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Identification of genetic loci associated with different responses to high fat diet induced obesity (DIO) in C57BL/6N and C57BL/6J substrains

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ABSTRACT

We have recently demonstrated that C57BL/6NTac and C57BL/6JRj substrains are significantly different in their response to high fat diet induced obesity (DIO). The C57BL/6JRj substrain seems to be protected from DIO and genetic differences between C57BL/6J and C57BL/6N substrains at 11 SNP loci have been identified. To define genetic variants as well as differences in parameters of glucose homeostasis and insulin sensitivity between C57BL/6NTac and C57BL/6JRj substrains which may explain the different response to DIO, we analysed 208 first backcross (BC1) hybrids of C57BL/6NTac and C57BL/6JRj [(C57BL/6NTacxC57BL/6JRj)F1xC57BL/6NTac] mice. Body weight, epigonadal and subcutaneous fat mass, circulating leptin, as well as parameters of glucose metabolism were measured after 10 weeks of high fat diet (HFD). Genetic profiling of BC1 hybrids were performed using TaqMan SNP genotyping assays. Furthermore, to assess if SNP polymorphisms could affect mRNA level, gene expression analysis was carried out in murine liver samples. Human subcutaneous adipose tissue was used to verify murine data of SNAP29. We identified four gender-specific variants which are associated with the extent of HFD induced weight gain and fat depot mass. BC1 hybrids carrying the combination of risk or beneficial alleles exhibit the phenotypical extremes of the parental strains. Murine and human SC expression analysis revealed *Snap29* as strongest candidate.

Our data indicate an important role of these loci in responsiveness to HFD induced obesity and suggest genes of the synaptic vesicle release system such as *Snap29* being involved in the regulation of high fat DIO.

Keywords: C57BL/6, high fat diet, DIO, genetic background, visceral fat mass, SNAP29, obesity

Background

Experimental animal models offer a great opportunity to overcome heterogeneity and various environmental factors influencing obesity and associated disorders. The C57BL/6J strain is the single most widely used inbred strain and its genome has been extraordinarily well categorized with the most complete sequence data available produced by the Mouse Genome Sequencing Consortium (33, 48). C57BL/6J mice have been extensively used as a control strain in the study of metabolic diseases or diet induced obesity (DIO) as well as background strain for transgenic and knockout mice (10). And while often different substrains are treated as identical (referred to as C57 or B6), determination of the exact background is essential when examining the genotype due to potential background effects on the phenotype. It is well known, that the genetic background influences the phenotype as demonstrated for the *ob/ob* mutation showing large differences in diabetes susceptibility on BTBR and C57BL/6J genetic backgrounds (43). Recently it was reported that mispairing of different C57BL/6 substrains can lead to a significant bias of results (5), reiterating the importance of the genetic background when designing and studying genetically engineered mice. Many recent studies reported phenotypical and genetic differences among B6J substrains (4, 8). In particular, differences in behavior (19, 40), alcohol and drug responsiveness (12, 18, 32) as well as glucose homeostasis (46) have been reported.

One extensively studied genetic difference between C57BL/6J and C57BL/6N mice was found in the nicotinamide nucleotide transhydrogenase (*Nnt*) gene on chromosome 13. In the C57BL/6J strain, a missense (methionine to threonine) mutation, in combination with the in-frame 5 exon deletion mutation (eliminating four putative transmembrane helices) results in a truncated Nnt variant and markedly lower Nnt protein expression in liver and islets (14, 46). Nnt activity has been linked to impaired glucose metabolism and insulin secretion (13, 46).

Recent genotyping of single nucleotide polymorphisms (SNPs) identified further genetic differences between C57BL/6J and C57BL/6N substrains at 11 loci (24, 31, 51). One SNP was found between C57BL/6J substrains while no genetic differences were detected among C57BL/6N substrains (31, 51). Five of the eleven loci map within known genes. The

genotype of fibroblast growth factor 14 *(Fgf14)*, LIM and senescent cell antigen-like domains 1 *(Lims1)*, amyloid precursor-like protein 2 *(Aplp2)* and soluble n-ethylmaleimide sensitive factor attachment protein 29 *(Snap29)* differs between C57BL/6N and C57BL/6J strains and a SNP associated with N-acetylated alpha-linked acidic dipeptidase-like 2 (*Naaladl2)* between certain C57BL/6J substrains.

Studies on high fat diet induced obesity revealed differences between core substrains C57BL/6NTac and C57BL/6JRj regarding their response to a HFD and the development of DIO (36). Recently, we reported phenotypical differences under HFD conditions between C57BL/6NTac and C57BL/6JRj substrains and found that the C57BL/6JRj strain is protected against DIO independently of physical activity and food intake (24).

To define the causal genetic differences which may underlie and explain the varying HFD responsiveness and DIO manifestation in C57BL/6J and C57BL/6N mice, we genetically and phenotypically characterized first backcross (BC1) hybrids of C57BL/6NTac and C57BL/6JRj mice [(C57BL/6NTacxC57BL/6JRj)F1 x C57BL/6NTac]. In addition, to assess if SNP polymorphisms could affect mRNA levels, real-time based gene expression analysis was carried out in murine liver samples and human subcutaneous adipose tissue.

MATERIAL AND METHODS

***Animals and phenotyping*.** In 2007, breeding pairs from Taconic Farms, Inc. (C57BL/6NTac; Hudson, New York, USA) and from Janvier (C57BL/6JRj; Le Genest Saint Isle, France) were obtained and bred in our animal facility under standardized environmental conditions. C57BL/6NTac mice were crossed with C57BL/6JRj and the (C57BL/6NTacxC57BL/6JRj)F1 hybrids were backcrossed onto C57BL/6NTac to generate first backcross hybrids (BC1). F1 hybrids (N=20 Female/Male 10/10) and all backcross hybrids (N= 208; Female: N=99, Male: N=109) were fed a diet containing 58% fat in total calories with sucrose (E15772-34, Ssniff, Soest, Germany; composition equals D12331 (Research Diets, New Brunswick, NJ, USA) as fed in (22)) for 8 weeks beginning at 4 weeks of age. All animals were kept in groups of 4 in Macrolon cages (Size 2, Ehret GmbH,

115 Emmerdingen Germany) in the same room and had free access to food and water. Body
116 weight was recorded weekly and at the end of observation period liver weight, visceral fat
117 mass, subcutaneous fat mass and HbA1c (%) were measured. Blood samples were obtained
118 after sacrifice. Serum leptin as well as insulin concentrations were measured using
119 commercially available ELISA kits (Crystal Chem, Downers Grove, IL). Organs and fat
120 depots were weighed and organ or depot mass was related to whole body mass to obtain
121 *relative organ* or *depot weights*, respectively.
122 All experiments were conform to the *Guide for the Care and Use of Laboratory Animals*
123 published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996)
124 and were approved by the local authorities (Regierungspräsidium Leipzig) of the state of
125 Saxony, Germany, as recommended by the responsible local animal ethics review board.
126 ***SNP genotyping.*** DNA was extracted from tail tips using the DNeasy kit (Qiagen, Hilden,
127 Germany). SNP genotyping was done using the TaqMan SNP Genotyping assay according
128 to the manufacturer's protocol (Applied Biosystems Inc., Foster City, CA). To assess
129 genotyping reproducibility, a random 5% selection of the sample was re-genotyped for all
130 SNPs; all genotypes matched initial designated genotypes. Furthermore, genomic DNA from
131 F1 hybrids and parental strains served as controls. Call rates of all SNPs ranged from 98 to
132 100%.
133 ***Expression analysis.*** Total RNA was isolated from snap-frozen liver (N=7 per genotype)
134 and subcutaneous adipose tissue (N=6 per genotype) samples using RNeasyMini Kit
135 (Qiagen, Hilden, Germany). RT-PCR was performed with the TaqMan 7500 system (ABI,
136 Darmstadt, Germany). *36B4* was used as an internal reference. The following primers were
137 used: *Snap29* 5´-AGGCTACAGGATGCAGAACTAGACT-3´ (forward) and 5´-
138 TGTCATCCTGTTCCTCAATTTCT -3´ (reverse); *Aplp2* 5´-CCGAATGGACAGGGTAAAGA-3´
139 (forward) and 5´-CACAAGCTGCTGCTTCTCAC-3´ (reverse); *Lims1* 5´-
140 GGAGCTGAAAGGGGAGCTAT-3´ (forward) and 5´- TGCCCAAGAAATGGTTTTTC-3´
141 (reverse); *Snca* 5´-CAGAGGCAGCTGGAAAGACA-3´ (forward) and 5´-

142 CACCACTGCTCCTCCAACAT-3´ (reverse). Relative gene expression was calculated using
143 the standard curve method.
144 ***Human subjects.*** Subcutaneous adipose tissue was obtained from 234 Caucasian men (*N* =
145 84) and women (*N* = 150) who underwent open abdominal surgery for cholecystectomy,
146 appendectomy, weight reduction surgery, abdominal injuries or explorative laparotomy
147 (Table 4). The age ranged from 18 to 89 years and body mass index from 14.1 to 71.0 kg/m^2.
148 Sixty nine subjects had type 2 diabetes. All subjects had a stable weight with no fluctuations
149 of more than 3% of the body weight for at least three months before surgery. Patients with
150 severe conditions including generalized inflammation or end stage malignant diseases were
151 excluded from the study. Samples of visceral and subcutaneous adipose tissue were
152 immediately frozen in liquid nitrogen after explantation. The study was approved by the
153 Ethics Committee of the University of Leipzig (Germany). All subjects gave written informed
154 consent before taking part in the study.
155 ***Measures of body fat content.*** BMI was calculated as weight (in kg) divided by the square
156 of height (in m). Waist and hip circumferences were measured, and the WHR was calculated.
157 Percentage body fat was measured by dual-energy X-ray absorptiometry (DEXA). In
158 addition, abdominal visceral and subcutaneous fat areas were calculated using computed
159 tomography (CT) scans at the level of L4–L5 (1).
160 ***Analysis of human SNAP29 gene expression.*** Human *SNAP29* mRNA was measured by
161 quantitative real-time RT-PCR using the TaqMan assay (Hs00191150_m1) and
162 hypoxanthine guanine phosphoribosyltransferase (HPRT, Hs01003267_m1) as house-
163 keeping gene and fluorescence was detected on an ABI PRISM 7000 sequence detector
164 (Applied Biosystems, Darmstadt, Germany). Human *SNAP29* gene expression was
165 determined by the standard curve method and normalized to the house-keeping gene HPRT
166 as previously described (25, 30). 1 µg of total RNA (TRIzol Reagent by Life Technologies,
167 Grand Island, NY) from paired subcutaneous and visceral adipose tissue samples was
168 reverse transcribed with standard reagents (Life Technologies, Grand Island, NY) as shown
169 elsewhere (25, 30). Quantitative real-time reverse transcription-PCR was performed for each

sample in duplicate with total RNA, 1×TaqMan Universal Master Mix no AmpErase UNG, 6.25 units of murine leukemia virus reverse transcriptase (both from Applied Biosystems) and gene-specific primers-probe sets, using an ABI PRISM 7500 sequence detector (Applied Biosystems). cDNA samples were incubated in the ABI PRISM 7500 sequence detector for an initial denaturation at 95°C for 10 min, followed by 40 PCR cycles, each cycle consisting of 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min. Accuracy of RNA quantitation was optimized by gene-specific primer-probe sets that span intron-exon boundaries. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

Data analysis and statistics. Data are given means ± SD. Datasets were analyzed for statistical significance using a two-tailed unpaired *t* test, or differences were assessed by one-way ANOVA using the Statistical Package for Social Science, version 20.0 (SPSS, Chicago, IL). All data are presented without correcting for multiple testing (38). To consider the findings significant, Bonferroni corrections for multiple testing would require a *P*-value of 0.005 (0.05 divided by the number of SNPs) for the SNP analyses and a P-value of 0.01 (0.05 divided by the number of considered genes) for the eQTL analyses.

RESULTS

DIO responsiveness of the F1 hybrids. The DIO responsiveness of the F1 hybrids (Fig 1A) revealed an intermediate response to the HFD between the DIO phenotypes of parental strains, indicating a polygenic cause of the observed DIO resistance in the C57BL/6JRj strain (24). As expected, all eleven SNP genotype distributions were obtained with the expected Mendelian frequency of 50 percent (Table 1). In a gender specific manner, BC1 hybrids indicated ~~significant~~ associations of SNPs rs13480122 (*Aplp2*), rs13481014, rs13478783 and rs4165065 (*Snap29*) with manifestation of DIO (Table 1). In males, we detected a significant association between rs13480122 (*Aplp2*) and a suggestive association rs13481014 between and body weight gain under HFD conditions. Male carriers of homozygous alleles of SNP rs13481014 (C/C) responded more pronounced to the HFD after 6 weeks of HFD while homozygous carriers of rs13480122 (C/C) featured significantly less body weight from beginning to end of the HFD. Homozygous carriers of rs13480122 (C/C) were already significantly leaner at the beginning of the HFD (Table 1). With respect to fat mass distribution under HFD, relative fat depot masses were suggestively associated with rs13480122 (C/C) and rs4165065 (C/C). In accordance to the body weight gain characteristics, homozygous carriers of rs13480122 (C/C) had a tendency for lower epigonadal and ~~significantly lower~~ subcutaneous fat mass. Male mice with the homozygous variant of SNP rs13481014 (C/C) had both lower epigonadal and subcutaneous adipose tissue mass (nominal p-value <0.05) (Table 2).

For female BC1 mice, homozygous carriers of SNPs rs13478783 (G/G) as well as rs4165065 (C/C) gained less weight after 8 weeks of HFD (Table 1), but exhibited only a non significantly lower epigonadal fat mass compared to heterozygous littermates (Table 2).

For both genders, we found suggestive/nominal associations between rs13478783 (G/G), rs13481014 (C/T) and lower serum leptin levels (Table 2). Additionally and only in males rs13478783 (G/G) was associated with lower serum insulin concentrations (nominal p-value <0.05) (Table 2). Combinations of respective beneficial or risk alleles (beneficial allele

combinations: male mice: (C/C) of rs13480122 and (C/T) of rs13481014; female mice: (G/G) of rs13478783 and (C/C) of rs4165065) result in an additive and even stronger effect on the extent of DIO manifestation both in male and female carriers (Table 1 and 2, Fig 1B, C).

Gene expression analysis. As mentioned, five of the eleven loci map within known genes. Therefore, we performed gene expression analysis of those known genes to elucidate if the SNP genotype affects mRNA levels. Here we detected an association between genotype and expression levels for *Snap29* (Fig 2A). Different levels of expression between genotypes were obtained in liver and subcutaneous adipose tissue samples for the *Snap29* gene (Fig 2A, B). In BL/6JRj mice *Snap29* mRNA expression is significantly higher than in BL/6NTac mice. Since *Snap29* seemed to be the strongest candidate in the murine model, we analyzed *SNAP29* expression in subcutaneous human adipose tissue. Comparison of *SNAP29* mRNA expression in subgroups of BMI <25 or >30kg/m² revealed significantly lower subcutaneous *SNAP29* mRNA expression in the obese subgroup (Fig 2C). Univariate correlation analysis of the entire study population (N=234) identified significant correlations between subcutaneous *SNAP29* gene expression and BMI (r=-0.267; p<0.001), % body fat (r=-0.241; p=0.002), CT ratio (r=0.204; p=0.004), waist and hip circumferences (waist: r=-0.268; p<0.001; hip: r=-0.063; p<0.001), subcutaneous and visceral fat area (sc: r= -0.265; p<0.001; vis: r=-0.217; p=0.002) as well as fasting plasma insulin (r=-0.211; p=0.005) and leptin serum concentrations (r=-0.258; p=0.001) (Table 3). After adjusting for age, gender and BMI, only CT ratio (r=0.191; p=0.032) remained suggestively associated with subcutaneous *SNAP29* mRNA expression.

DISCUSSION

We found that the C57BL/6JRj substrain from Janvier differed at 11 SNPs from the C57BL/6NTac substrain as it has been reported for other C57BL/6J substrains (24, 31). SNP rs13477019 within the *Naaladl2* gene represents a unique allele found in some C57BL/6J strains (31, 51). The C57BL/6JRj strain shares the same rs13477019 allele as C57BL/6N substrains (A) and thus differs from the “original” C57BL/6J (Jackson Labs or Charles River)

strain (T-allele), as it has been reported for two C57BL/6J substrains commonly used in Japan (C57BL/6JJcl and C57BL/6JmsSlc) (31). The Japanese substrains C57BL/6JJcl and C57BL/6JmsSlc were separated from the C57/BL/6J strain from The Jackson Laboratory in the late 1980s (1989 and 1987) and C57BL/6JRj mice were transferred from Centre de Service des Animaux de Laboratoire (Orleans, France) to Janvier at F_{172} in 1993, according to the Janvier product catalog. The strain has been frequently used in various studies as control and background strain for knock-out mice (2, 3, 6, 23, 28, 29).

We have recently demonstrated that substrains of C57BL/6, C57BL/6NTac and C57BL/6JRj have a heterogeneous responsiveness to HFD and manifestation of DIO (24). These data support previous findings of gender- and substrain-related differences in the response to DIO in C57BL/6NJ mice and C57BL/6J mice (both from Jackson Laboratory) (36). In addition, DIO was dependent on the diet itself (36). Under a high fat diet (60% of calories), C57BL/6J mice gained significantly more weight, while under a moderate fat diet (10% of calories) C57BL/6NJ mice outgained their C57BL/6NJ counterparts (36).

Since different response to DIO between C57BL/6JRj and C57BL/6N mice could not be explained by increased physical activity or differences in food intake, we investigated the impact of the identified eleven SNPs on manifestation of DIO by generating first backcross hybrids [(C57BL/6NTacxC57BL/6JRj)F1xC57BL/6NTac].

Here, we demonstrate that 4 SNPs (rs13481014, rs13480122 (*Aplp2*), rs13478783, rs4165065 (*Snap29*) differing between C57BL/6NTac and C57BL/6JRj are associated with body weight gain under HFD in a gender specific manner (Table 1). Furthermore and reflecting the HFD induced body weight gain differences, relative fat depot mass was associated with SNPs rs13480122, rs13481014 and rs4165065 (nominal p-value <0.05). In both genders, we found suggestive associations between rs13478783, rs13481014 and serum leptin levels and additionally rs13478783 showed effects on serum insulin levels in a gender specific manner (Table 2).

Since rs13481014 obviously maps within a “gene desert”, functional consequences and links to DIO are elusive. However, rs13480122 is mapping within the *Aplp2* gene, which has been

linked to glucose homeostasis and growth (35). Amyloid precursor like-protein 2 (Aplp2) belongs to the amyloid precursor protein (APP) family together with Aplp1 and APP, with APP representing the source of the neurotoxic amyloid *β* peptide involved in Alzheimer's disease (17). Knockout mice studies have shown that single disruptions of *App*, *Aplp1*, or *Aplp2* only caused minor abnormalities (20, 47, 50) While $Aplp2^{-/-}/App^{-/-}$ mice and $Aplp2^{-/-}/Aplp1^{-/-}$ both showed a lethal phenotype, $Aplp1^{-/-}/App^{-/-}$ mice were viable (20) and triple knockout mice showed a 100% lethal phenotype (21). These results suggest that APLP2 exhibits a key physiological role among the mammalian APP family members. Functions of APP family proteins and especially APLP2 are poorly understood. *Aplp2* deficiency in mice ($Aplp2^{-/-}$) results in significantly lower body weight, lower plasma glucose and increased plasma insulin levels in 13 weeks old $Aplp2^{-/-}$ mice compared to control mice (C57BL/6J x 129/Sv) (35). In contrast to that, Koch et al. reported $Aplp2^{-/-}$ mice to be normal in size and healthy up to 22 months of age in the original publication on the generation of the $Aplp2^{-/-}$ model and mice had the same genetic backgrounds in both studies (47). Noteworthy, besides resistance to HFD induced weight gain, we found male BC1 hybrids homozygous for rs13480122 (C/C) already significantly lighter than heterozygous (C/T) allele carriers at 4 weeks of age before the switch to the HFD (Table 1) indicating a diet independent effect of this variant on body weight possibly related to altered *Aplp2* gene activity.

In addition, SNP rs4165065 exhibiting a female specific effect on DIO is mapping within the *Snap29* gene which has been linked to cerebral dysgenesis, neuropathy, ichthyosis and keratoderma (CEDNIK) syndrome and schizophrenia (15, 39, 42). Soluble n-ethylmaleimide sensitive factor attachment protein (SNAP) 29 is a member of the SNAP receptor (SNARE) family of proteins which are required for vesicle trafficking and thus are essential in numerous physiological processes (7, 49). SNARE proteins seem to be mainly responsible for mediating fusion between vesicles and their target membrane (11).Two loss of function mutations in the Snap29 gene result in the neurocutaneous CEDNIK syndrome in humans (15, 42). On the molecular level, the loss of SNAP29 resulted in impaired recycling of transferrin and β1-integrin demonstrating the importance of SNAP29 mediated membrane

fusion in endocytic recycling and consequently cell motility (37). It has been reported, that SNAP29 is present at synapses and inhibits disassembly of the SNARE complex and seems to modulate synaptic transmission and postfusion recycling of SNARE components (44).

SNP rs13478783 is located 150kb upstream of the *alpha synuclein* (*Snca*) gene. SNCA, is primarily known for its prominent role as molecular hallmark of several neurodegenerative conditions such as Parkinson's disease now termed synucleinopathies (41). There is strong evidence on the role of SNCA in the regulation of synaptic-vesicle release and indicate a stabilising effect on complexes of SNARE family proteins (9, 26, 34). In this context, SNCA has been shown to be a cytoplasmic ligand of the insulin-secretory granule and to interact with K_{ATP} channels and in consequence exhibited an inhibitory action on insulin secretion (16). Exogenous overexpression of α-synuclein inhibited insulin secretion in INS1-832/13 cells, while loss of SCNA expression potentiated insulin secretion in SNCA deficient ASKO islets (16). Additionally, recent studies demonstrated inflammatory stimuli induced SNCA expression in macrophages and an SNCA inherent ability of macrophage activation depending on N-terminal and C-terminal domains of the protein (27, 45). A possible regulatory role of SCNA in processes of inflammation might present a link between SCNA and adipose tissue and obesity, as obesity is well established as a state of systemic and chronic, low-grade inflammation.

To assess if SNP differences could affect *Snap29, Aplp2*, *Snca* expression, we performed real-time based gene expression from C57BL/6JRj, C57BL/6NTac and heterozygous mice in liver samples. Here we detected that *Snap29* mRNA is significantly up-regulated in liver and adipose tissue of C57BL/JRj mice, indicating association between genotype and mRNA level. Since, Snap29 seems to have strongest effects in mice; we performed gene expression analysis in subcutaneous human adipose tissue as well. Comparison of *SNAP29* mRNA expression in subgroups of BMI <25 or >30kg/m² revealed significantly lower SC *SNAP29* mRNA expression in the obese subgroup (Fig 2B), indicating an association between *SNAP29* mRNA level and measures of obesity. Univariate correlation analyses of the entire study population identified significant correlations primarily related to parameters of

obesity and fat distribution. These correlations did not remain significant after adjusting for age, gender and BMI and correcting for multiple testing. These expression data suggests a potential role of SNAP29 in manifestation of obesity and or DIO.

Conclusion

In summary, we have identified gender specific SNPs between C57BL/6NTac and C57BL/6JRj substrains which are associated with body weight gain and relative fat depot mass under HFD in first backcross hybrids of C57BL/6NTac and C57BL/6JRj mice [(C57BL/6NTacxC57BL/6JRj)F1 x C57BL/6NTac]. For the SNPs mapping within or nearby known genes, *Aplp2*, *Snap29* and *Snca* all are involved in severe neurodegenerative or neurocutaneous diseases or syndromes. Gene expression analysis in mice and human tissue identified *Snap29* as strongest candidate for parameters related to obesity and fat distribution. And although knowledge and understanding of molecular functions of these genes are in general limited, SNP concerning genes reported in this study as associated with manifestation of DIO all have certain common functional ground as being involved in synaptic vesicle release and SNARE complex stability.

Our data demonstrate that DIO responsiveness is associated with genetic disparity between C57BL/6NTac and C57BL/6JRj substrains and suggests genes of the synaptic vesicle release system being involved in the regulation of high fat DIO.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: NK MB. Performed the experiments: MK AK1 AK2 GF JK JTH. Analyzed data: NK JTH MS PK. Contributed reagents/materials/analysis tools: PK Wrote the paper: NK JTH; Edit manuscript: NK, JTH, MB, PK

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Figure legends

Figure 1. Manifestation of high fat diet induced obesity in parental substrains, F1 and BC1 hybrids. Body weight gain of parental C57BL/6JRj and C57BL/6NTac in comparison with F1 mice (females, 6 week HFD) (A) or in comparison with beneficial or risk alleles carrying BC1 hybrids (male mice;8 week HFD) (B). Body weight of male parent C57BL/6JRj and C57BL/6NTac as well as BC1 carriers of beneficial and risk alleles after 8 weeks under HFD (C). Beneficial allele combination: (C/C) of rs13480122 (*Aplp2*) and (C/T) of rs13481014; risk allele combination: (C/T) of rs13480122 (*Aplp2*) and (C/C) of rs13481014. Data is presented as mean ± SD.

Figure 2. Relative gene expression analysis in mouse liver (A) and subcutaneous adipose tissue (B) of *Snap29, Snca, Aplp2* in different genotypes and *SNAP29* mRNA levels in human subcutaneous adipose tissue (C). Results present means ± SE from n=6 liver and subcutaneous adipose tissue samples per genotype. Results present means ± SE from lean subjects, N=44 vs. obese subjects, N=190. * $P<0.01$ and ** $P<0.001$ indicate statistical significance after Bonferroni corrections for multiple testing. AU, arbitrary units.

Figure 1.

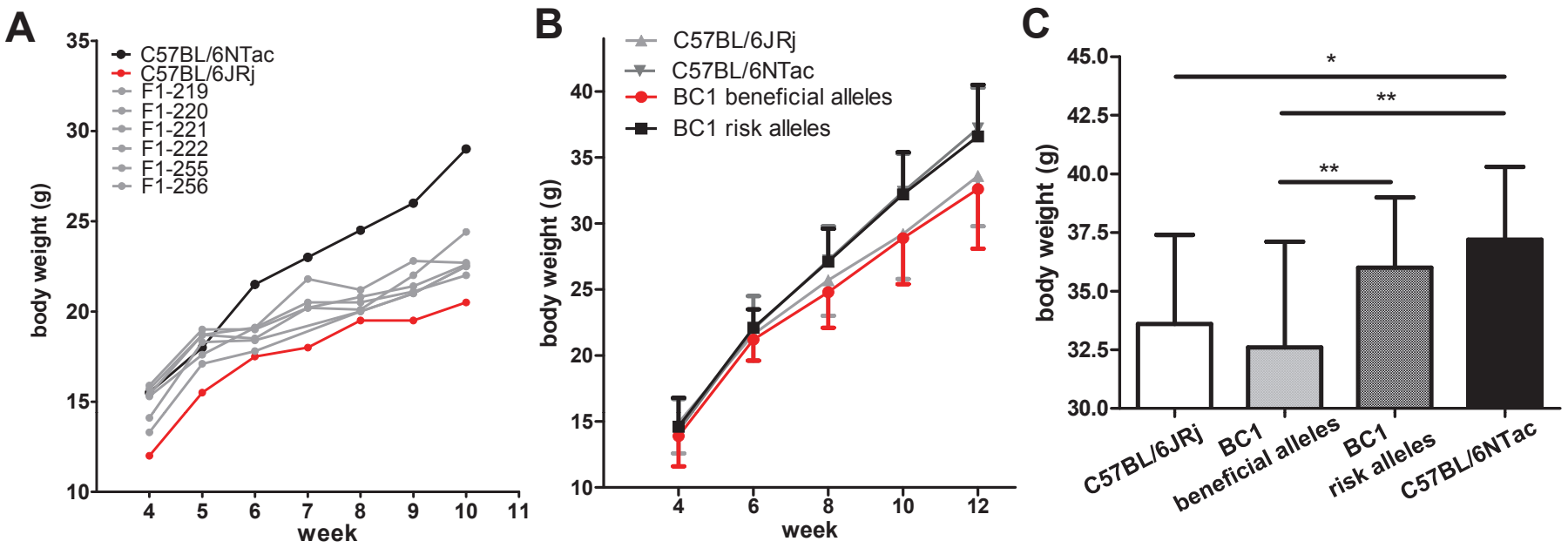
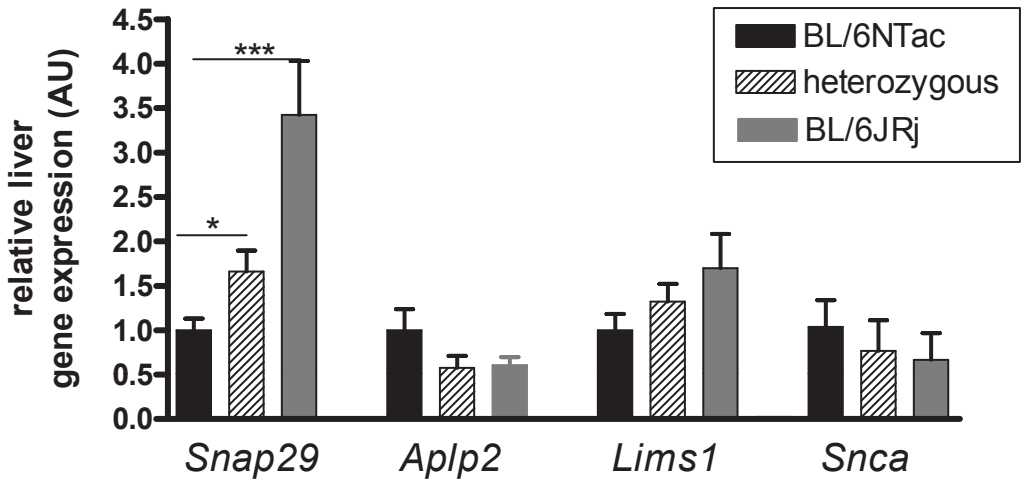
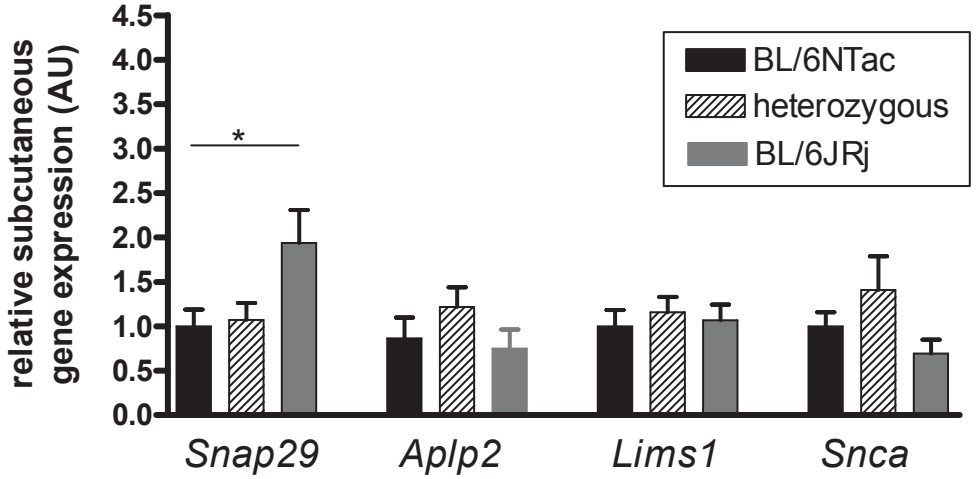

A
C57BL/6NTac
C57BL/6JRj
F1-219
F1-220
F1-221
F1-222
F1-255
F1-256
body weight (g)
week
B
C57BL/6JRj
C57BL/6NTac
BC1 beneficial alleles
BC1 risk alleles
body weight (g)
week
C
body weight (g)
*
**
**
C57BL/6JRj
BC1 beneficial alleles
BC1 risk alleles
C57BL/6NTac

Figure 2.

A


relative liver gene expression (AU)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
*
BL/6NTac
heterozygous
BL/6JRj
Snap29
Aplp2
Lims1
Snca

B


relative subcutaneous gene expression (AU)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
BL/6NTac
heterozygous
BL/6JRj
Snap29
Aplp2
Lims1
Snca

C

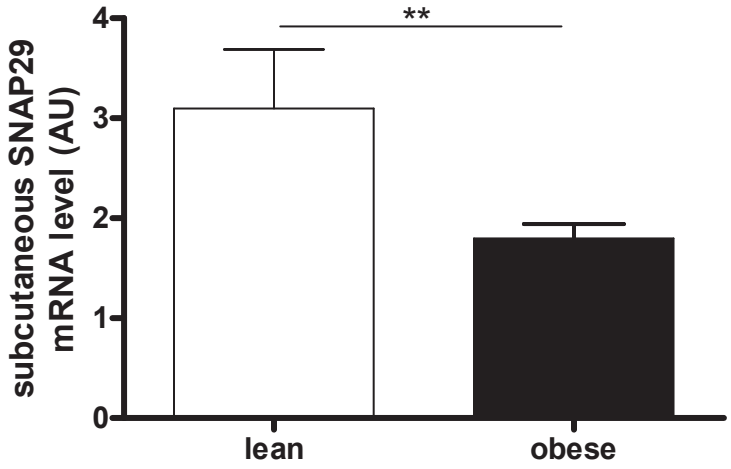

subcutaneous SNAP29 mRNA level (AU)
4
3
2
1
0
**
lean
obese

Table 1. High fat diet induced body weight gain in backcross hybrids and parental control strains.

SNP or strain	Chr.	Sex	Genotype	N	Body weight (g)				
					week 4	week 6	week 8	week 10	week 12
rs13480122 *(Aplp2)*	9	m	homo (C/C)	52	**13.7 ± 2.3#**	**21.4 ± 1.6***	**25.3 ± 2.6#**	**29.5 ± 3.4#**	**33.4 ± 4.1#**
			het (C/T)	55	**15.1 ± 2.4**	**22.1 ± 1.4**	**26.8 ± 2.4**	**31.4 ± 3.2**	**35.6 ± 3.8**
rs13481014	11	m	homo (C/C)	47	14.1 ± 2.3	22.0 ± 1.5	26.6 ± 2.1	**31.3 ± 3.2***	**35.6 ± 3.8****
			het (C/T)	62	14.8 ± 2.5	21.7 ± 1.6	25.6 ± 2.6	**29.8 ± 3.3**	**33.6 ± 4.1**
allele combination		m	beneficial	30	13.9 ± 2.3	**21.2 ± 1.6***	**24.8 ± 2.7****	**28.9 ± 3.5****	**32.6 ± 4.5****
			risk	25	14.6 ± 2.2	**22.1 ± 1.4**	**27.1 ± 2.5**	**32.2 ± 3.2**	**36.6 ± 3.9**
C57BL/6JRj		m		9	14.9 ± 2.3	21.6 ± 2.0	25.7 ± 2.7	**29.2 ± 3.4***	**33.6 ± 3.8***
C57BL/6NTac		m		10	14.3 ± 2.4	22.0 ± 2.5	27.2 ± 2.6	**32.4 ± 2.9**	**37.2 ± 3.1**
rs13478783 *(Snca)*	6	f	homo (G/G)	46	12.8 ± 1.6	17.6 ± 1.2	**19.8 ± 1.3***	**22.2 ± 1.9****	**25.0 ± 3.0***
			het (G/A)	53	12.7 ± 1.8	17.9 ± 1.4	**20.4 ± 1.8**	**23.5 ± 2.7**	**26.6 ± 3.6**
rs4165065 *(Snap29)*	10	f	homo (C/C)	48	12.8 ± 1.6	17.5 ± 1.3	**19.8 ± 1.5***	**22.4 ± 2.1***	**25.2 ± 3.0***
			het (C/T)	49	12.6 ± 1.9	18.0 ± 1.3	**20.5 ± 1.7**	**23.4 ± 2.7**	**26.6 ± 3.6**
allele combination		f	beneficial	24	12.9 ± 1.5	17.4 ± 1.2	**19.6 ± 1 4***	**21.9 ± 1.9****	**24.6 ± 3.0****
			risk	29	12.6 ± 2.0	18.1 ± 1.4	**20.8 ± 3.0**	**24.0 ± 3.0**	**27.3 ± 3.9**
C57BL/6JRj		f		6	13.4 ± 1.5	18.6 ± 1.6	20.5 ± 1.6	22.5 ± 2.2	24.5 ± 2.7
C57BL/6NTac		f		6	13.1 ± 1.8	18.3 ± 1.6	20.8 ± 2.0	23.8 ± 3.0	27.8 ± 3.3

Data represent means ± SD. *suggestive association between genotype variants (uncorrected p-values at *0.05, **0.01 level). # significant association between genotype variants (p-values < #0.005). homo: homozygous; het: heterozygous. beneficial allele combination: male mice:(C/C) of rs13480122 and (C/T) of rs13481014; female mice: (G/G) for rs13478783 and (C/C) for rs4165065; risk allele combination: male mice: (C/T) for rs13480122 and (C/C) for rs13481014; female mice: (G/A) for rs13478783 and (C/T) for rs4165065;

Table 2. Specific organ masses and fasting serum concentrations in BC1 hybrids and parental control strains.

SNP or strain	Chr.	Sex	Genotype	N	Tissue weight (% from body weight)			Fasting serum levels	
					liver	epi	sc	leptin (ng/ml)	insulin (ng/ml)
rs13478783 *(Snca)*	6	m	homo (G/G)	59	3.8 ± 0.4	4.9 ± 1.1	2.6 ± 0.6	**14.0 ± 6.5***	**1.03 ± 0.48***
			het (G/A)	50	3.7 ± 0.4	5.0 ± 1.0	2.7 ± 0.6	**16.8 ± 7.5**	**1.37 ± 0.84**
rs13480122 *(Aplp2)*	9	m	homo (C/C)	52	3.7 ± 0.3	4.7 ± 1.1	**2.5 ± 0.5***	14.3 ± 6.7	1.08 ± 0.56
			het (C/T)	55	3.8 ± 0.5	5.1 ± 1.0	**2.8 ± 0.7**	16.2 ± 7.5	1.30 ± 0.79
rs13481014	11	m	homo (C/C)	47	3.8 ± 0.5	**5.3 ± 1.0****	**2.8 ± 0.6***	**17.0 ± 6.4***	1.19 ± 0.62
			het (C/T)	62	3.8 ± 0.3	**4.7 ± 1.0**	**2.6 ± 0.7**	**14.0 ± 7.3**	1.18 ± 0.74
allele combination		m	beneficial	30	3.8 ± 0.3	**4.5 ± 1.1****	**2.5 ± 0.6****	**13.0 ± 7.2***	1.12 ± 0.60
			risk	25	3.9 ± 0.6	**5.5 ± 1.0**	**3.0 ± 0.6**	**17.9 ± 7.0**	1.34 ± 0.68
C57BL/6JRj		m		9	3.7 ± 0.2	4.7 ± 1.2	**2.5 ± 0.5***		
C57BL/6NTac		m		10	3.7 ± 0.4	4.8 ± 0.4	**3.0 ± 0.4**		
rs13478783 *(Snca)*	6	f	homo (G/G)	46	3.9 ± 0.6	2.3 ± 1.0	2.1 ± 0.8	**6.3 ± 4.7***	0.67 ± 0.45
			het (G/A)	53	3.7 ± 0.4	2.6 ± 0.9	2.1 ± 0.6	**8.8 ± 5.1**	0.69 ± 0.39
rs13481014	11	f	homo (C/C)	51	3.8 ± 0.6	2.6 ± 1.0	2.2 ± 0.7	**8.8 ± 5.6***	0.68 ± 0.38
			het (C/T)	46	3.8 ± 0.4	2.4 ± 0.9	2.1 ± 0.7	**6.4 ± 4.2**	0.68 ± 0.46
allele combination		f	beneficial	24	3.9 ± 0.5	2.3 ± 1.1	2.0 ± 0.8	6.0 ± 4.4	**0.59 ± 0.33***
			risk	29	3.7 ± 0.3	2.8 ± 0.6	2.3 ± 0.6	9.0 ± 5.7	**0.67 ± 0.37**
C57BL/6JRj		f		6	**4.4 ± 0.1***	**1.5 ± 0.7***	**1.8 ± 0.5***		
C57BL/6NTac		f		6	**3.9 ± 0.1**	**2.7 ± 0.9**	**2.5 ± 0.6**		

Data represent means ± SD. *suggestive association between genotype variants (uncorrected p-values at *0.05, **0.01 level). homo: homozygous; het: heterozygous; epi: epigonadal; sc: subcutaneous. beneficial allele combination: male mice:(C/C) of rs13480122 and (C/T) of rs13481014; female mice: (G/G) for rs13478783 and (C/C) for rs4165065; risk allele combination: male mice: (C/T) for rs13480122 and (C/C) for rs13481014; female mice: (G/A) for rs13478783 and (C/T) for rs4165065.

Table 3. Univariate correlations between human *SNAP29* mRNA expression and parameters of obesity, fat distribution, glucose and lipid metabolism and adipokines.

	SC *SNAP29* (r; p-value)	***after adjustment for age, gender and BMI***
Age (years)	0.128; 0.051	
Visceral fat area (cm²)	**-0.217; 0.002**	
SC fat area (cm²)	**-0.265; <0.001**	
CT ratio	**0.204; 0.004**	0.191; 0.032
Body weight (kg)	-0.043; 0.514	
BMI (kg/cm²)	**-0.267; <0.001**	
WHR	-0.099; 0.131	
Waist (cm)	**-0.268; <0.001**	
Hip (cm)	**-0.063; <0.001**	
% body fat	**-0.241; 0.002**	
HbA1c (%)	-0.107; 0.112	
Fasting plasma glucose (mmol/l)	-0.052; 0.433	
Fasting plasma insulin (pmol/l)	**-0.211; 0.005**	
2h OGTT (mmol/l)	0.022; 0.833	
Glucose infusion rate (mg/kg/min)	-0.017; 0.870	
Total cholesterol (mmol/l)	-0.155; 0.072	
HDL-cholesterol (mmol/l)	0.075; 0.394	
LDL-cholesterol (mmol/l)	-0.235; 0.010	
Triglyceride (mmol/l)	0.055; 0.514	
FFA (mmol/l)	-0.161; 0.041	
hsCrp (mmol/l)	-0.044; 0.522	
Serum leptin (pg/ml)	**-0.258; 0.001**	
Serum adiponectin (µg/ml)	0.111; 0.149	
IL-6 (pg/ml)	-0.147; 0.056	

SC, subcutaneous; corrected P values <0.01 were considered significant (bold); r, Spearman´s correlation coefficient.

Table 4. Anthropometric and metabolic characteristics of the study groups. (n=234).

	Male (N=84)	***Female (N=150)***
Age (years)	53 ± 14	49 ± 15
Visceral fat area (cm²)	251 ± 170	250 ± 178
SC fat area (cm²)	1034 ± 819	1256 ± 779
CT ratio	0.53 ± 0.70	0.33 ± 0.42*
Body weight (kg)	125 ± 48	116 ± 39
BMI (kg/cm²)	39 ± 13	42 ±13
Waist (cm)	128 ± 35	117 ± 29**
Hip (cm)	128 ± 35	129 ± 28
WHR	0.99 ± 0.10	0.90 ± 0.09***
Body fat (%)	33.8 ± 13.7	37.8 ± 10.1
HbA1c (%)	6.0 ± 0.9	5.9 ± 1.0
FPG (mmol/l)	6.3 ± 1.7	5.9 ± 1.6
FPI (pmol/l)	97 ± 121	75 ± 94
2h OGTT (mmol/l)	7.0 ± 2.8	6.8 ± 2.8
Clamp (mg/kg/min)	71.4 ± 37.1	81.4 ± 29.3
Total cholesterol (mmol/l)	4.9 ± 1.0	5.0 ± 0.9
HDL-cholesterol (mmol/l)	1.2 ± 0.3	1.4 ± 0.4*
LDL-cholesterol (mmol/l)	3.2 ± 0.9	3.0 ± 0.8
Triglyceride (mmol/l)	1.8 ± 1.1	1.4 ± 0.7**
Free fatty acids (mmol/l)	0.53 ± 0.40	0.50 ± 0.42
Serum leptin (pg/ml)	22.4 ± 15.9	42.3 ± 21.3***
Serum adiponectin (µg/ml)	6.4 ± 4.3	7.9 ± 4.7
IL-6 (pg/ml)	5.9 ± 6.4	6.7 ± 5.0

Data are means ± SD, statistically significant differences between genders at p-value * < 0.05, ** < 0.01; *** < 0.001