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The juxtaparanodal proteins CNTNAP2 and TAG1 regulate diet-induced obesity

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Abstract

Despite considerable effort, the identification of genes that regulate complex multigenic traits such as obesity has proven difficult with conventional methodologies. The use of a chromosome substitution strain-based mapping strategy based on deep congenic analysis over-came many of the difficulties associated with gene discovery and led to the finding that the juxtaparanodal proteins CNTNAP2 and TAG1 regulate diet-induced obesity. The effects of a mild *Cntnap2* mutation on body weight were highly dependent on genetic background, as both obesity-promoting and obesity-resistant effects of *Cntnap2* were observed on different genetic backgrounds. The more severe effect of complete TAG1 deficiency, by decreasing food intake, completely prevented the weight gain normally associated with high-fat-diet feeding. Together, these studies implicate two novel proteins in the regulation of diet-induced obesity. Moreover, as juxtaparanodal proteins have previously been implicated in various neurological disorders, our results suggest a potential genetic and molecular link between obesity and diseases such as autism and epilepsy.

Introduction

Obesity as a genetic disorder continues to gain acceptance as the underlying genetic factors continue to be identified. These include genes such as leptin, proopiomelanocortin (POMC), and melanocortin 4 receptor (MC4R), which when mutated result in severe monogenic forms of obesity (Farooqi 2011). Our understanding of the more prevalent and genetically complex forms of obesity had lagged until recently when the use of genome-wide association studies identified numerous genes that regulate body weight, such as fat mass and obesity associated (FTO), SH2B adaptor protein 1 (SH2B1), potassium channel tetramerization domain containing 15 (KCTD15), and others (Hofker and Wijmenga 2009). Although much has been learned, studies of both monogenic and genetically complex forms of obesity have thus far been unable to account for the vast majority of heritability, suggesting that much of the genetic basis for body weight regulation remains to be discovered.

Animal models continue to be useful resources to further our understanding of the genetics of obesity. For example, leptin and SH2B1 were both initially recognized as regulators of obesity from studies in mice (Coleman 1973; Zhang et al. 1994; Ren et al. 2005). In addition, as it is becoming increasingly clear that the genetics of obesity and many other traits are more complex than originally hypothesized (Eichler et al. 2010), the use of animal models allows for many different types of defined crosses and stable genetic resources (Nadeau et al. 2000; Aylor et al. 2011) that can be used to investigate the multigenic and epigenetic nature of obesity in ways that would be difficult or impossible in humans.

Our laboratory has focused on the use of chromosome substitution strains (CSSs) and congenic strains derived from them to study the genetic architecture of complex traits such as obesity, and to identify the underlying genetic factors. Collectively, analyses of these strains suggest a genetic architecture comprising many large-effect QTLs that act in a nonadditive, context-dependent manner (Shao et al. 2008). Further analysis of one such quantitative trait locus (QTL) has recently suggested a novel role for the *Slc35b4* solute receptor in the regulation of body weight and glucose homeostasis (Yazbek et al. 2011), thus demonstrating the utility of the CSSs for gene discovery despite remarkably complex genetic architecture. In addition, studies of CSSs can also identify heritable transgenerational epigenetic effects such as the obesity QTL *Obrq2a*, the effect of which is dependent on the paternal and grandpaternal genotype rather than on the individual's genotype (Yazbek et al. 2010). The present study provides further evidence of the utility of CSSs to identify epistatic interactions and genes underlying complex traits, and implicates

contactin associated protein-like 2 (Cntnap2) and contactin 2 (Cntn2 or Tag1) in the regulation of body weight.

Materials and methods

Mice

Congenic 6C2 and 6C3 mice were generated as previously described (Shao et al. 2008) and were maintained with brother–sister matings. Tag1 knockout mice were previously generated in W9.5 ES cells derived from strain 129S1/Sv (Law et al. 2008). All Tag1 mice used in the experiments described were backcrossed to C57BL/6J for more than ten generations and were obtained from breeding colonies at Case Western Reserve University (CWRU). Mice were housed in ventilated racks, had access to food and water ad libitum, and were maintained at 21 °C on a 12-h light/12-h dark cycle. All strains were maintained on LabDiet 5010 chow (PMI Nutrition International, St. Louis, MO, USA). For studying the response to low- and high-fat diets, 5-week-old mice were placed on either a high-fat simple-carbohydrate diet (HFSC, D12331, Research Diets, New Brunswick, NJ, USA) or a low-fat complex-carbohydrate diet (LFCC, D12328, Research Diets). All test and control mice used for each experiment were born within 3 months of each other. The Institutional Animal Care and Use Committee approved all procedures.

Generation of sub-subcongenic panel

A panel of subcongenic strains spanning Obrq3 was derived from a cross between strain 6C2 and strain 6C3 (Fig. 1). The resulting (6C2 & 6C3) F1 offspring were intercrossed, with the F2 offspring genotyped for the polymorphic markers D6CWRU2 and D6MIT273 (Supplementary Table 1) to identify mice carrying a chromosome that was recombinant within the Obrq3 interval. The recombinant F2 mice were then backcrossed to B6 and the resulting offspring that were heterozygous for the recombinant chromosome were intercrossed to make the A/J-derived segment homozygous. In addition to genotyping with markers within the Obrq3 interval to test for homozygosity, the mice were genotyped with D6MIT138, D6MIT264, and D6MIT159 to test for homozygosity of the proximal region of chromosome 6 that contains Obrq2. Once made homozygous, brother–sister mating was used to maintain the congenic strains in their homozygous state. To further localize recombination breakpoints within the Obrq3 interval, mice were genotyped with a panel of polymorphic markers located between D6CWRU2 and D6MIT273 (Supplementary Table 1).

Food intake

Twenty-four-hour food intake was determined as previously described (Yazbek et al. 2010). Briefly, female mice were housed two to three per cage and fed the HFSC diet beginning at 35 days of age. At the time of measurement, mice were placed in a clean cage and the food was weighed. The food and mice were weighed after 24 h, and the average food intake per mouse per cage was calculated. Mice remained group-housed throughout the experiment to minimize stress.

Lipid adsorption

Lipid adsorption was determined as previously described (Jandacek et al. 2004) in mice fed a custom diet identical to the HFSC diet, except that 5% of the dietary fat was replaced with the nondigestible food additive sucrose polybehenate (Research Diets). Fecal analysis of fatty acids was analyzed by gas chromatography at the University of Cincinnati Mouse Metabolic Phenotyping Center.

Nodal immunostaining

The sciatic nerve was isolated from 6C2 and 6C3 mice. Sections of the nerve were teased with a needle to isolate single fibers on superfrost-plus slides, allowed to dry, and then fixed in 4% paraformaldehyde for 1 h. The slides were pretreated with 1% bovine serum albumin (BSA) (Jackson ImmunoResearch Laboratories, West Grove, PA), 10 % normal donkey serum, and 0.3 % Triton X-100 (TX-100) in phosphate-buffered saline (PBS) to block nonspecific staining. The splayed nerve fibers were incubated for 18–24 h with a PBS cocktail of goat anti-Ezrin (1:50) (C-19, sc-6407, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit anti-Kv1.2 (1:200) (APC-010, Alomone, Jerusalem, Israel) with 0.3% TX-100 and 1% BSA. After washing, the slides were incubated with PBS containing donkey anti-rabbit Rhodamine Red X and donkey anti-mouse FITC (1:400) (Jackson ImmunoResearch) and 10 % normal donkey serum with 0.3 % TX-100. The slides were washed and coverslipped using Vectashield (Vector Laboratories, Burlingame, CA). The nerve fibers were imaged with a Nikon E600 equipped with a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI). Staining was absent on control slides when the Kv1.2 or Ezrin antibody was omitted.

Body composition

Total body composition was determined by quantitative magnetic resonance at the University of Cincinnati Mouse Metabolic Phenotyping Center as described (Tinsley et al. 2004).

Telemetry

Mice were surgically implanted with a telemetric transponder (E-Mitter, Mini-Mitter Inc., Bend, OR) in the intraperitoneal cavity and allowed to recover from surgery for 5 days. Mice were anesthetized during surgery with isoflurane to minimize pain and discomfort. A receiver platform (ER4000 Energizer Receiver, Mini-Mitter Inc.) placed under each cage continually recorded activity data for the 1-week duration of the study. Singly housed female mice were used for this study because male Tag1 knockout mice are better breeders and were therefore used primarily in the breeding colony. Since the E-Mitter transponder also records body temperature, these same mice were used to investigate thermal balance during a cold challenge. Mice were subjected to 4 °C for 4 h and core body temperature was recorded every 30 s by the E-Mitter device. Data were collected for analysis with the VitalView software platform (Mini-Mitter Inc.). These studies were conducted by the Case Mouse Metabolic Phenotyping Center (MMPC).

Indirect calorimetry

Female mice were acclimated to single housing for 1 week prior to the experiment. The mice were acclimated for 2 days in the indirect calorimetry cages before data were collected. Indirect calorimetry was performed at room temperature without bedding using a four-chamber open-circuit Oxymax system (CLAMS, Columbus Instruments, Columbus, OH) at the CWRU MMPC. Energy expenditure was calculated for light cycle 1 (12:00 p.m.–6:00 p.m.), dark cycle (6:00 p.m.–6:00 a.m.), and light cycle 2 (6:00 a.m.–12:00 p.m.) as described (Kaiyala et al. 2010).

RNA isolation

Brain tissue was stored in RNAlater (Ambion, Austin, TX, USA), and RNA from whole brain was extracted using the RNeasy mini kit (Qiagen, Valencia, CA).

Quantitative PCR

RNA from brain was reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA). Quantitative PCR was performed in triplicate using TaqMan assay Mm00470553_m1 (Applied Biosystems, Carlsbad, CA). The expression level for *Cntnap2* was calculated using the DDCT method relative to the Eukaryotic 18S rRNA endogenous control (Hill-Baskin et al. 2009).

Additivity test

Body weights from strains B6, 6C2, 6C3b, and 6C3b-R1 were used to compare an additive versus interaction model for QTLs *Obrq2* and *Obrq3b*. It was assumed that only two QTLs were present. Body weight was the dependent variable and the QTLs were treated as independent variables. Both the additive model and interaction model for *Obrq2* and *Obrq3b* were generated using linear regressions implemented in R (Everitt and Hothorn 2006). The interaction model had smaller residual mean square values than the additive model. A subsequent ANOVA test to compare these two models showed a *p* value <0.0001, suggesting a significant difference between the two models, with the interaction model providing a better explanation of the data.

Statistics

Measurements are presented as mean $\pm$ standard error. Comparisons of multiple groups (i.e., WT, Het, and KO mice) were done using a one-way ANOVA with Tukey's correction for multiple observations. Unadjusted comparisons between two groups were performed using Student's *t* test. Analysis of congenic strains for QTL detection was performed as previously described (Shao et al. 2010). Energy expenditure was also adjusted for group differences in body mass using analysis of covariance (Kaiyala and Schwartz 2011) with PASW Statistics (IBM, Somers, NY). Body mass index (BMI) was calculated as g/cm² based on the nose–anus length of the mouse.

Results

Subcongenic panel

Obrq3 was identified using a congenic mapping strategy (Shao et al. 2010), which located three additional body weight QTLs on chromosome 6, including the neighboring QTL *Obrq2* (Shao et al. 2008). *Obrq3* is a body weight QTL that was mapped to a 19.0-Mb interval on mouse chromosome 6 between D6MIT159 (located at 29.7 Mb) and D6MIT274 (located at 48.7 Mb) (Shao et al. 2008). Although B6 mice are widely used as a model of diet-induced obesity, the *Obrq3*^{B6} allele was found to promote obesity resistance, whereas the *Obrq3*^{A/J} allele was found to promote obesity.

To further refine the QTL interval by localizing the recombination breakpoints in strains 6C2 and 6C3 that define *Obrq3*, both strains were genotyped with additional polymorphic markers (Fig. 1b). *Obrq3* was thus reduced from a 19.0-Mb interval to a 4.6-Mb interval located between markers rs30218447 (42.5 Mb) and rs30496331 (47.1 Mb). This interval contains 26 annotated genes. Higher-resolution genetic mapping of *Obrq3* was undertaken by first analyzing offspring from a (6C2 & 6C3) F1 intercross to identify mice carrying a chromosome with a recombination event within the *Obrq3* interval that could be used to further narrow the QTL. The design of this cross ensured that only recombination events within the *Obrq3* interval would alter the sequence of the recombinant chromosome, whereas the sequence within the *Obrq2* interval could not change. This cross also retained the association between *Obrq2* and *Obrq3*, which was necessary because of the possibility that the effect of *Obrq3* was context-dependent (Shao et al. 2008). The genotypes of 1,080 (6C2 & 6C3) F2 mice were analyzed to identify mice carrying a recombinant chromosome

within the *Obrq3* interval. Only 15 recombinants were identified, which is slightly fewer than the expected number of 26, based on the size of the interval and the average recombination rate in mice (Jensen-Seaman et al. 2004; Paigen and Petkov 2010). The recombination events largely clustered into two intervals, one between markers rs29970601 and rs30457651 (6 recombinants) and the second between markers rs13478735 and D6MIT273 (8 recombinants). Given the proximity of the recombination event between rs13478735 (46.18 Mb) and D6MIT273 (46.89 Mb) to the distal border of *Obrq3* (47.06 Mb), subcongenic mice were not generated using this recombination breakpoint. Therefore, only two new subcongenic strains, 6C3a and 6C3b, were generated spanning the *Obrq3* interval.

The obesity phenotypes of the newly derived subcongenic strains 6C3a and 6C3b were both evaluated by determining body weight and BMI in the male mice of these strains following 100 days of being fed the HFSC diet, beginning at 35 days of age. Both subcongenic strains were compared to the 6C2 and 6C3 control strains and found to be significantly more obese than strain 6C2 and not significantly different than strain 6C3 (Fig. 1b). Therefore, both subcongenic strains are susceptible to obesity relative to strain 6C2. The imprecise localization of recombination breakpoints in strains 6C3a and 6C3b leaves open the possibility that these strains share a maximum of 67 kb of A/J-derived sequence between markers rs29970601 and rs30457651, which could imply that the susceptibility to obesity of both strains is due to polymorphisms within this shared interval. However, this seems unlikely for two reasons. First, there are no known genes within this interval and there is only a single expressed sequence tag (EST) (BY678477) that has been identified (Konno et al. 2001). However, the EST is not spliced, does not contain an open reading frame, and is not evolutionarily conserved, even between mouse and rat, suggesting that it may represent contamination or genomic noise rather than a functional gene. Second, the clustering of recombination breakpoints in this interval suggests the presence of a recombination hotspot, as does a recent genome-wide analysis that identified two hotspots within this 67-kb interval (Smagulova et al. 2011). Thus, if the recombination events in strains 6C3a and 6C3b both occurred in the same hotspot, there would in fact be no overlap between the strains, suggesting two independent, tightly linked QTLs. Importantly, a high density of QTLs across the genome may be a common feature of complex traits (Steinmetz et al. 2002; Yazbek et al. 2011). Therefore, the data collectively suggest that strain 6C3a defines the sub-QTL *Obrq3a* that is located in the 1.5-Mb interval between markers rs30218447 and rs30457651, and that the 6C3b strain defines the sub-QTL *Obrq3b* that is located in the 3.1-Mb interval between markers rs29970601 and rs30496331. Therefore, although *Obrq3a* and *Obrq3b* each contain a QTL with a similar magnitude effect on weight gain, *Obrq3b* was chosen for further study because it contains only two known genes (Ensembl v59) that are further discussed below. We then performed a replicate study measuring the body weight of strains 6C2 and 6C3b when fed the HFSC diet. Strain 6C3b (39.81 ± 0.56 g, $n = 73$) again was more obese than strain 6C2 (36.51 ± 0.54 g, $n = 64$, $p < 0.0001$), thus confirming the phenotypic effect and location of *Obrq3b*.

Nonadditive interactions between *Obrq3b* and *Obrq2*

The structure of the congenic panels used to identify *Obrq3b* and *Obrq2* prevents one from distinguishing whether the effect of *Obrq3b* on body weight is additive or nonadditive to the effects of *Obrq2*. This is because *Obrq3b* was analyzed only in the context of *Obrq2*. We therefore sought to examine the independent effect of *Obrq3b* on body weight by generating a new congenic strain that contains the A/J-derived allele of *Obrq3b* but has been separated from the A/J-derived allele of *Obrq2* by recombination. Among the 346 offspring of a cross between (6C3b & B6) F1 and B6 mice, three independent recombination events were identified that separated *Obrq3b* and *Obrq2* as determined by genotyping a panel of markers

spanning the two QTL intervals. Two of the mice carrying the recombinant chromosomes were bred to make the *Obrq3b^{A/J}* allele homozygous as described (Yazbek et al. 2011), thereby generating the new congenic strains 6C3b-R1 and 6C3b-R2. Both strains were then tested on the HFSC diet for 100 days. Strains 6C3b-R1 and 6C3b-R2 did not differ (6C3b-R1: 41.47 ± 0.95 g, $n = 31$; 6C3b-R2: 41.36 ± 0.75 g, $n = 38$, $p > 0.99$) with respect to body weight so the data were pooled for comparison to 6C2 (*Obrq2*) and 6C3b (*Obrq2* + *Obrq3b*). It was surprising that the strains carrying the *Obrq3b^{A/J}* allele on an otherwise B6 background (6C3b-R1 and 6C3b-R2) weighed less than the control B6 mice (Fig. 1) because the *Obrq3b^{A/J}* allele has an obesity-promoting effect on *Obrq2^{A/J}*. To examine whether this phenotypic divergence could be explained by nonadditive interactions, both additive and nonadditive (epistatic) interaction models (details given in the Methods section) were compared to test which best accounted for the effects of *Obrq2* and *Obrq3b*. The non-additive interaction model better explained the body weight data of strains 6C2, 6C3b, and 6C3b-R1 ($p < 0.0001$). Thus, the effects of *Obrq2* and *Obrq3b* are due to an epistatic interaction between the two QTLs, with *Obrq3b^{A/J}* promoting increased body weight in the context of *Obrq2^{A/J}* and reduced body weight in the context of *Obrq2^{B6}*.

Evaluation of *Cntnap2* as an *Obrq3b* candidate gene

One of the only two known genes in the *Obrq3b* interval, AC158680, is a known pseudogene of *Rbpsi* (Klein et al. 2000) and was therefore not further evaluated. The second gene, *Cntnap2*, spans 2.2 Mb of genomic DNA and is involved in the localization of voltage-gated potassium channels within the nervous system (Poliak et al. 1999). Mutations in *Cntnap2* have been associated with speech disorders, autism, and epilepsy, although no previous association with metabolic disease has been described (Strauss et al. 2006; Alarcon et al. 2008; Arking et al. 2008; Bakkaloglu et al. 2008; Vernes et al. 2008; Newbury and Monaco 2010; O’Roak et al. 2011). Sequence analysis of the *Cntnap2* gene identified two nonsynonymous variants between strains B6 and A/J (Fig. 2). The first variant, V161I (rs48516420), falls within a FV/FVIII protein binding domain; however, this substitution is conservative, is not highly conserved evolutionarily as both isoleucine and valine are found in different species at this position (Fig. 2a), and is computationally predicted to be “tolerated” using the SIFT algorithm (Kumar et al. 2009). In contrast, the H538Q variant (rs13478735) changes a highly evolutionarily conserved residue with the histidine consistently present in species as distantly related as tetraodon (Fig. 2b). This nonconservative substitution is computationally predicted by SIFT to be “damaging” to protein function. The expression of *Cntnap2* in whole-brain tissue was also evaluated from congenic strains carrying the *Cntnap2^{B6}* allele and the *Cntnap2^{A/J}* allele but was found not to differ (data not shown). Thus, *Cntnap2* is the only known protein-coding gene within the *Obrq3b* interval and it contains two coding variants between B6 and A/J, one of which is predicted to be deleterious to protein function. Although this does not rule out subtle or tissue-specific expression differences in *Cntnap2* between strains B6 and A/J, it suggests that the phenotypic effects of *Obrq3b* are due to alterations in the protein function of *CNTNAP2*.

Impaired nodal Kv channel localization associated with *Cntnap2^{B6}* allele

CNTNAP2 is localized to the juxtaparanodal region at the nodes of Ranvier in myelinated axons, where it is required for proper localization of Kv1.1 (potassium voltage-gated channel, shaker-related subfamily, member1; *Kcna1*) and Kv1.2 (potassium voltage-gated channel, shaker-related subfamily, member2; *Kcna2*) to the juxtaparanodes (Poliak et al. 1999, 2003). To test whether the allelic variation in *Cntnap2* between strains 6C2 (*Cntnap2^{B6}*) and 6C3 (*Cntnap2^{A/J}*) was associated with alterations in Kv channel localization, immunohistochemistry was performed on the sciatic nerve in mice fed the HFSC diet. Localization of ezrin, which is found in Schwann cells at the nodes of Ranvier

(Melendez-Vasquez et al. 2001), and Kv1.2 was examined. Although the majority of nodes in strains 6C2 and 6C3 demonstrated normal Kv channel distribution, a higher percentage of nodes in strain 6C2 demonstrated abnormal Kv channel localization (heminodal) or an absence of detectable Kv channels ($p < 0.0001$). This suggests that the *Cntnap2*^{B6} allele, which contains the H538 allele that was predicted to damage protein function, has a subtly impaired ability to maintain proper Kv channel localization at the nodes of Ranvier. As *Cntnap2* $-/-$ mice have altered Kv channel localization in the absence of motor nerve conduction velocity defects in the sciatic nerve (Poliak et al. 2003), it remains to be determined how altered localization of Kv channels in 6C3 mice will translate to functional neuronal signaling defects.

Tag1 regulates adiposity

Cntnap2 $-/-$ mice have been generated and described; however, they exist on a mixed genetic background, having originated from a mouse embryonic stem (ES) cell line derived from strain 129, which was then backcrossed to strain ICR (Poliak et al. 2003; Peñagarikano et al. 2011). Although the phenotype of the OBRQ QTLs in 129 mice is not known, it is possible that the 129-derived congenic interval flanking the *Cntnap2* knockout allele may contribute to the body weight of the knockout strain, which precludes using these mice to evaluate the role of *Cntnap2* in the regulation of body weight. Therefore, we sought to evaluate the body weight phenotype of knockout mice for the interacting gene *Tagl*. *CNTNAP2* directly interacts with *TAG1*, and both are required for the proper localization of voltage-gated potassium channels at the juxtaparanodal region of the nodes of Ranvier (Poliak et al. 2003; Traka et al. 2003). Loss of *CNTNAP2* function causes a failure of *TAG1* and Kv channels to localize to the juxtaparanodes, whereas loss of *TAG1* function causes a failure of *CNTNAP2* and Kv channels to localize to the juxtaparanodes.

Both male and female *Tagl* knockout ($-/-$), heterozygous ($+/-$), and control ($+/+$) mice were fed the HFSC diet for 100 days at which time adiposity was assessed. No significant difference in body weight or BMI was found between wild-type (WT) and *Tagl* $+/-$ mice of either sex, although *Tagl* $-/-$ mice weighed significantly less, had reduced body length, and had lower BMIs than control mice (Table 1). To confirm that these differences were due to adiposity, body composition analysis was performed. Consistent with measures of total body weight, the percent body fat of male *Tagl* $+/+$ mice did not differ significantly from *Tagl* $+/-$ mice, whereas *Tagl* $-/-$ mice had a significant reduction (Table 1). Similarly, female *Tagl* $+/+$ and $+/-$ mice did not differ with respect to percent body fat, while the *Tagl* $-/-$ mice were considerably leaner (Table 1). These data confirm that *Tagl* $-/-$ mice were resistant to diet-induced obesity relative to both $+/-$ and WT control mice. Lean mass was also lower in male and female *Tagl* $-/-$ mice relative to controls (Table 1), which is consistent with their reduced body length. As no difference in adiposity was observed between *Tagl* $+/+$ and $+/-$ mice, only *Tagl* $+/-$ mice were used as controls in future experiments.

The *Tagl* $-/-$ and control mice fed a HFSC diet were also compared to mice of the same genotypes that were fed a low-fat, complex-carbohydrate diet (LFCC). Although the *Tagl* $+/-$ mice gained considerable body weight on the HFSC diet relative to the LFCC diet (males: $p < 0.01$; females: $p < 0.001$, Table 1), the *Tagl* $-/-$ mice on the HFSC diet gained no additional weight relative to their counterparts on the LFCC diet (males: $p > 0.3$; females: $p > 0.2$, Table 1). Collectively, these data demonstrate that *Tagl* $-/-$ mice are completely protected from the normal weight gain associated with an obesogenic diet.

Energy expenditure differences do not account for the obesity resistance of Tag1-deficient mice

The resistance of Tag1 $-/-$ mice to diet-induced obesity must be due to changes in either energy intake or expenditure (Spiegelman and Flier 2001). To compare energy expenditure (EE) levels in Tag1 $+/-$ and $-/-$ mice, EE was recorded over a 24-h period in mice that had been fed the HFSC diet for 100 days. Respiratory quotient did not differ between Tag1 $+/-$ and $-/-$ mice, suggesting a similar utilization of fatty acids and carbohydrates for energy production (Fig. 3a). However, both absolute EE and EE adjusted for body mass were significantly lower in Tag1 $-/-$ mice relative to control mice in both the light and dark phases (Fig. 3b, c). Thus, the metabolic phenotype of Tag1 $-/-$ mice does not likely account for their reduced adiposity relative to controls. The relatively hypometabolic phenotype of Tag1 $-/-$ mice suggested by the adjusted analysis is consistent with the lower activity level observed in this group (Fig. 3d) (Savvaki et al. 2008). While the more pronounced hypothermic excursion during cold challenge of the Tag1 $-/-$ animals compared to controls (Fig. 3e) might also seem consistent with a lower EE phenotype in Tag1 $-/-$ mice, their smaller total mass and fat mass would favor greater heat loss and hence a greater propensity for hypothermia in these animals.

Tag1 deficiency reduces food intake

The hypometabolic phenotype of Tag1 $-/-$ mice suggests that their obesity resistance is due to reduced caloric intake. To test this, we first measured the amount of energy extracted from food. However, the levels of intestinal lipid absorption did not differ ($p > 0.3$) between Tag1 $+/-$ ($97.5 \pm 0.7\%$, $n = 5$) and Tag1 $-/-$ mice ($98.2 \pm 0.4\%$, $n = 5$). Although the energy extracted from food did not differ, the Tag1 $-/-$ mice ate significantly less food than their $+/-$ littermates after 2 days on the HFSC diet (Table 2). Additionally, after 56 days on the HFSC diet, the $-/-$ mice again trended toward reduced food intake, although the difference did not reach statistical significance ($p = 0.1$). Although the food intake data after 56 days on the diet is difficult to interpret given the differences in body mass at this time, the data after 2 days on the diet demonstrates reduced caloric intake during the early dynamic phase of differential mass and fat gain. Therefore, these data together with the reduced EE phenotype suggests that decreased food intake accounts for the obesity resistance of Tag1 $-/-$ mice.

Discussion

The proteins that comprise the juxtaparanodal complex, including CNTNAP2, TAG1, Kv1.1, Kv1.2, and others, have been well characterized, although the role of these proteins in action potential propagation and in health and disease remain poorly understood (Rasband 2010). CNTNAP2 mutations in humans are associated with speech disorders, autism, and epilepsy, whereas KCNA2 (Kv1.2) mutations cause episodic epilepsy and myokemia, together demonstrating a clear link between components of the juxtaparanode and a spectrum of neurological disorders (Browne et al. 1994; Strauss et al. 2006; Alarcon et al. 2008; Arking et al. 2008; Bakkaloglu et al. 2008; Vernes et al. 2008; Newbury and Monaco 2010; O'Roak et al. 2011). These disorders are likely due to the resulting abnormalities in nerve conduction within the nervous system (Rasband 2011). In addition, a recent study of Cntnap2 knockout mice found that complete Cntnap2 deficiency causes epilepsy and an autism-like phenotype (Peñagarikano et al. 2011). However, no overt neurological disorders were observed in any of the mice used in this study, which is consistent with previous reports of Tag1 and Cntnap2 deficiency (Poliak et al. 2003; Traka et al. 2003). The absence of neurological symptoms in the Tag1 and Cntnap2 mutant mice in this study suggests that the obesity-resistance phenotype described in these studies was not secondary to neurological defects. In support of this, the body weight of Tag1 $-/-$ mice fed a standard

chow diet did not differ from that of control mice, proving that they are not simply sick mice. This can also be shown by behavioral phenotyping using the cued Morris water maze test in which mice swim in a large tank of water to an elevated platform. The *Tag1* $-/-$ mice were able to swim to the platform as efficiently as control mice, demonstrating the absence of severe movement disorders (Savvaki et al. 2008). Finally, the *Cntnap2* $-/-$ mice that were described with an autism-like phenotype did not demonstrate a body weight phenotype, suggesting that *Cntnap2*-related neurological disorders do not lead to resistance to obesity (Peñagarikano et al. 2011).

This study implicates components of the juxtaparanodal complex in the regulation of diet-induced obesity. Previous studies found no body weight differences between *Cntnap2* $-/-$ and control mice, which is consistent with our studies of *Tag1* $-/-$ mice, which also showed no weight difference from controls when fed a normal chow diet (Poliak et al. 2003; Peñagarikano et al. 2011). This suggests that the role of the juxtaparanodes in neuronal signaling may be particularly important in an obesogenic environment. The *Cntnap2* variants associated with *Obrq3* have a subtle effect on Kv channel localization at the nodes of Ranvier (Fig. 4), but nonetheless alter total body weight by more than 10%. An even larger effect on body weight was observed in the *Tag1* deficient mice, likely due to the increased severity of the mutation relative to *Cntnap2*. *Tag1* $-/-$ mice fed the HFSC diet weighed 35–45 % less than the control mice fed the same diet and remarkably were not significantly heavier than *Tag1* $-/-$ mice fed the LFCC diet, demonstrating that they were completely protected from diet-induced obesity (Table 1). The reduction in body weight was consistent with a reduction in food intake, although as EE was measured only after months on the HFSC diet, an early increase in EE following the initiation of the HFSC diet cannot be ruled out as also contributing to the phenotype. In addition, the magnitude of the reduction in food intake is less than that for the weight difference, suggesting that factors beyond food intake likely contribute to the obesity resistance of *Tag1*-deficient mice.

The subtle abnormalities in Kv channel localization detected at the nodes of Ranvier in mice carrying the *Cntnap2*^{B6} allele suggest that obesity, like many neurological disorders, may also result from signaling abnormalities within the nervous system. In support of this, a number of recent epidemiological human studies have demonstrated a positive association between obesity and both autism and epilepsy (Daniels et al. 2009; Seidenberg et al. 2009; Chen et al. 2010; Curtin et al. 2010; Rimmer et al. 2010; Walters et al. 2010), which together with our study and the study linking *Cntnap2* knockout mice to epilepsy and autism (Peñagarikano et al. 2011), suggest that these distinct disorders may have a common underlying genetic basis. In addition to the associations between obesity and neurological disease in humans, evidence from animal studies suggests that the pathogenesis of diet-induced obesity requires intact Kv channel signaling. The relationship between Kv channels and obesity has been best studied in mouse models of Kv1.3 deficiency, although Kv1.3 has not been reported to localize to the juxtaparanodes or otherwise interact with CNTNAP2 or TAG1 (Rasband 2010). Nonetheless, Kv1.3 knockout mice are more insulin-sensitive and resistant to diet-induced obesity than control mice (Xu et al. 2003, 2004). In addition, Kv1.3 deficiency reduces the adiposity and improve the life span of MC4R-deficient mice (Tucker et al. 2008), thereby demonstrating an effect on both diet-induced and genetically induced models of obesity. However, the mechanism of action is likely to be different between Kv1.3 and both CNTNAP2 and TAG1, as Kv1.3 is not found at the juxtaparanodes and Kv1.3 mutants have increased activity levels, whereas *Tag1* mutants have decreased activity levels, suggesting that each mutant regulates different components of the energy balance equation (Savvaki et al. 2008; Tucker et al. 2008). Beyond Kv1.3, antisense knockdown of the juxtaparanodal potassium channel Kv1.1 was found to decrease food intake (Ghelardini et al. 1997). As food intake levels were reduced in *Tag1* knockout mice, this suggests that the juxtaparanodal Kv1.1 may be critical for regulating food intake. Another important

mediator of food intake is leptin, which has been shown to negatively regulate *Cntnap2* mRNA expression levels in the hypothalamic paraventricular nucleus (Tung et al. 2008), where leptin expression regulates both energy intake and expenditure (Bagnasco et al. 2003). Thus, perhaps the juxtaparanodal Kv channels are key mediators of the leptin-signaling pathway. Further studies will be required to discern the sites of *Cntnap2* and *Tag1* expression that are relevant for the regulation of food intake, including both the relevant cell types and the regions within a cell, since, in addition to the juxtaparanodes, both *TAG1* and *CNTNAP2* are also found at the axon initial segment (Rasband 2010).

In addition to implicating two novel genes, *Cntnap2* and *Tag1*, in the regulation of diet-induced obesity, this study also highlights the complex underlying genetic architecture. Although B6 mice are widely used as a model of diet-induced obesity, the B6-derived allele of *Obrq3b* was associated with a 4.89-g reduction in body weight (Fig. 1, strain 6C2 vs. 6C3b), which is 12.5 % of the total body weight of 6C3b mice. Thus, B6 mice carry in their genome this potentially potent obesity-resistance allele and yet are remarkably susceptible to diet-induced obesity. However, due to the nature of the congenic panel used to identify these QTLs, both these findings were made in the context of the *Obrq2^{A/J}* allele. The same B6-derived *Obrq3b* allele, when removed from the context of *Obrq2^{A/J}* and studied on an otherwise B6 background, was found to increase body weight by 5.15 g (11.1 % of total body weight) relative to control mice (Fig. 1, B6 vs. strains 6C3b-R1 and 6C3b-R2). The fact that a single QTL can have dramatically opposite effects on body weight depending on the genomic context highlights the difficulty in trying to identify the genetic basis of complex traits in humans using single-locus models to detect associations. As listed in the dbSNP database, the two amino acid variants found in the *Cntnap2^{B6}* allele are also found in other commonly used inbred strains, including C3H/HeJ, DBA/2J, and 129S1/SvImJ, whereas the *Cntnap2^{A/J}* allele is found in strains FVB/NJ, BALB/cByJ, and AKR/J. Thus, it would be of interest to test the effect of each *Cntnap2* allele on other genetic backgrounds.

There are currently a number of computational protocols in development that seek to extend association models by attempting to identify interacting loci, with most models focusing on two-locus interactions (Gilbert-Diamond and Moore 2011). These models have been used to identify interacting SNP pairs missed using traditional association methods that influence susceptibility for type 2 diabetes, Crohn's disease, bipolar disorder, hypertension, and rheumatoid arthritis (Emily et al. 2009; Liu et al. 2011). However, as two-loci interaction studies are already computationally intensive and require a considerable statistical penalty for multiple observations, higher-order interactions are even more difficult to detect (Cordell 2009; Zuk et al. 2012). This represents a major limitation toward the identification of genetic risk factors as our previous analyses of chromosome substitution and congenic strains demonstrated the widespread nature and important contributions of epistatic interactions to complex disease (Shao et al. 2010; Yazbek et al. 2011). As the majority of genetic susceptibility alleles for complex human traits remain undiscovered, improved statistical methods for the detection of complex epistatic interactions guided by insight gained from studies in model organisms may greatly aid in the identification of disease genes and improve our understanding of their context-dependent effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Schematic of subcongenic strains derived from strains 6C2 and 6C3. a Locations of *Obrq2* and *Obrq3* are indicated relative to chromosome 6. b Subcongenic panels that define the body weight QTLs *Obrq3a* and *Obrq3b*. Strain names are indicated on the left. Body weight data are presented as mean ± SEM. c Strains 6C3bR1 and 6C3b-R2 carry the A/J-derived allele of *Obrq3b* on an otherwise B6 background and were used to test for epistasis between *Obrq2* and *Obrq3b*

		V161I	
A			
Mouse (B6)	QHAVVARYVR	V	VPLDWNGEGH
Mouse (A/J)	QHAVVARYVR	I	VPLDWNGEGH
Human	QHPIIARYVR	I	VPLDWNGEGR
Lemur	QHPIIARYVR	I	VPLDWNGEGR
Cat	XXXVIARYVR	I	VPLDWNGEGR
Armadillo	QHPITARYVR	M	VPLDWNGEGR
Rabbit	QHPVIARYVR	I	VPLDWNGEGH
Microbat	QHPVARYVR	F	VPLDWNGEGR
Squirrel	QHPVARYVR	M	VPLDWNGEGR
Elephant	QHPVTARYVR	L	VPLDWNAEGR
Rat	QHAVVARYVR	I	VPLDWNGEGH
Orangutan	QHPVIARYVR	V	VPLDWNGEGR
Chimpanzee	QHPIIARYVR	I	VPLDWNEEGR
Tetraodon	QHAILARYIR	L	IPVDWSREGR
B		H538Q	
Mouse (B6)	DQLVNLYEVA	H	RKPGSFANVT
Mouse (A/J)	DQLVNLYEVA	Q	RKPGSFANVT
Human	DQLVNLYEVA	Q	RKPGSFANVS
Cat	DQLVNLYEVA	Q	RRPGSFANVS
Rabbit	DQLVNLYEVA	Q	RKPGSFANVS
Microbat	DQLVNLYEVA	Q	RKPGSFANVS
Squirrel	DQLVNLYEVA	Q	RKPGSLANVS
Tree Shrew	DQLVNLYEVA	Q	-KPGSFANVS
Rat	DQLVNLYEVA	Q	RKPGSFANVT
Orangutan	DQLVNLYEVA	Q	RKPGSFANVS
Chimpanzee	DQLVNLYEVA	Q	RKPGSFANVS
Macaque	DQLVNLYEVA	Q	RKPGSFANVS
Chicken	DQLVDLQAVE	Q	GQLGSFANVS
Tetraodon	DHLADLRAVE	Q	GLIGTFENVS

Fig. 2.

Location of amino acid variants in CNTNAP2 between B6 and A/J. Amino acid sequence of the CNTNAP2 protein flanking the a V161I or b H538Q variant is indicated. All sequences were obtained from the Ensembl genome browser

Fig. 3.

Decreased absolute and adjusted energy expenditure in Tag1-deficient mice. Respiratory quotient (a), unadjusted energy expenditure (b), and energy expenditure adjusted for body mass (c) were measured in control and Tag1 knockout mice fed the HFSC diet as indicated. Measurements were taken during the light phase (light 1), the dark phase (dark), and then again in the light phase (light 2) over a 24-h period; n = 8 per group. d Total activity was measured with an implanted E-Mitter over 5 days in mice (n = 4 per group) fed the HFSC diet. e Core body temperature was recorded (n = 4 per group) every 30 s with an implanted E-Mitter during a 4-h cold challenge at 4 °C. *p < 0.05; **p < 0.001; ***p < 0.0001

Fig. 4.

Localization of ezrin and Kv1.2 in sciatic nerve. Sciatic nerve from adult mice fed the HFSC diet was labeled with antibodies to ezrin (green) and Kv1.2 (red). Nodes were identified by ezrin staining and scored as demonstrating normal, heminodal, or an absence of Kv1.2 staining (n = 100 per strain). The distribution of Kv1.2 staining patterns was compared using a χ^2 test and shown to be significantly different between strains 6C2 and 6C3b ($p < 0.0001$) (Color figure online)

Table 1

Measures of adiposity in Tag1-deficient and control mice

Diet	Tag1 genotype	Body weight (g)	Length (cm)	BMI (g/cm ²)	Body fat (%)	Fat mass (g)	Lean mass (g)
HFSC	Males						
	+/+	45.74 ± 1.34 (n = 16)	105.5 ± 0.9 (n = 6)	0.41 ± 0.02 (n = 6)	24.85 ± 1.55 (n = 4)	11.30 ± 1.33 (n = 4)	32.59 ± 1.66 (n = 4)
	+/-	41.06 ± 1.48 (n = 25)	104.3 ± 0.7 (n = 11)	0.40 ± 0.01 (n = 11)	24.58 ± 0.99 (n = 9)	10.86 ± 0.68 (n = 9)	32.12 ± 0.93 (n = 9)
	-/-	22.11 ± 0.69* (n = 16)	95.6 ± 1.6* (n = 5)	0.23 ± 0.01* (n = 5)	7.76 ± 0.88* (n = 2)	1.73 ± 0.12* (n = 2)	20.01 ± 1.22* (n = 2)
LFCC	Females						
	+/+	28.50 ± 0.63 (n = 33)	100.2 ± 0.7 (n = 13)	0.27 ± 0.01 (n = 13)	17.58 ± 2.14 (n = 5)	5.52 ± 1.01 (n = 5)	23.90 ± 1.19 (n = 5)
	+/-	29.93 ± 0.68 (n = 41)	101.1 ± 1.0 (n = 9)	0.31 ± 0.01 (n = 9)	21.56 ± 1.93 (n = 9)	7.91 ± 1.21 (n = 9)	26.37 ± 1.19 (n = 9)
	-/-	19.04 ± 0.27* (n = 34)	95.1 ± 0.7* (n = 8)	0.22 ± 0.01* (n = 8)	9.70 ± 0.76* (n = 7)	1.94 ± 0.18* (n = 7)	17.12 ± 0.23* (n = 7)
LFCC	Males						
	+/+	28.66 ± 2.69 (n = 3)	103.0 ± 1.2 (n = 3)	0.27 ± 0.02 (n = 3)	ND	ND	ND
	+/-	20.66 ± 0.66* (n = 4)	97.0 ± 0.9* (n = 4)	0.22 ± 0.01* (n = 4)	ND	ND	ND
	Females						
LFCC	+/+	23.50 ± 0.76 (n = 7)	100.0 ± 0.6 (n = 3)	0.23 ± 0.01 (n = 3)	10.10 ± 2.34 (n = 4)	2.48 ± 0.67 (n = 4)	20.30 ± 0.56 (n = 4)
	-/-	19.76 ± 0.48* (n = 9)	95.8 ± 1.2* (n = 5)	0.22 ± 0.01 (n = 5)	7.81 ± 0.66 (n = 4)	1.56 ± 0.14 (n = 4)	16.98 ± 0.52* (n = 4)

ND not done

* p < 0.05 relative to +/+ controls (HFSC diet) or +/- controls (LFCC diet)

Table 2

Food intake of Tag1 knockout and control mice

Tag1 genotype	Days on HFSC diet	Food intake per mouse (g)
+/-	2	3.13 ± 0.08
-/-	2	2.79 ± 0.10 *
+/-	56	4.00 ± 0.31
-/-	56	3.22 ± 0.33

*
p < 0.05 relative to +/- control

n = 6–8 cages of mice