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Burly1 is a mouse QTL for lean body mass that maps to a 0.8-Mb region of chromosome 2

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Abstract
To fine map a mouse QTL for lean body mass (*Burly1*), we used information from intercross, backcross, consomic, and congenic mice derived from the C57BL/6ByJ (host) and 129P3/J (donor) strains. The results from these mapping populations were concordant and showed that *Burly1* is located between 151.9 and 152.7 Mb (*rs33197365* to *rs3700604*) on mouse chromosome 2. The congenic region harboring *Burly1* contains 26 protein-coding genes, 11 noncoding RNA elements (e.g., lncRNA), and 4 pseudogenes, with 1949 predicted functional variants. Of the protein-coding genes, 7 have missense variants, including genes that may contribute to lean body weight, such as *Angpt41, Slc52c3*, and *Rem1*. Lean body mass was increased by the B6-derived variant relative to the 129-derived allele. *Burly1* influenced lean body weight at all ages but not food intake or locomotor activity. However, congenic mice with the B6 allele produced more heat per kilogram of lean body weight than did controls, pointing to a genotype effect on lean mass metabolism. These results show the value of integrating information from several mapping populations to refine the map location of body composition QTLs and to identify a short list of candidate genes.

Introduction

An average adult mouse weighs about 25–30 g, and most of that weight is lean body mass. Lean body mass can differ almost threefold among common inbred strains, and this trait is highly heritable (Reed et al. 2007, 2011). We and others have discovered dozens of influential body-mass-related quantitative trait loci (QTLs), many of which are cataloged in the Mouse Genome Database (Eppig et al. 2005). Identifying the underlying causal genetic variants responsible for lean body mass (Drinkwater and Gould 2012), while challenging, is an important scientific goal, because lean body mass affects many tissues and functions of the body, including basal metabolic rate, glucose metabolism and metabolic health (Brochu et al. 2008; Fukushima et al. 2016), the immune system, and bone development (Crabtree et al. 2004).

Several genes and their variants greatly affect body size or lean body mass composition. Perhaps the best known are alleles of the myostatin gene that markedly increase muscle mass in mice (McPherron et al. 1997; Szabo et al. 1998), cows (Grobet et al. 1997; Kambadur et al. 1997; McPherron and Lee 1997), sheep (Clop et al. 2006), and other animals (Mosher et al. 2007), including humans (Schuelke et al. 2004). Other well-known variants are components of the growth hormone pathway, such as the *little* mutation (Donahue and Beamer 1993; Lin et al. 1993) and *dwarf* (Snell 1929). *Little* mice are small, with reduced lean body mass, whereas *dwarf* mice are tiny (Li et al. 1990) but have the usual proportion of lean and fat mass (Benson and Chada 1994). Beyond these single-gene mouse mutations, natural variation in lean body mass has a complex genetic architecture encompassing numerous genes. Exactly how many genes contribute is unclear: QTL experiments suggest scores of loci (Wuschke et al. 2006), and knockout experiments suggest that almost a third of viable strains have reduced body weight (Reed et al. 2008; White et al. 2013), indicating

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that many thousands of genes may participate. Meta-analysis of human genome-wide association approaches indicates that there are five reproducible loci for human lean body mass (Zillikens et al. 2017), but even collectively, these studies are underpowered. One reason for this is that direct measures of lean body mass are time-consuming and require specialized equipment relative to other measures like height. We speculate that when comparable numbers of subjects have been studied, common variation in hundreds or even thousands of genes will determine individual differences in human lean mass, similar to human height (Field et al. 2016; Wood et al. 2014).

In addition to genotype, the amount of lean body mass of individual mice is affected by sex, age, and maternal characteristics. Male mice have more lean body mass than do females of the same strain (Reed et al. 2007). Lean body mass peaks in middle to late adulthood and then declines (Gargiulo et al. 2014). Maternal effects include the dam's age, diet, behavior, and litter size (Funk-Keenan and Atchley 2005). Another factor that affects body composition is the parental origin of particular inherited alleles (imprinting) (Cheverud et al. 2010; Lawson et al. 2013), with paternally transcribed alleles generally favoring rapid growth. In the current work, we strove to keep these factors constant. We compared 180-day-old male littermates segregating a particular genetic variation, so that age, sex, and maternal effects were similar but the genotype differed. However, for some specialized mouse strains, such as consomics, it is impractical to compare littermates differing in genotype. The reason it is impractical to compare littermates in this case is because breeding heterozygous consomic mice (i.e., those parents that have a full length donor and a full length host chromosome) produce offspring that have a recombinant chromosome. Thus in these cases, we compared homozygous consomic mice to inbred mice of the host strain, e.g., (Singer et al. 2004; Spiezio et al. 2014).

With these points in mind, our goals here were to map a particular lean body mass QTL, *Burly1*, and to identify the underlying genetic variants using a pair of contrasting inbred mouse strains. We began by intercrossing the heavier C57BL/6ByJ (B6) strain with the lighter 129P3/J (129) strain (Bachmanov et al. 2001b; Reed et al. 2003, 2006) and found a QTL on chromosome 2 for body weight, *Bwq5*. Mouse chromosome 2 harbors many related QTLs [e.g., (Gularte-Merida et al. 2015; Kobayashi et al. 2014; Mollah and Ishikawa 2011; Rocha et al. 2004b)]. This density makes it especially hard to dissect particular genetic effects on body composition, and thus this intercross population did not provide sufficient mapping resolution to narrow the genomic interval. Also, the phenotype, body weight as a proxy measure of lean body mass, was imprecise. Therefore, our next steps were to dissect this QTL by creating and studying additional mapping resources and by using direct measures of body composition rather than body weight. To this end, we studied a second intercross population, two reciprocal consomic strains (Lin et al. 2015), several congenic strains, and the backcross mice produced during the breeding of these strains.

We conducted metabolic assessments for the *Burly1* congenic mice and measured food intake and locomotor activity because energy intake, oxidation, and expenditure are linked to lean body mass (Tschop et al. 2012). We also conducted oral glucose tolerance tests (OGTT) because of an association between insulin resistance and lean body mass (Gysel et al. 2014).

Here we report all the *Burly1* mapping data we obtained from 2053 mice derived from two intercrosses, four backcross generations, one consomic and one sub-consomic strain (defined below), and 25 congenic strains. To compare these mapping populations, we assayed or imputed genotypes for a common set of markers on mouse chromosome 2 and analyzed the genotype-phenotype associations using several approaches. To gain insight about how the *Burly1* locus affects lean body mass, we characterized its effects in mice at different ages and measured related traits.

Methods

Animal husbandry

We bred all mice in a vivarium at the Monell Chemical Senses Center, located in Philadelphia, Pennsylvania (USA), using inbred B6 and 129 mice originally purchased from the Jackson Laboratory. Husbandry practices were stable throughout the study, with nearly the same vivarium personnel, cages, and type of bedding (Aspen Shavings, Northeastern Products Corp, Warrensburg, NY). All mice were fed Rodent Diet 8604 (Harlan Teklad, Madison, WI) and lived in a 12:12-h light/dark cycle, with lights off at 7 pm (barring unusual circumstances, such as power outages). For most mapping populations, we studied body composition of male mice only, to reduce overall trait variation and increase mapping power. However, in some experiments, we collected data from female mice for other reasons, and we report those data here as well. The Monell Institutional Animal Care and Use Committee approved these study procedures.

Intercrosses

We have previously reported the body weight results from the first F_2 intercross (McDaniel et al. 2006; Reed et al. 2003, 2006), but we also include them here to facilitate comparison with results from later mapping populations. We bred a second intercross population with the same parental strains and measured lean and fat mass using dual X-ray

absorptiometry (DEXA). For this second intercross, we previously reported the results for fat but not lean body mass (Lin et al. 2013).

Backcrosses and consomics

To make consomic strains, we produced reciprocal N_2 ($F_1 \times$ B6 and $F_1 \times$ 129) and then N_3 backcross generations, followed by serial backcrossing and intercrossing of male and female mice to create the consomic B6-Chr2^{129} and 129-Chr2^{B6} strains. We were successful in creating one of these two reciprocal consomic strains (B6-Chr2^{129}), which is publicly available and listed in Table S1 (Lin et al. in preparation). The other reciprocal strain (129-Chr2^{B6}) was difficult to breed, so instead we created a strain with a partial- rather than full-length donor chromosome, which we refer to as 'sub-consomic.' We measured lean body mass in male and female mice from both the fully consomic and the sub-consomic strain, the inbred host strains or the littermate controls, and male mice from the backcross generations used to create them (i.e., the N_2 and N_3 generations).

Congenics

We bred congenic mice by backcrossing N_8F_2 males (heterozygous males with a partial donor chromosome 2 generated from the consomic strain B6-Chr2^{129}) to the B6 background to obtain 129-derived donor regions of various lengths. The goal was to identify male breeders with a donor region that overlapped the QTL location from the first F_2 intercross. We named the congenic strains with codes that reflect their lineage; for instance, all congenic strains had the prefix 'C' for congenic and a number 1 (e.g., C1.1) to indicate descent from common progenitors. We bred all congenic mice from B6 (inbred) mothers and from fathers that were heterozygous for the 129-derived donor region. This approach reduced maternal effects (all mothers of the congenic mice had the same genotype) and reduced imprinting variation (only fathers contributed the congenic donor region). This strategy allowed us to compare littermates with one copy of the donor region (heterozygous; 129/B6) to those without the donor region (homozygous; B6/B6; Fig. 1). Each congenic mouse potentially was genetically unique (because the paternal donor region could shorten due to meiotic recombination). Therefore, we genotyped each congenic mouse to ensure we could define the donor region breakpoints. In addition to these congenic strains, we bred homozygous mice from a congenic strain with a small donor fragment. This strain is also now publicly available (strain C2.5; Table S1). We produced additional mice from the littermates of strain C2.5 without the 129-derived donor fragment for use as a comparison group.

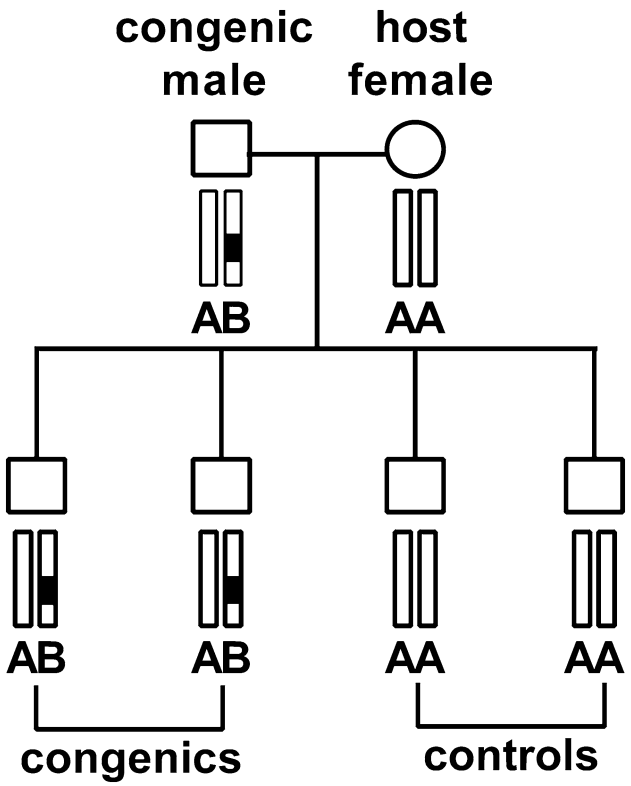

Fig. 1 Congenic experimental design to reduce variation due to imprinting and maternal effects. Littermates with one copy of the donor region from the male parent are compared to littermates with the host genotype

Body composition

Our primary outcome measure was lean body mass as assessed by DEXA (PIXImus II densitometer; GE software, version 2.00; Lunar Corp., Madison, WI). We also weighed the body of each mouse to the nearest 0.1 g and measured some, but not all, congenic mice at 90, 120, 150, and 180 days for lean body mass using magnetic resonance (MR) methods (Bruker mini-spec LF110, Horizontal Whole-Body Composition Rat and Mice Analyzer; Bruker BioSpin Corporation, Billerica, MA). We obtained this MR instrument in 2012, during the course of this study, so mice tested prior to that date have no MR data. For some congenic mice (from C1 and C2; $N = 198$), we also performed a necropsy using anatomic landmarks to define organs (Cinti 1999; Hayakawa et al. 2001). We weighed organs for the spleen, heart, pancreas, brain, kidney, and liver to the nearest 0.01 g.

Genotyping

We assayed the genotype of markers on chromosome 2 in a number of ways because the technology changed over the course of the study. A list of genotyped markers is presented

in Table S2. We evaluated simple sequence-length polymorphism markers by polyacrylamide gel electrophoresis after polymerase chain reaction amplification by locus-specific primers (Dietrich et al. 1992) in our laboratory. We assayed single-nucleotide polymorphisms (SNPs) at three locations: the Genotyping and RNA Analysis Core of the Monell Chemical Senses Center, the Center for Inherited Disease Research (see Electronic Resources), and a commercial vendor (LGC, Beverly, MA; formerly KBiosciences). When assaying variants in the Monell genotyping core, we used primers and allele-specific dye-labeled probes (Life Technologies, Carlsbad, CA). Irrespective of genotyping location and method, controls (blank samples, and genomic DNA from inbred progenitors and their F_1 hybrids) were included with all assays, and we retested unlikely genotypes as needed. We did not type all mice for all markers, so we imputed missing data by tracing the parental origin of the marker alleles (where applicable) and assumed that no double recombination occurred between markers separated by 26 Mb or less. We cite all genomic base pair positions here relative to GRCm38.

Data analysis overview

Several goals guided the statistical analysis plan. We wanted to confirm the validity of the lean body mass measurement methods, map the *Burly1* locus to the smallest possible physical location, analyze the broader *Burly1* phenotype, and find most or all genes and variants in the *Burly1* region. We describe each goal in turn below.

Before performing parametric statistical analyses, we checked the distribution of the phenotype for normality within each mapping population using the Lilliefors test; non-normal data were then transformed as appropriate (Delignette-Muller et al. 2014). ANOVAs and general linear models were all type 1 (sequential sum of squares) which we selected because it tests the effects of the fixed factor sequentially (in this case genotype was the first factor and our most important dependent variable). All post hoc tests were Fisher's least square mean tests unless otherwise indicated. For all data analyses, we computed the statistical tests with R (version 3.3.3) and R-studio (version 1.0.136) and graphed the results using either R or GraphPad Prism 6 (version 6.05; GraphPad Software, La Jolla, CA). All data are available for download on the Center for Open Science (https://osf.io/7tfjr/).

Validity of measure of lean body composition

We compared the MR, DEXA, and body weight data of the mice when they were 180 days of age using Pearson correlation coefficients to determine if these methods gave similar estimates of lean body mass, assuming that congruence indicates the validity of each. We focused on male mice for this analysis because we made most of these measures on males.

Age

We analyzed the age-related increase in lean body mass expected at 90, 120, 150, and 180 days of age using a repeated-measures ANOVA with these age categories as the repeating measures. (Scheduling issues precluded measuring mice at exactly these ages; we measured some a few days earlier or later; we grouped mice by age category if they were within 8 days of the target age.) For this analysis, we separated all mice with MR measures into two groups based on genetic background (B6 vs. 129), because mice with a predominantly 129 genetic background are obviously smaller than are those with the B6 background regardless of *Burly1* genotype, and these large differences could mask smaller age effects.

Mapping *Burly1* to a chromosome location

To map *Burly1* to its physical location, we conducted a general linear model analysis on each of the mapping populations (Table 1), as described below. For congenic strains, we used the common segment analysis method to analyze results obtained (Shao et al. 2010).

Intercross, backcross, and congenic analysis

Within each segregating mapping population, we conducted a general linear model analysis with genotype as a fixed factor and body weight as a covariate. For all populations and for each marker, we calculated (a) the genotype means, (b) the p-value test statistic as the negative base 10 logarithm, and (c) the effect size using Cohen's *D* (Cohen 1988). We report these values, including confidence intervals (defined by 2 units of $-\log_{10} p$-value drop), for the peak marker from each mapping population. Statistical thresholds were computed with a Bonferroni correction to an α level of 0.05 for the number of markers ($N = 122$, $-\log 10\frac{\infty}{N.} = \sim 3.39$). For the first F_2 population, we used body weight as a proxy measure of lean body mass. We included age as a covariate in any population where age differed by more than a month, and if we measured female as well as male mice, we included sex in the model.

Consomic analysis

Our analysis methods differed between the consomic (B6-Chr2^{129}) and sub-consomic (129-Chr2^{B6}) strains. For the consomic strain, we analyzed lean body mass using strain (consomic vs. inbred B6) and sex as fixed factors

Table 1 Characteristics of the 2053 mice used in mapping studies

Population	*N*	Age range (days)	Tests performed[a]			Age at MR (days, $M \pm SD$)	Period of study (mm/dd/year)		References
			BW	DEXA	MR		Start	End	
F_2-First	397	94–354	√	x	x	195 ± 87	09/13/94	05/28/96	Reed et al. (2003)
F_2-Second	113	231–270	√	√	x	241 ± 9	05/28/02	07/01/02	Lin et al. (2013)
N_2 ($F_1 \times 129$)	100	287–298	√	√	x	293 ± 2	08/29/05	11/23/05	NA
N_2 ($F_1 \times B6$)	92	288–298	√	√	x	292 ± 2	09/06/05	12/12/05	NA
N_3 [($F_1 \times 129$) × 129]	79	322–335	√	√	x	327 ± 3	07/17/06	01/17/07	NA
N_3 [($F_1 \times B6$) × B6]	49	322–335	√	√	x	325 ± 3	07/17/06	01/22/07	NA
129-Chr2^{B6}	81	178–190	√	√	p	180 ± 2	12/30/09	02/22/13	Lin et al. (2015)
B6-Chr2^{129}	63	175–209	√	√	p	187 ± 11	11/28/10	09/26/14	Lin et al. (2015)
Inbred	49	179–207	√	√	p	183 ± 5	03/27/10	01/04/14	NA
Congenic	1030	161–351	√	√	√	188 ± 28	11/16/11	04/05/16	NA

Population type of mapping resource, *F_1 and F_2* derived from inbred 129 and B6 strains, *N* number of mice, *Age range* age in days for the last test of mice, *BW* body weight, *DEXA* dual X-ray absorptiometry, *MR* magnetic resonance, *Age at MR* final age point only, mean ± standard deviation (*SD* most of mice underwent MR at 180 days, but some were a few days older or younger), *Start and End* start and end dates for breeding, by month/day/year, *Ref* reference that describes breeding of the mapping population (*NA* not applicable)

[a] √, measured in all mice; p (partial), measured in some but not all mice; x, not measured in that population

and body weight as a covariate followed by post hoc tests to determine the significance of strain effects. For the sub-consomic strain, we analyzed the data the same way except using individual genotype at each marker location, that is, for the sub-consomic mice (129/B6) versus their homozygous littermates (129/129). We calculated the genotype effect size in Cohen's *D* of each sex for the consomic and sub-consomic strains to compare the QTL effect size across mapping populations.

Congenics and the common segment method

The common segment method is a way to map genes underlying QTL and is based on the idea that congenic strains that differ from the host strain in the trait of interest should all share a specific donor region which congenic strains that do not differ from the host strain should not share, i.e., there should be a perfect match between trait and donor region (Lin et al. 2017; Shao et al. 2010). We analyzed the data derived from congenic mice using this common segment method with the strains listed in Table 2. Some strains had very few mice, so we performed two analyses: more broadly, we analyzed all potentially informative congenic strains, defined as strains with at least three mice of each genotype group within a congenic strain; more narrowly, we included only those strains with at least 38 mice in each of the two possible genotypes. These choices of number of mice per group are arbitrary but were suggested by natural breakpoints in the number of mice we obtained per strain. In addition, we analyzed the effect of the *Burly1* genotype on lean body mass by comparing homozygous congenic mice (strain C2.5; 129/129) with control mice without the 129-derived donor fragment (B6/B6). We conducted this analysis for lean body mass using genotype of marker *rs3666533* as a fixed factor and with body weight and age as covariates followed by post hoc tests to determine significance of genotype effects, using $p < 0.01$ as the significance threshold. We chose this particular marker as a proxy for the donor fragment because it was most strongly associated with lean body mass in the *Buly1* QTL region; we included age as a covariate in this model (Table 1).

Age by genotype

To determine how early in adult life we could detect the effects of *Burly1*, we applied a repeated-measure ANOVA using lean body mass in grams (MR data) for mice from the relevant congenic strains. (By 'relevant' we mean the congenic strains with donor regions that overlapped with the *Burly1* locus, defined as genotype variation at the *Burly1* peak marker *rs3666533*.) In total, 21 *Burly1* congenic strains were included in this analysis. Four congenic strains were excluded because of incomplete MR data (strains C1, C1.1, C1.3, and C2). Next, to check whether the lean body mass was accounted for by a specific organ, we calculated Pearson correlations between body weight and weight of each organ weight (kidney, pancreas, spleen, liver, heart, and brain) within the congenic mice (from strains C1 and C2) grouped by the *Burly1* genotypes (129/B6 and 129/129). We tested whether the relationship between body weight and organ

Table 2 Characteristics of congenic strains

Strain	*N*	Age at DEXA (days)		MR[a]
		Range	Mean ± SD	
C1	218	165–200	180 ± 3	x
C1.1	2	181–181	181 ± 0	x
C1.10	8	179–180	179 ± 0	p
C1.11	4	180–180	180 ± 0	√
C1.11.1	77	179–182	180 ± 0	p
C1.12	8	179–181	180 ± 0	p
C1.13	12	180–181	180 ± 0	p
C1.14	1	180–180	180 ± 0	√
C1.2	14	180–181	180 ± 0	p
C1.3	3	180–180	180 ± 0	x
C1.4	9	180–181	180 ± 0	p
C1.5	10	180–180	180 ± 0	√
C1.6	1	180–180	180 ± 0	√
C1.7	17	180–183	180 ± 0	p
C1.7.1	29	179–224	187 ± 16	√
C1.8	11	180–181	180 ± 0	p
C1.9	8	179–180	179 ± 0	p
C2	164	177–192	180 ± 2	x
C2.1	16	180–250	204 ± 32	p
C2.2	2	181–181	181 ± 0	√
C2.3	27	180–350	195 ± 34	p
C2.4	171	179–183	180 ± 0	p
C2.5	195	161–351	216 ± 50	p
C2.6	10	180–181	180 ± 0	√
C2.7	10	180–180	180 ± 0	√

N number of mice, *DEXA* dual X-ray absorptiometry, *SD* standard deviation (an SD of 0 indicates less than 1 day), *MR* magnetic resonance at four time points (90, 120, 150, and 180 days), *Metab* metabolism measures using the LabMaster equipment, *OGTT* oral glucose tolerance test.

[a]√ measured in all mice; p (partial), measured in some but not all mice; x, not measured in that strain. Owing to husbandry errors, we made DEXA measures outside of our normal procedures that are not reflected in the table totals: strain 2.2, $n=2$ measured at 120 days old (not included in this table); strain 2, $n=1$ measured at 100 days old (not included in this table); homozygous strain C2.5, $n=36$ measured at 8 months of age and $n=15$ at 161–163 days old; $n=7$ for homozygous congenic with donor (129/129) and $n=44$ their littermates without donor (B6/B6). For the metabolism measures, we measured 52 mice in total, $n=30$ with the B6/129 genotype and $n=20$ with the B6/B6. For the OGTT, we measured 47 mice in total, $n=25$ with the B6/129 genotype and $n=22$ with the B6/B6. All mice measured for metabolism and for OGTT were from the C2.5 strain. For necropsy, we measured 367 mice in total, $N=209$ from C1 strain and $N=158$ from C2 strain

size differed by genotype (allometry) using the Fisher r-to-z transformation with two-tailed p value, $p<0.05$ was set as a significance level (Revelle 2018).

Metabolism

We examined whether mice that differed in lean body mass as function of *Burly1* genotype also differed in food intake, activity, or metabolism. Using a TSE LabMaster (version 5.0.6; TSE Systems, Inc., Chesterfield, MO, USA), we measured the phenotypes of mice from congenic strain C2.5 and a control group comprising littermates without the 129 donor fragment (B6/B6). We chose this congenic strain because it contained the smallest donor region that contained the *Burly1* locus. To measure these traits, we trained mice for several days in cages that mimicked the experimental cages to ensure they learned to eat and drink appropriately. When we transferred the mice to the experimental cages, we quantified food intake and water intake corrected for lean body mass, physical activity in three dimensions (including rearing as well as walking), and increases in carbon dioxide production and decreases in oxygen consumption. We used these values to compute heat produced per hour for each mouse, correcting for lean body mass rather than total body weight (Speakman et al. 2013), and we expressed all data as the mean of four 24-h data acquisition cycles. To analyze these data, we used *t*-tests to compare genotype groups of marker *rs3666533* (129/B6 vs. B6/B6 in the congenics).

Oral glucose tolerance test (OGTT)

To determine if the *Burly1* genotype affects oral glucose tolerance, we measured 22 heterozygous congenic mice (strain C2.5; 129/B6) and a control group of their 25 homozygous (B6/B6) littermates. We deprived most mice of food for 4 h. However, three congenic mice and five mice from the control group were deprived for 18 h owing to a technical error, but including or excluding these eight mice did not affect the results. We gavaged them with 0.2 g/ml glucose solution for a final dose of 2 g/kg mouse body weight. We collected tail blood samples at baseline and 5-, 15-, 20-, 25-, 30-, 45-, 60-, 75-, 90-, 105-, and 120-min postgavage and measured blood glucose concentrations (mg/dL) using an Accu-Chek Avia Plus meter (Roche Diagnostics, Indianapolis, IN, USA). Missing data points, due to difficulty collecting blood at every time point from some mice, were interpolated using the average values within each genotype group at the same time point. We analyzed the data using a repeated one-way ANOVA followed by post hoc Tukey's HSD tests.

Evaluating genes and variants in the *Burly1* region

Drawing on the genomic coordinates suggested by the congenic results from the common segment analysis method, we found all previously annotated genes within the *Burly1* region using an online database (NCBI) (Anonymous 2015b). We used another online database (WellcomeTrust

Sanger Mouse) (Anonymous 2011) to find genomic variants among inbred mouse strains related to the B6 and 129 stains studied here (Keane et al. 2011; Yalcin et al. 2011). We formatted this information using an online tool (Variant Effect Predictor; VEP) (McLaren et al. 2016), and we identified those regulatory, nonsense-mediated mRNA decay (NMD) and coding variants with the potential to cause functional changes (Anonymous 2012). In addition, we identified human genes and their variants associated with body mass that are located in the region of conserved synteny with the mouse *Burly1* region by searching an online catalog of human genome-wide association results (Anonymous 2015a) using the key word 'lean body mass' as well as the less specific term 'body mass index.'

Results

Overview

Table 1 lists the number of mice studied per mapping population, their age range, and which of the three lean body mass methods we used to measure this trait: body weight, and lean body mass measured by DEXA and by MR. Table 2 lists the individual congenic strains, number of mice studied per strain, and their ages at DEXA analysis, as well as which additional measures we made on these mice. The wide range in the number of mice bred for each congenic strain owes to breeding difficulties and practical constraints on the size of our animal colony. Here, we show details of every congenic strain we bred, even those that were potentially uninformative because of the sample size. However, we eliminated the data from three mice from these tables: two were pregnant (owing to husbandry errors), and one had a large kidney tumor.

Normality

We assessed whether lean body mass data were distributed normally within each mapping population. Significant deviations from normality were present in the first F_2 population and the pooled congenic population. For the first F_2 population, no transformation was effective at normalizing the distribution of the data, and because no method achieved the desired result, we report the analysis of the untransformed data after confirming that the results were similar using all of the transformations attempted (Table S3).

Validity

Lean body mass was validly measured by both DEXA and MR. We draw this conclusion because the three measures: body weight, and lean body mass measured by DEXA and by MR—while not identical, were highly correlated (Fig. 2; *r*-values, 0.62–0.95; $p < 0.0001$). The exact r-statistics varied depending on the mapping population. Figure S1 shows all body weight, DEXA, and MR correlation data; Table S4 provides all correlation test statistics by mapping population. For the primary end-point measure, we used the DEXA data, which provided a more complete analysis because we did not have MR data for all mice.

Age

As we expected, mouse lean body mass increased over time until ~ 150 days of age and was stable thereafter (Fig. S2). We also noted that, independent of the *Burly1* locus, mice with a 129 genetic background differed from those with a B6 background in the pace of lean body mass growth between 120 and 150 days of age. Mice with the B6 background were still adding lean body mass during this period, whereas those with the 129 background plateaued.

Mapping

For the intercross, backcross, and congenic mice, we show the association test statistics and the QTL locations, with confidence intervals, for each mouse mapping population (Fig. 3a–e), as well as the genotype mean of lean body mass (Fig. 3f–j) and the effect sizes for the peak linked marker (Fig. 3k–o). We found a single common genomic region (around 152 Mb) responsible for lean body mass in all mapping populations (Fig. 3a–e). There is a consistent direction of allelic effect, with the B6 allele increasing lean body mass relative to the 129 allele (Fig. 3f–j). The means of the genotype groups differed by over 0.5 standard deviations for the backcrosses with the B6 background

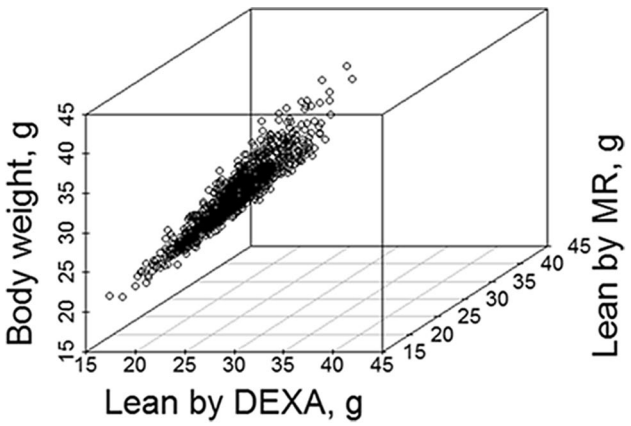

Fig. 2 3D scatter plot of body weight and lean body mass measured by DEXA and MR in male mice. These measures are highly correlated (*r*-values of 0.62–0.95, $p < 0.0001$) within the mapping populations (Fig. S1)

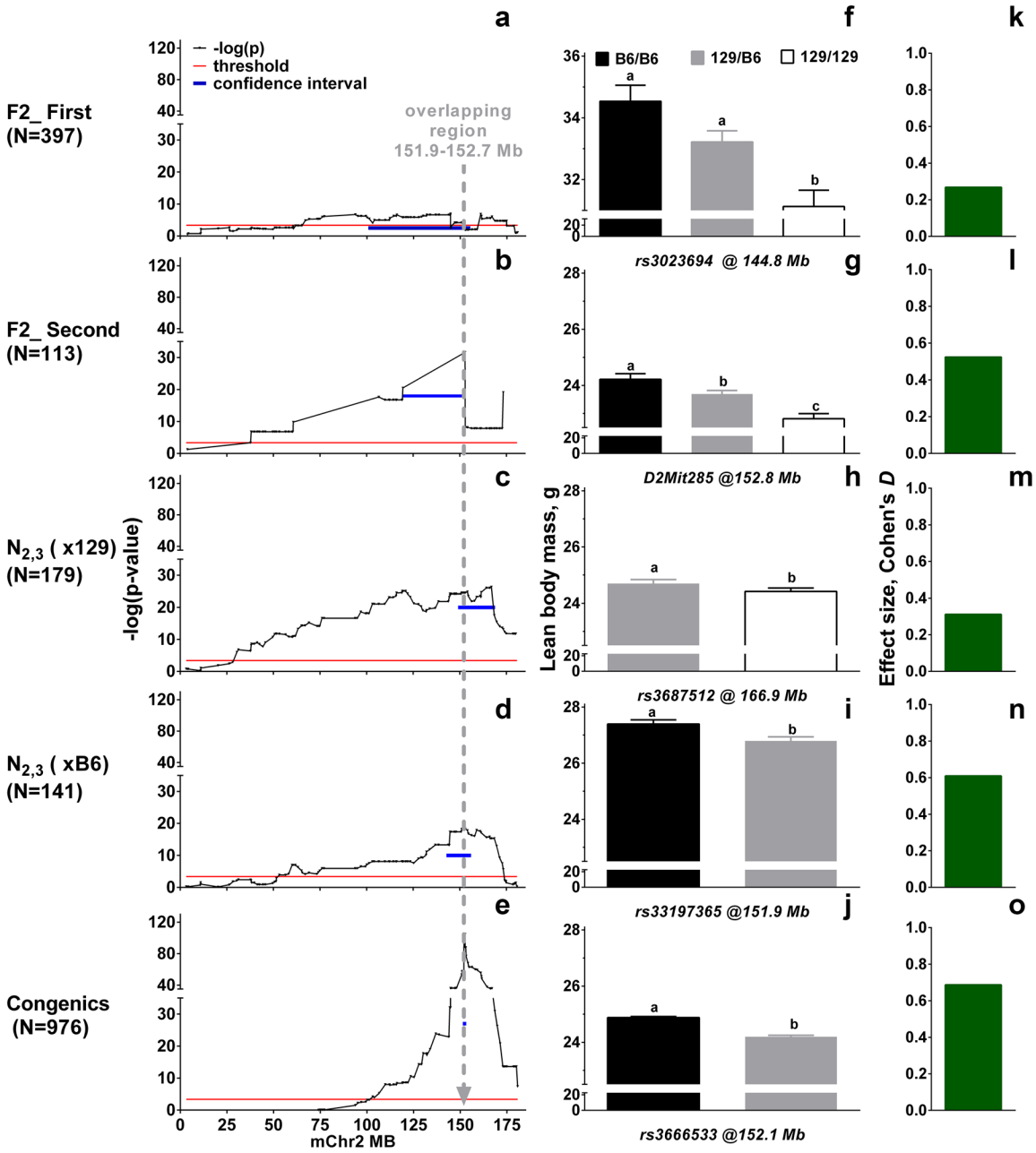

Fig. 3 The genomic location of mouse QTL *Burly1* identified in multiple mapping populations. **a**–**e** Association test statistics and QTL locations, with confidence intervals, for each mouse mapping population. The *x*-axis is the location of the markers in Mb on mouse chromosome 2 (mChr2); the *y*-axis is the −log of the statistic test by each marker genotype. The blue lines labeled chromosome regions indicate the confidence intervals of the QTLs that were supported by 2 units of $-\log_{10}$ *p*-value drop. The red horizontal line shows a Bonferroni-corrected statistical threshold. The gray shading arrow indicates the overlapping confidence interval (from 151.9 to 152.7 Mb) across the mapping populations. **f**–**j** Mean and standard error of lean body mass of mice grouped by peak marker genotype. For the first F_2 population, the results are for body weight, not lean body mass. Letters above the bars (*a, b*, and *c*) show significant differences between genotypes ($p < 0.00001$, post hoc tests, general linear model). **k**–**o** Effect sizes of lean body mass at the peak marker for each mapping population shown in **f**–**j**. For the two F_2 populations, the effect size was calculated in Cohen's *D* using least square means of genotypes of B6/B6 versus 129/B6, which allows us to compare it with the congenic mice with 129 donor fragment onto the B6 host inbred strain

and among the congenic strains (Fig. 3n, o). The reciprocal backcrosses have a similar *Burly1* effect, but the effect is larger in mice with the B6 genetic background than with the 129 background (Fig. 3c, h, m compared with Fig. 3d, i, n, respectively). The *Burly1* effect was also similar in both reciprocal consomic/sub-consomic strains. The introgression

of chromosome 2 from the 129 strain (B6-Chr2^{129}) reduced lean body weight relative to the inbred parent strain mice (Fig. 4a). These results further confirm the observation that the B6-derived allele increases lean body mass (Fig. 3f–j), and also demonstrates that it does so in both male and female mice. In fact, the effect size is larger in females than in males (Fig. 4b). We observed similar genotype and sex effects from the reciprocal sub-consomic mice (Fig. 4d, e).

Isolation of *Burly1* in congenics

We bred 1030 congenic mice from 25 congenic strains with donor regions of varying lengths and breakpoint locations (Table S5), which we confirmed by genotyping each congenic mouse. Not all strains were equally informative for the common segment method because of sample size, so we did two analyses, one narrow and one broad. (For a list of strains included and excluded from each analysis, see Table S6). We conclude based on both the narrow and broad approaches that there is a 0.8-Mb region of chromosome 2 that contains the *Burly1* locus (151.9–152.7 Mb; Fig. 5a, Table S5). We draw this conclusion because, using the general linear model, there is distinct and highly significant peak at that location (Fig. 5a), and because from the common segment approach this region is shared among the strains with the *Burly1* genotype effect and not shared with the strain C2.4 without this effect (Fig. 5b). We show the results by individual congenic strains in Fig. 5c and all post hoc tests (including those for the broad analysis below) in Table S7. Figure 5d shows all known noncoding RNA, protein-coding genes, pseudogenes, and processed transcripts within this region obtained from the Ensemble Mouse Genome Brower. The broader results also pointed to the same region (Fig. S3). Likewise, the broader analysis of the common segment method pointed to the same physical location on the chromosome with the same direction of allelic effect (Figs. 5c, S3c).

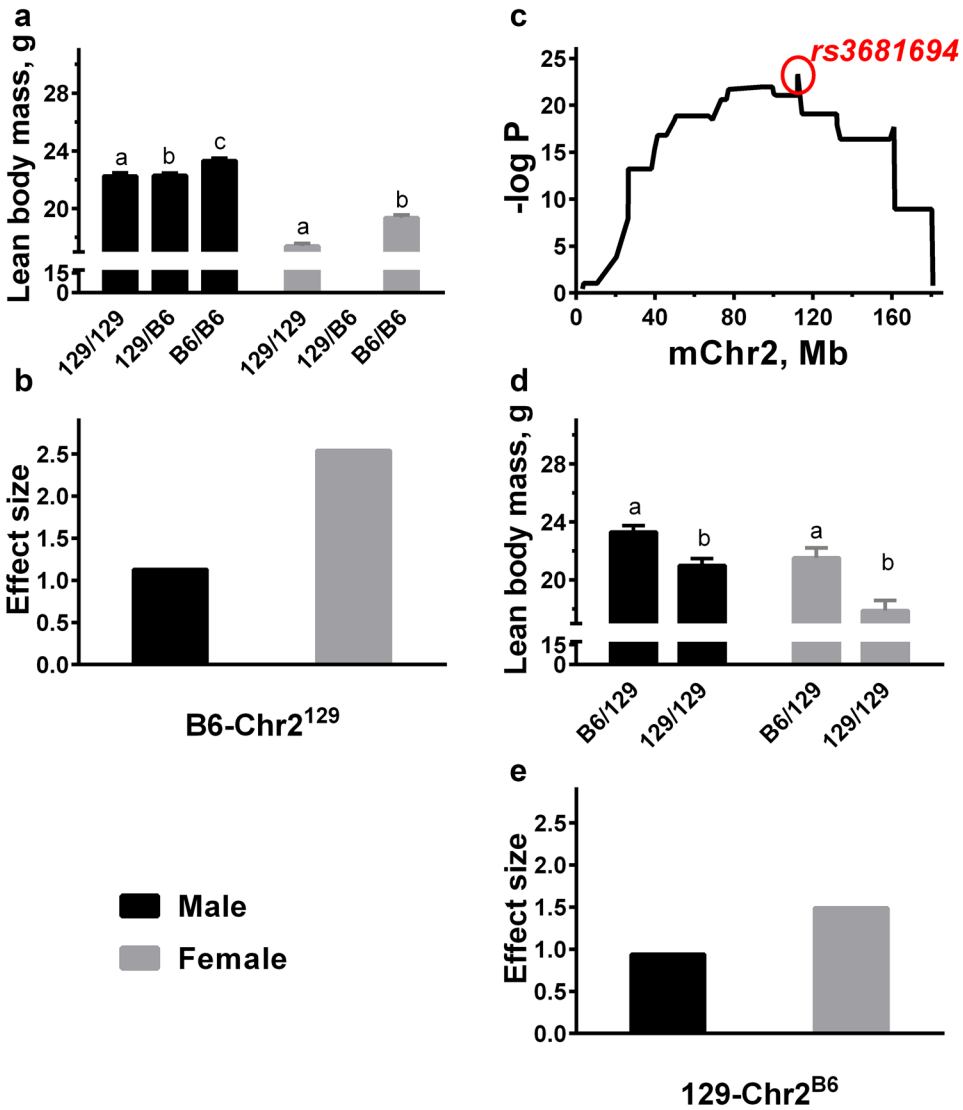

Fig. 4 The *Burly1* locus has consistent effects in the reciprocal consomic and sub-consomic strains. **a, b** B6-Chr2^{129} consomic strain: homozygous, heterozygous (male only) consomic, and inbred host strain (B6/B6). Lean body mass (**a**) is shown as means ± SEM; effect size in Cohen's *D* (**b**) was computed using B6/B6 versus 129/129. **c** Mapping of the sub-consomic 129-Chr2^{B6} mice (N_6, N_7, and N_{10}) created as part of the consomic process shows broad linkage peaks. The *x*-axis is the location of the markers in Mb on mouse chromosome 2 (mChr2); the *y*-axis shows −log *p*-values from a general linear model using genotype and sex as fixed factors and body weight as a covariate for lean body mass as the (black line). The strongest associated marker is *rs3681694* (red). **d, e** 129-Chr2^{B6} sub-consomic strain: backcross mice grouped by *rs3681694* genotype-heterozygous mice with one copy of the B6 allele (129/B6) versus homologous littermates without a B6 allele (129/129). Lean body mass (**d**) is shown as means ± SEM; the effect size in Cohen's *D* (**e**) was computed using B6/129 versus 129/129. In panels **a** and **d**, letters *a, b, c* show significance at $p < 0.05$ by post hoc testing

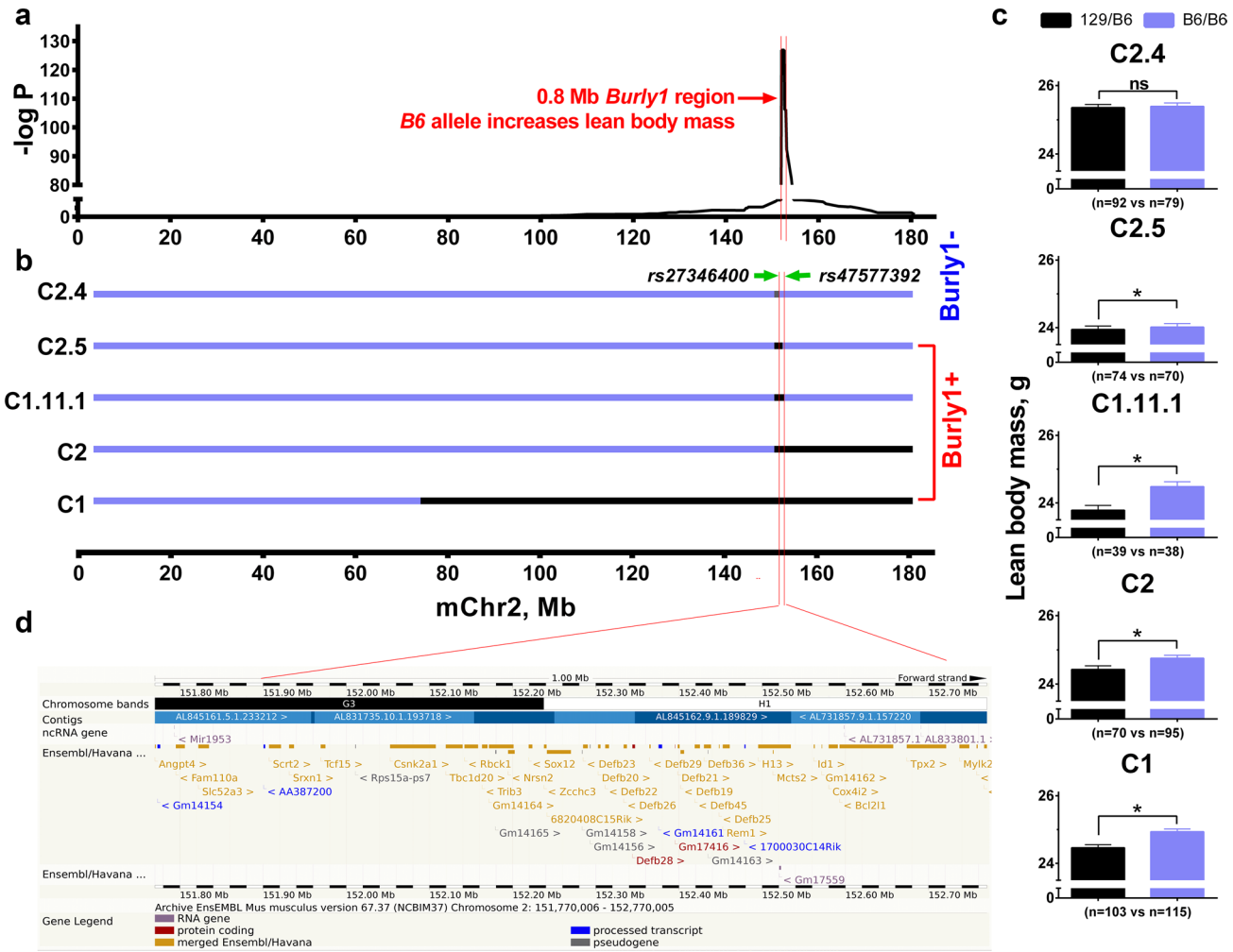

Fig. 5 The *Burly1* locus region isolated by comparing the five most informative congenic strains. **a** Average lean body weight compared using a general linear model with body weight as a covariate among all congenic mice grouped by genotype at each marker. The *x*-axis is marker positions in Mb on chromosome 2 (mChr2); *y*-axis, $-\log_{10}$-transformed *p*-values. **b** Donor region of each congenic strain: black bar indicates donor region retained the *Burly1* locus; gray bar in strain 2.4, donor region did not retain the locus; blue bar, region contributed by the host strain. We determined whether congenic strains (shown at left) retained the *Burly1* locus (i.e., were 'positive') by comparing within each strain the average lean body mass of littermates with and without the donor fragment. **c** Strain comparisons with sample size (*n*) of each genotype within each strain. $*p < 0.05/18 = 0.002778$ except for strain C2.5, $p = 0.013416$; *ns* not significant, $p > 0.05$. **d** *Burly1*-positive strains share a common region (red line; 0.8 Mb from *rs33197365* at 151.9 Mb to *rs3700604* at 152.7 Mb) that the *Burly1*-negative strains do not share. The allele effect direction matches that from the consomic mice, with the B6 strain allele increasing the trait. We show noncoding RNA genes, protein-coding genes, pseudogenes, and processed transcripts within the 0.8 Mb *Burly1* region, which we obtained from the Ensemble Mouse Genome Brower

Analysis of the homozygous *Burly1* congenic strain and control mice showed consistent results for the *Burly1* location and direction of effect (strain C2.5, Table S5). Mice homozygous for the 129 allele had 1.5 g less lean body mass than did mice with the B6/B6 genotype [$F(1,47) = 10.477$, $p = 0.002$; 129/129 vs. B6/B6]. We used this homozygous strain (C2.5) because of its small donor region to demonstrate that the *Burly1* effect is on lean and not on fat mass [$F(1,47) = 0.064$, $p = 0.801$; Fig. 6a], which reveals a distinct fat locus that differed from the lean body mass locus (Fig. S4).

To check whether the *Burly1* locus affected fat mass, and organ weight, we reanalyzed congenic strains using fat mass as the outcome measure. Consistent with results from the homozygous congenic strain, these additional results show that the *Burly1* locus is independent of fat mass (Fig. 6), although there is a nearby fat mass QTL (Fig. S4). Congenic strain C2.5 (Table S5) retains the *Burly1* locus but no loci that affect fat mass (Fig. 6, Fig. S4). We also queried whether the lean weight changes might be due to the overgrowth of specific organs. From the necropsy data of the congenic mice, we found that there was no difference between two

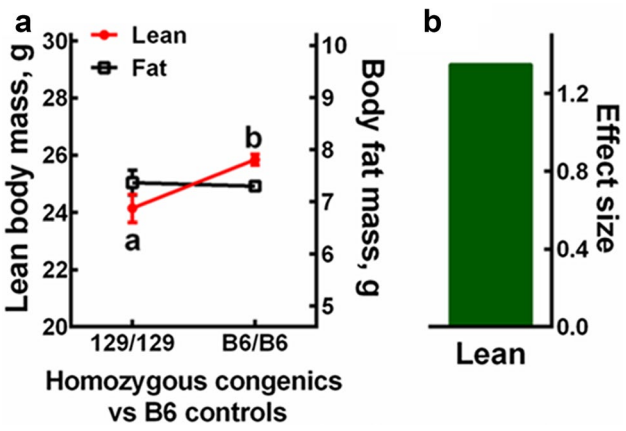

Fig. 6 The phenotype effect of the lean-body-mass-specific *Burly1* locus is confirmed in homozygous congenic strain C2.5. **a** Mean ± SEM of lean body mass (red circles) and body fat mass (black squares) for homozygous congenic mice versus mice descended from their littermates. We found a significant genotype effect on lean body mass ($p < 0.0001$, post hoc tests) but not body fat mass. **b** Effect size in Cohen's *D* for lean body mass

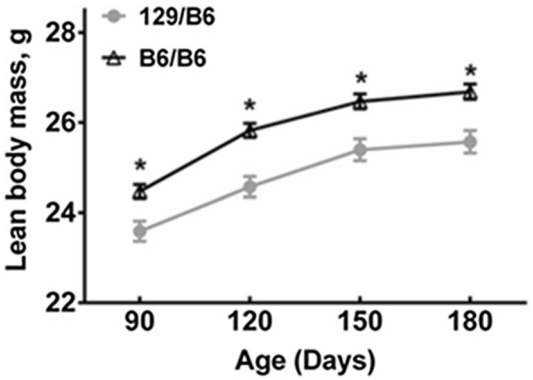

Fig. 7 The *Burly1* genotype effect on lean body mass in male mice at ages 90, 120, 150, and 180 days. Sample sizes for genotypes of marker *rs3666533* are $n = 209$ for B6/B6 and $n = 96$ for 129/B6. *Burly1* significantly affects lean body mass, with the B6 allele consistently increasing the trait (*$p < 0.001$, repeated-measures ANOVA with post hoc tests)

correlation coefficients for body weight and each organ weight (Fig. S5) except for kidney ($z = -2.6$, $p = 0.0093$), which is only ~ 1.5% of body weight. For the congenic mice, kidney weight was more related to body weight than the control mice (129/B6: $r = 0.72$, $p < 2.2e\text{-}16$; B6/B6: $r = 0.56$, $p < 2.2e\text{-}16$). For the other organs including the liver, organ weights were equally in proportion to body weight in mice of both genotypes (pancreas: $z = -1.1$, $p = 0.2713$; spleen: $z = -1.6$, $p = 0.0969$; liver: $z = 0.85$, $p = 0.3953$; heart: $z = 0.33$, $p = 0.7414$; brain: $z = 0.71$, $p = 0.4777$).

Age by genotype

The *Burly1* locus affected lean body mass at every time point we measured, 90, 120, 150, and 180 days of age, with the B6 allele consistently increasing the phenotype ($p < 0.001$, repeated-measures ANOVA with post hoc comparison) and a similar effect size (Cohen's *D*) at all age points (0.40, 0.56, 0.45, and 0.45, respectively; Fig. 7).

Metabolism

We studied the metabolism of 52 congenic mice and littermates with opposing genotypes (stain C2.5; Table 1). Mice with the 129-derived allele consumed less the oxygen [ml/kg lean, hr; $t(1,49) = 2.143$, $p = 0.037$] and produced less the heat per kilogram lean body weight [kcal/kg lean, hr; $t(1,50) = 2.179$, $p = 0.034$] and less carbon dioxide per kilogram lean body weight [ml/kg lean, hr; $t(1,50) = 2.032$, $p = 0.047$] than did their littermates without the 129-derived allele (Fig. 8). However, there were no significant *Burly1* genotype effects on food [g/kg lean, 24 h; $t(1,49) = 1.073$, $p = 0.289$], water [ml/kg lean, 24 h; $t(1,49) = 0.625$, $p = 0.535$], respiratory exchange ratio [$t(1,50) = 0.743$, $p = 0.461$], or activity [beam breaks/hr; $t(1,50) = 0.278$, $p = 0.782$]. Genotype also did not account for differences among mice in plasma glucose concentration at any time point after they were gavaged with glucose [$F(1, 45) = 0.93$, $p = 0.347$; Fig. S6].

Candidate genes

Using the coordinates suggested by the congenic strain mapping results, we surveyed the Mouse Genome Database (Eppig et al. 2005) for other body weight and lean body mass QTLs on chromosome 2. The eight previously reported QTLs (*Gnf1, Wg2d, Bwq9, BWq5, Pwbwq1, Pwgrq1, Pwgrq2*, and *Pwbwq5*) had an overlapping confidence interval with the *Burly1* QTL reported here (Table S8). We extracted 1,949 variants between the B6 and 129 strains (Fig. S7). Of these variants, the in silico analysis predicted that 7% change some aspect of mRNA regulation (Table S9) and 2.5% affect nonsense-mediated mRNA decay (Table S10). There are 26 protein-coding genes (Anonymous 2015b) (Table S11), seven of which contain missense variants (Table S12). To the best of our knowledge, none of these variants is within a gene previously studied for its effect in the development or regulation of lean body mass, as determined by searching a publicly available database of experimental studies using the gene symbols as search terms (Coordinators 2016). Using the genomic coordinates of the *Burly1* region (Chr2: 151926390–152665217), we identified the homologous region of the human chromosome (Chr20:

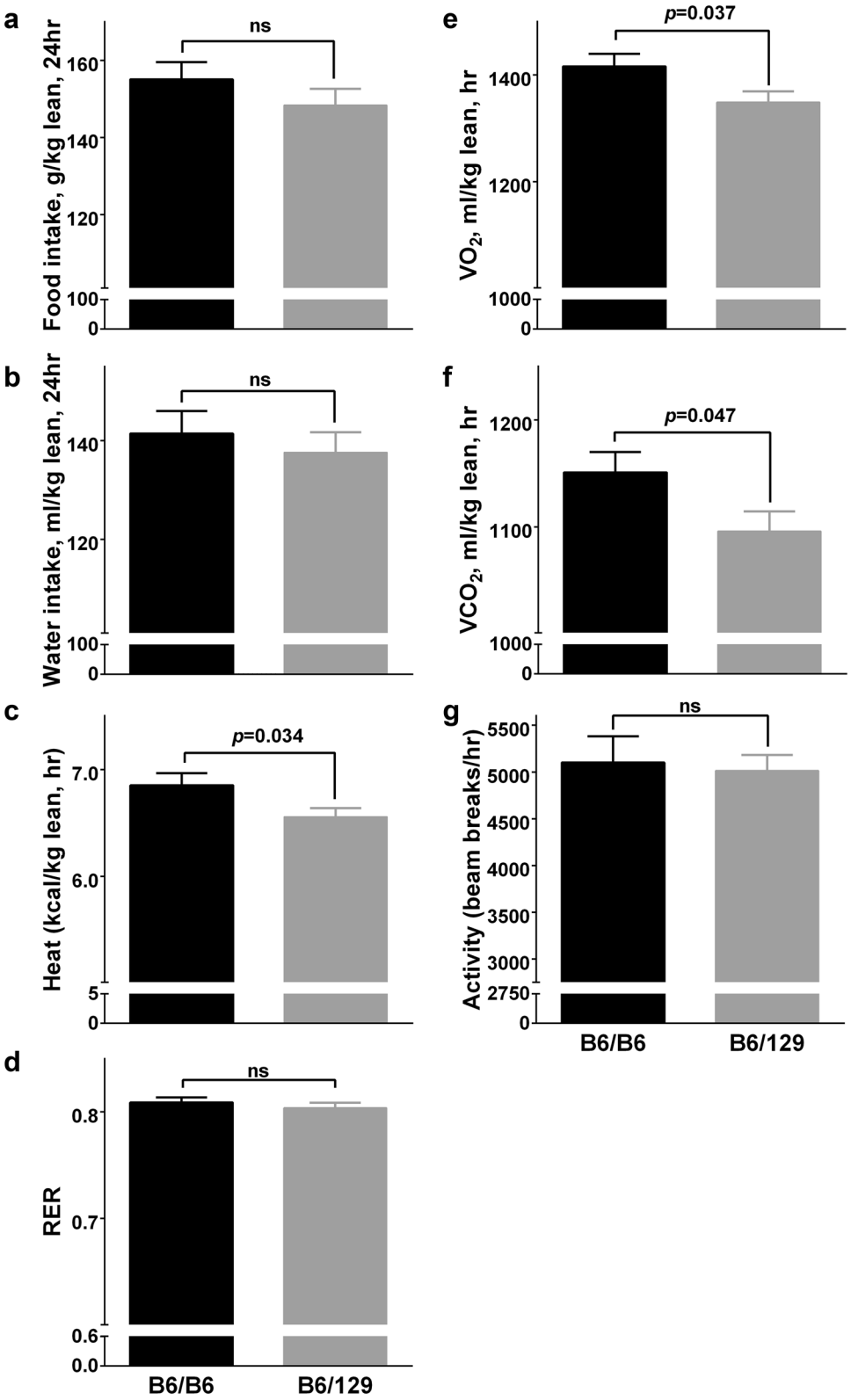

Fig. 8 Metabolic assessments in *Burly1* congenic strain C2.5 and control mice: food intake (**a**), water intake (**b**), heat production (**c**), respiratory exchange ratio (RER; **d**), oxygen consumption (VO_2; **e**), carbon dioxide production (VCO_2; **f**), and activity over the entire 24-h light/dark cycle (**g**). Data are mean ± SEM

142056–888846) and compared recent human genome-wide association results for lean body mass or body mass index to determine if the regions contained variants in common genes, but we found none.

Discussion

Overview

Positional cloning of body composition loci during the genomic era was initially quick, most notably the identification of several obesity genes, including leptin (Zhang et al. 1994), the leptin receptor (Tartaglia et al. 1995), tubby (Kleyn et al. 1996; Noben-Trauth et al. 1996), and agouti (Bultman et al. 1992); however, progress identifying QTLs with smaller effect sizes or with complex architecture has been slower. For example, we found in an attempt to narrow the causal allele for an adiposity QTL (*Adip20*) that it decomposed into several linked QTLs (Lin et al. 2017). We were surprised that the *Burly1* phenotype mapped to a single region because adiposity and related traits have many interacting and sub-QTLs on mouse chromosome 2. However, the *Burly1* locus has an easily detected and consistent effect on body composition that maps to a small region on chromosome 2, with the B6 allele increasing lean mass compared with the 129 allele.

Control of nongenetic factors

Lean body mass is affected by many factors, such as age, sex, and diet (McMullan et al. 2016), so we took steps to reduce these sources of variation whenever possible. For the strains studied here, we measured male mice almost exclusively and took care to compare mice that were close in age whenever practically possible. In retrospect, our choice to study adult mice from 3 to 6 months of age was fortuitous because the *Burly1* genotype effects are large and consistent during this time window. Therefore, the choice of age to study was not a limitation; however, studying mostly male mice was a limitation, and this choice reduced our ability to generalize our findings to female mice (Clayton and Collins 2014), especially because the two sexes differ in similar studies (Farber and Medrano 2007). However, we did examine a few female mice and learned that the *Burly1* phenotype of females was similar and perhaps even surpassed that of males.

Like age and sex, diet is another source of variation. To control these effects, we fed mice food ordered from the same manufacturer with the same catalog number for the duration of this project. However, we acknowledge that chow diets change over time because of their natural constituents (e.g., grains) (Tordoff et al. 2002). Therefore, the vicissitudes of diet composition could contribute to nongenetic sources of variation, especially for mice studied years apart.

Challenges in assessing body composition loci on mouse chromosome 2

Mouse chromosome 2 has posed special challenges for the genetic study of body composition because of its QTL density (Diament et al. 2004; Farber and Medrano 2007a, b; Gularte-Merida et al. 2015; Kobayashi et al. 2014; Rocha et al. 2004a, b; Warden et al. 2004; Yuan et al. 2012) and the interdependence of body composition traits. QTL density was a consideration when we initially chose among the available fine-mapping approaches (Kang et al. 2008; Solberg Woods 2014). We adopted the congenic approach because this method seemed most suitable for isolating very closely linked QTLs (Rapp and Joe 2012), and because we had positive outcomes with it (Bachmanov et al. 2001a). The tight interdependence of body composition traits was also a challenge. Lean and fat mass are correlated in mice (Brockmann et al. 2009), as they are in humans (Kemp et al. 2016; Zhao et al. 2008), so we chose methods that measure lean and fat weight separately. One additional challenge was that, while we measured lean body mass and the weight of many organs, we did not measure muscle weight directly. The lack of a direct measure was unfortunate, especially considering the presence of a nearby QTL for muscle weight (Lionikas et al. 2010).

Biological basis of breeding problems

We attempted to create and study reciprocal consomic strains to compare the effect of the *Burly1* allele on two different genetic backgrounds. This is an important goal for the study of body composition because many nonspecific genetic effects reduce body size. Therefore, we wanted to determine not only whether the 129-derived allele reduces body size but also whether the B6-derived allele increases it. However, our breeding plan for these reciprocal strains was only partially successful, because we found it nearly impossible to breed consomic mice with a B6 donor chromosome 2 on a 129 genetic background. These breeding difficulties were unsurprising because prior studies show that the agouti region on chromosome 2 interacts with other loci to reduce reproductive performance (Hrbek et al. 2006; Peripato et al. 2004). However, we did successfully produce heterozygous mice with a donor sub-chromosome 2; from those mice, we learned that the B6-derived allele from the *Burly1* region increased lean body mass. Thus, this sub-consomic strain, while imperfect, was informative and further confirmed that *Burly1* has specific effects that, depending on the allele, increase or decrease lean body weight.

Mouse human homology

Investigators who have conducted human genome-wide association studies of lean body mass, measured using methods similar to those used here in mice, report no associations to the homologous *Burly1* region (Comuzzie et al. 2012; Guo et al. 2013; Hai et al. 2012; Liu et al. 2009; Pei et al. 2014; Urano et al. 2014). However, this may be due to low power of human studies to detect genes with smaller effect sizes because, relative to studies of body mass index, far fewer human subjects have been measured for lean body weight. Again, drawing on body mass index as an example, many additional loci are uncovered as sample size increases (Berndt et al. 2013). Other explanations for this lack of human-mouse agreement would be that the causal gene has few or no functional variants in humans or may not have the same function between mice and humans (Gharib and Robinson-Rechavi 2011). Genetic studies in mice can point to functional roles of genes, and their value is due in part to this knowledge even in the absence of comparable human variation (Attie et al. 2017).

Methodology of measuring body mass

In this study to measure lean body mass, we used both dual-energy X-ray absorptiometry (DEXA) and magnetic resonance (MR). To choose these methods, we considered the four common ways to measure lean body mass in rodents: total body weight, chemical extraction (Reynolds and Kunz 2001), DEXA (Nagy and Clair 2000), and MR (Jones et al. 2009; Nixon et al. 2010). As mentioned above, total body weight is an imperfect proxy measure of lean body mass because it includes adipose tissue (fat mass), which can differ among rodent strains up to 20-fold (Reed et al. 2007, 2011). Most investigators consider chemical extraction the gold standard (Halldorsdottir et al. 2009; Nagy and Clair 2000), but it has at least two limitations: it can be conducted on dead mice only, and it is time-consuming (Reynolds and Kunz 2001). DEXA and MR are more direct measures of lean body mass than body weight, are more valid compared to chemical extraction methods, and can be conducted with living mice. DEXA requires mice to be anesthetized, whereas MR can be used with mice that are awake. Thus, MR requires less time and fewer resources than does DEXA. Overall using both methods was efficient and allowed us to compare the methods and to practically measure lean body mass of mice over time.

Candidate genes

Within the 0.8-Mb *Burly1* region, there are at least seven protein-coding genes (*Angpt4, Fam110a, Slc52a3, Zcchc3, 6820408C15Rik, Defb25, Rem1*) with missense variants between two mouse strains closely related to the parental strains we studied here (Beck et al. 2000). To the best of our knowledge, investigators have not reported a role for any of these genes in lean body weight, and there have been only a few functional studies of any type. However, there are clues about how a few of these genes might affect lean body weight. Perhaps most compelling, the protein product of the *Angpt4* gene is a secreted growth factor that promotes the growth of blood vessels. Inborn differences in the function or abundance of this growth factor may affect the amount of lean tissue, although there is no direct evidence for this hypothesis that we are aware of, except that the degree of *Angpt4* gene methylation differs by body weight in humans (Wilson et al. 2017). The *Slc52a3* gene codes for a protein that transports riboflavin (Yao et al. 2010), and treatment with riboflavin in humans with mutations of this gene improves the strength of their muscles by acting at the motor neuron (Bashford et al. 2017). It is possible that improving or reducing the nerve-muscle junction will increase or decrease overall lean body mass. The *Rem1* gene codes for a GTP-binding protein that inhibits a particular type of voltage-dependent calcium channel in muscle (Beqollari et al. 2014). Following the logic applied above, reducing muscle function might change lean body mass. The protein produced from the *Fam110a* gene is a member of a small family of proteins that form part of the centrosome when cells divide, and while it is not specific to muscle cells, it might affect the pace of cell division and perhaps final cell number (Hauge et al. 2007).

From the characterization of the congenic mice, we learned that *Burly1* genotype has no effect on food and water intake when expressed per unit of lean body mass, which is similar to the results of our previous studies of the progenitor strains (Bachmanov et al. 2002, 2001b). However, the *Burly1* congenic mice with a 129-derived allele produced significantly less heat per kilogram of lean body weight than did controls. This current result is consistent with a prior observation that 129 versus B6 strain variation affects heat-generating mitochondria within brown adipocytes in muscle (Almind et al. 2007).

Future work

The benefit of the positional cloning method is that it can be used to find out what genes do, which is a priority because many or even most genes have an unknown functions (Su and Hogenesch 2007) and the majority of biomedical research focuses on only a fraction of known genes (Dolgin 2017). But this benefit of uncovering new functions for genes also comes with a risk. This risk is that even when a small genomic region is identified, if the genes therein have no prior function or no previous connection to the trait, there may still be too many genes and variants to test individually

even with advances in gene editing technology (Thompson et al. 2017). This risk of positional cloning has reduced its popularity especially with the rise of other methods to identify gene function, particularly systematic gene knockout (Koscielny et al. 2014). Despite this risk, we view positional cloning as especially valuable for discovering genes effecting lean body mass because the alternative method of creating knocked out (null) alleles often has nonspecific effects on body composition (Reed et al. 2008; White et al. 2013). Our hope is that short list of candidate genes derived from the present work will aid pursuing large-scale approaches such as tissue transcript sequencing (Mele et al. 2015) or genome-wide association studies (Sudlow et al. 2015) to help pinpoint a high priority candidate.

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Compliance with Ethical Standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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