software package R/qtl (version 1.04-8) using the expectation–maximization (EM)-algorithm and 1000 permutations (52). Two-way interactions (epistasis) were estimated with a two-QTL scan implemented by the R/qtl package.

Bioinformatic packages

A detailed description of the different bioinformatics tools used in the study is described in Supplementary Material, Table S1.

Expression analysis in pigs

Paired samples from s.c. and omental fat tissue of lean and obese adult Göttingen minipigs ($n = 5$–6 per group) were used for expression analysis (Renner, S. *et al.*, Papers in preparation). Total RNA was isolated with TRIZOL reagent (Invitrogen) and 0.5 μg of RNA samples were reverse-transcribed in a final volume of 20 μl using RevertAid Reverse Transcriptase (ThermoFisher Scientific, Darmstadt, Germany) according to the manufacturer's instructions. For quantitative RT-PCR a TaqMan gene-expression analysis was carried out in a StepOne machine (ThermoFisher Scientific) using primers (0.5 μM), 2 μl cDNA and the PowerUp SYBR Green Master Mix (ThermoFisher Scientific) in a final volume of 10 μl. Transcript copy numbers were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA copies.

Expression analysis in humans

Paired samples of s.c. and omental visceral adipose tissue were obtained from 55 individuals (38 women, 17 men). The age ranged from 18 to 85 years and the BMI from 16.1 to 75.5 kg/m^2. All adipose tissue samples were collected during laparoscopic abdominal surgery as described previously (53). Adipose tissue was immediately frozen in liquid nitrogen and stored at -80°C. RNA from adipose tissue was extracted by using RNeasy Lipid tissue Mini Kit (Qiagen). Quantity and integrity of RNA was monitored with NanoVue plus Spectrophotometer (GE Healthcare, Freiburg, Germany). One microgram of total RNA from SC and Vis adipose tissue (305 ng RNA from adipocytes and SVF) was reverse-transcribed with standard reagents (Life technologies, Darmstadt, Germany). Expression of PLA2G4A, CEP350 and SOAT1 was measured with HT12v4 expression bead chips (Illumina, Inc., San Diego, CA, USA). The study was approved by the Ethics Committee of the University of Leipzig (approval no: 159-12-21052012), and performed in accordance to the declaration of Helsinki. All subjects gave written informed consent before taking part in this study.

Statistical analysis

Statistical analysis was performed by either unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by post hoc Bonferroni test as appropriate. Statistical analyses were conducted using the SPSS software. The Pearson correlation test was used to determine the relationship between the expression and different metabolic parameters in pigs and human subjects. A *P*-value <0.05 was considered significant and values are expressed as means ±SEM.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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