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Fat maintenance is a predictor of the murine lifespan response to dietary restriction

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Summary

Dietary restriction (DR), one of the most robust life-extending manipulations, is usually associated with reduced adiposity. This reduction is hypothesized to be important in the life-extending effect of DR, because excess adiposity is associated with metabolic and age-related disease. Previously, we described remarkable variation in the lifespan response of 41 recombinant inbred strains of mice to DR, ranging from life extension to life shortening. Here, we used this variation to determine the relationship of lifespan modulation under DR to fat loss. Across strains, DR life extension correlated inversely with fat reduction, measured at midlife (males, $r = -0.41$, $P < 0.05$, $n = 38$ strains; females, $r = -0.63$, $P < 0.001$, $n = 33$ strains) and later ages. Thus, strains with the least reduction in fat were more likely to show life extension, and those with the greatest reduction were more likely to have shortened lifespan. We identified two significant quantitative trait loci (QTLs) affecting fat mass under DR in males but none for lifespan, precluding the confirmation of these loci as coordinate modulators of adiposity and longevity. Our data also provide evidence for a QTL previously shown to affect fuel efficiency under DR. In summary, the data do not support an important role for fat reduction in life extension by DR. They suggest instead that factors associated with maintaining adiposity are important for survival and life extension under DR.

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Introduction

The life-extending effect of dietary restriction (DR) has long been known (McCay *et al.*, 1935; Weindruch & Walford, 1988), but the underlying molecular and physiological mechanisms are still obscure (Masoro, 2005). Reduction in fat mass has been argued to be a key factor (Barzilai, 1999; Barzilai & Gupta, 1999; Masoro, 1999; Barzilai & Gabriely, 2001; Das *et al.*, 2004). The case favoring a role for reduced fat is based on the role of excess visceral adiposity in insulin resistance, type II diabetes, and metabolic syndrome (Despres & Lemieux, 2006). Furthermore, removal of visceral fat not only improves insulin sensitivity (Barzilai *et al.*, 1998; Das *et al.*, 2004) but also extends the lifespan of one strain of rat (Muzumdar *et al.*, 2008). As acknowledged (Muzumdar *et al.*, 2008), this view does not account for DR-induced lifespan extension in strains that do not exhibit metabolic disease or obesity (Masoro *et al.*, 1992; McCarter *et al.*, 2007) nor the beneficial effects of DR on pathological conditions and age-related dysfunctions (e.g., lymphoma and cataracts) not primarily linked to metabolic disease or obesity (Weindruch & Walford, 1988).

The argument against a role for reduced fat mass in the life-extending effect of DR is based on several observations. In a study using inbred rats, the DR rats with the greatest peak fat reserves had the greatest extension of life (Bertrand *et al.*, 1980). Also, positive correlations have been observed in DR mice between lifespan and body weight (BW) (Weindruch *et al.*, 1986; Goodrick *et al.*, 1990; Harper *et al.*, 2006; Rikke *et al.*, 2010). In addition, the lifespan of genetically obese mice (*ob/ob*) under DR was found to be the same as the lifespan of their wild-type littermates under DR, even though the obese mice on DR had much higher levels of fat than their wild-type *ad libitum* (AL) controls (Harrison *et al.*, 1984).

Although absolute fat mass and life extension were positively correlated in one strain of rat under DR (Bertrand *et al.*, 1980), no study has asked whether fat reduction *per se* is associated with life extension by DR. We therefore conducted a systematic, unbiased screen to determine the relationship between DR lifespan and adiposity across 41 recombinant inbred (RI) mouse strains. These strains exhibit extensive genetic variation in the lifespan response to DR (Liao *et al.*, 2010a), ranging from lifespan lengthening to lifespan shortening. We also examined the relationship between lifespan and lean body mass.

Quantitative trait loci (QTL) mapping screens the genome for statistical associations between phenotypic and genotypic

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information, which facilitates finding genes that underlie quantitative traits (Flint & Mott, 2001; Glazier *et al.*, 2002; Korstanje & Paigen, 2002; Flint *et al.*, 2005). Although a large number of QTLs and potential genes have been identified for BW, body composition, and lifespan under AL feeding (Gelman *et al.*, 1988; de Haan *et al.*, 1998; Miller *et al.*, 1998; Jackson *et al.*, 2002; Henckaerts *et al.*, 2004; de Haan & Williams, 2005; Wuschke *et al.*, 2007), no study has searched for genetic loci modulating adiposity and lean mass under DR. Earlier studies using ILSXISS RI mouse strains have reported marked genetic variation in the response of BW, growth, fertility, and body temperature to DR and have begun to identify the genetic basis for this variation using QTL mapping (Rikke *et al.*, 2006, 2010; Rikke & Johnson, 2007). Here, we searched for QTLs in the ILSXISS RI panel specifying the modulation by DR of adiposity, lean mass, and lifespan as a first step to identify genes and pathways mediating the responses of these traits to DR.

Results

To test the hypothesis that fat reduction under DR is important for life extension, we measured the response of fat and lean mass in the ILSXISS RI mouse strains (Williams *et al.*, 2004): 38 strains for males and 33 for females. Mice were fed AL and DR (60% of AL) diets beginning at 2–5 months of age, and body composition was measured using quantitative magnetic resonance (QMR) at 15–17 months of age. Correlation analysis was used to evaluate the association between body composition and lifespan (the lifespan data are the same as in Liao *et al.*, 2010a). Quantitative trait locus (QTL) mapping was used to identify the genetic regions that contribute to strain variation in the response of body composition and lifespan to DR.

Genetic variation in the response of body composition

The RI strains exhibited marked genetic variation in absolute fat mass under both AL and DR conditions (Fig. 1A,B). Mean AL fat mass varied 4.8-fold in males and 3.9-fold in females. The strain variation under DR was even greater, varying 6.2-fold in males and 6.3-fold in females. The effect of strain on fat mass was highly significant for both sexes under both feeding conditions (all $P < 0.0001$, one-way ANOVA). Heritability under AL feeding was 52% for males (95% CI of 39–63%) and 56% for females (95% CI of 42–67%). Under DR, heritability was 75% for males (95% CI of 63–82%) and 64% for females (95% CI of 48–74%). Surprisingly, fat mass under DR did not correlate with fat mass under AL (Table S1), indicating that the genetic modulation of adiposity differs under the two feeding conditions. Adiposity was positively correlated between males and females (Table S2).

Overall, 40% DR reduced fat mass by 38% in Insets males and 33% in females (strains equally weighted, Fig. 1A,B). However, the extent of reduction varied markedly among strains (Fig. 1C,D), ranging from an 80% reduction to an unexpected ~60% increase (observed in ILSXISS 3 in both males and

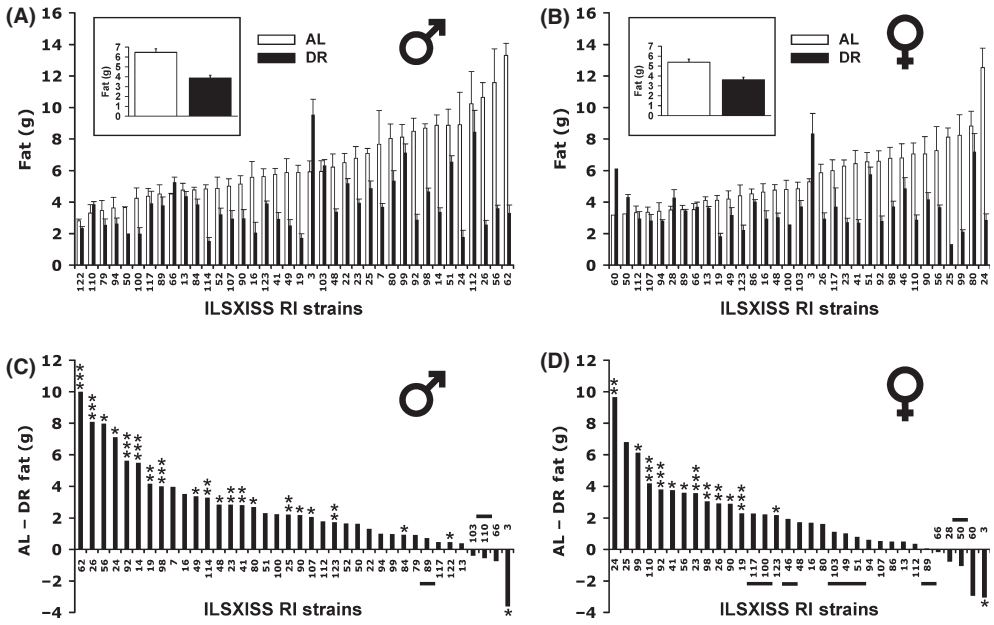

Fig. 1 Strain variation in fat mass of ILSXISS recombinant inbred (RI) mice under *ad libitum* (AL) and 40% dietary restriction (DR) diets. Fat mass was obtained using quantitative magnetic resonance (QMR) from ILSXISS RI mice aged 15–17 months after they were under AL or DR diet since 2–5 months of age. Mean fat mass in the upper two panels is shown for each strain [AL (□) and DR (■)], ranked in ascending order according to the AL means (A: males, 38 strains; B: females, 33 strains). Insets in A and B: The mean fat mass of all strains. C (males) and D (females) illustrate the difference of fat mass between AL and DR groups, ranked from the strain with the greatest reduction in fat mass under DR to the strain with the least reduction (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by *t*-test; no experiment-wise Bonferroni correction). The strains that showed significantly increased lifespan under DR (Liao *et al.*, 2010a) are underlined or overlined. Error bars represent SEM.

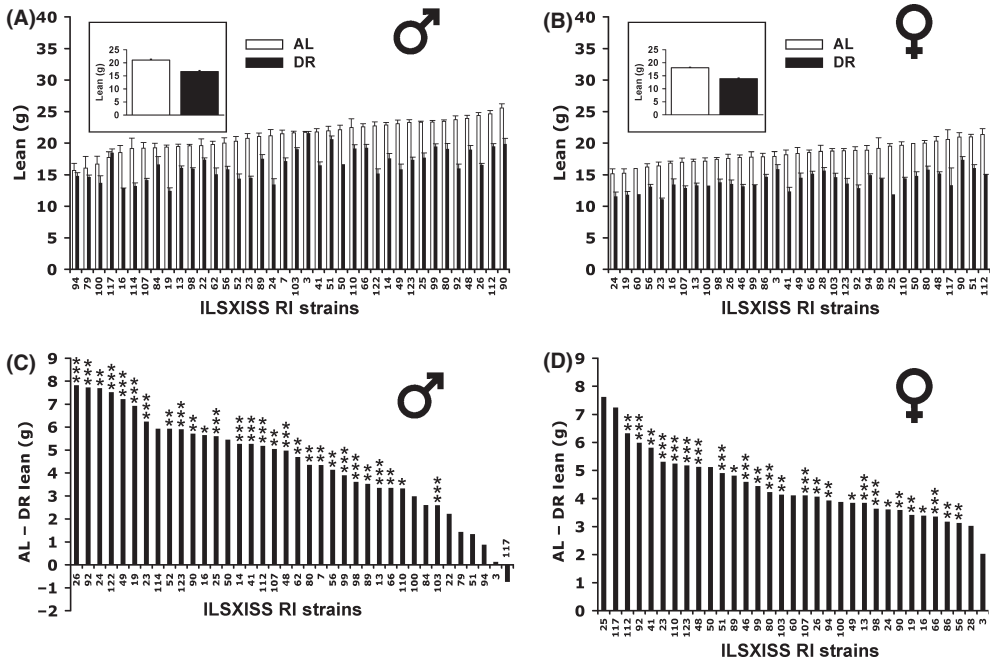

Fig. 2 Strain variation in lean mass of ILSXISS recombinant inbred (RI) mice under *ad libitum* (AL) and 40% dietary restriction (DR) diets. Lean mass was obtained using quantitative magnetic resonance (QMR) from ILSXISS RI mice aged 15–17 months after they were under AL or DR diet since 2–5 months of age. The mean lean mass in the upper two panels is shown for each strain [AL (□) and DR (■)], ranked in ascending order according to the AL means (A: males, 38 strains; B: females, 33 strains). Insets in A and B: The mean lean mass of all strains. C (males) and D (females) illustrate the difference of lean mass between AL and DR, ranked from the strain with the greatest reduction in lean mass under DR to the strain with the least reduction (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by *t*-test; no experiment-wise Bonferroni correction). Error bars represent SEM.

females, Fig. 1C,D). Remarkably, many strains showed no appreciable reduction in adiposity under DR. Fat reduction measured again at 20–22 months of age was comparable and significantly correlated with fat reduction assessed at 15–17 months of age (males: $r = 0.74$; females: $r = 0.79$; all $P < 0.0001$; Fig. S1).

The strain variation in lean body mass under AL and DR diets was also significant, although less than that observed for adiposity ($P < 0.0001$, one-way ANOVA; Fig. 2A,B). The AL mean varied 1.8-fold in males and 1.7-fold in females. The DR mean varied 1.8-fold in males and 1.6-fold in females. Heritability under AL was 64% in males (95% CI of 52–73%) and 46% in females (95% CI of 32–58%), and under DR, it was 72% in males (95% CI of 58–80%) and 61% in females (95% CI of 44–72%). Lean mass under DR, in contrast to fat mass, was correlated with lean mass under AL (Table S1), indicating partial genetic co-regulation under the two feeding conditions. Lean mass was positively correlated between males and females (Table S2).

Overall, 40% DR reduced lean mass by 17% in Insets males and 21% in females (strains equally weighted, Fig. 2A,B), with most strains exhibiting a reduction that was statistically significant (74% and 79% of the strains for males and females, respectively, using single strain *P* values < 0.05). The reduction also varied markedly among strains (Fig. 2C,D). This genetic variation was also present at 20–22 months of age (Fig. S2) and correlated significantly with the variation at 15–17 months of age (males, $r = 0.81$; females, $r = 0.70$; all $P < 0.0001$). BW also varied considerably among strains under both AL and DR feeding (Fig. S3, $P < 0.0001$, one-way ANOVA) as reported previously at earlier ages (Rikke *et al.*, 2006).

Relationships among adiposity, lean mass, BW, and lifespan

We calculated the correlations between absolute fat mass and lifespan across strains under AL and DR diets to determine whether absolute fat mass was a predictor of lifespan across inbred mouse strains. In DR mice, fat mass correlated positively with mean lifespan in both sexes (Table 1; Fig. 3C,D). BW under DR also correlated positively with lifespan, reflecting the fact that both lean mass and fat mass were positively correlated with

Table 1 Correlation coefficients among strain means between mean lifespan and absolute fat mass, lean mass, and body weight (BW) at 15–17 months of age in both sexes and diets

		Fat	Lean	BW
AL lifespan	♂ ($n = 41$)	0.47**	–0.19	0.18
AL lifespan	♀ ($n = 39$)	0.46**	–0.36*	0.11
DR lifespan	♂ ($n = 38$)	0.35*	0.43**	0.42**
DR lifespan	♀ ($n = 33$)	0.47**	0.37*	0.51**

n, number of RI strains; AL, *ad libitum*; DR; dietary restriction.
*$P < 0.05$; **$P < 0.01$: Pearson correlation coefficient (*r*), two-tailed tests.
The lifespan data are from Liao *et al.* (2010a).

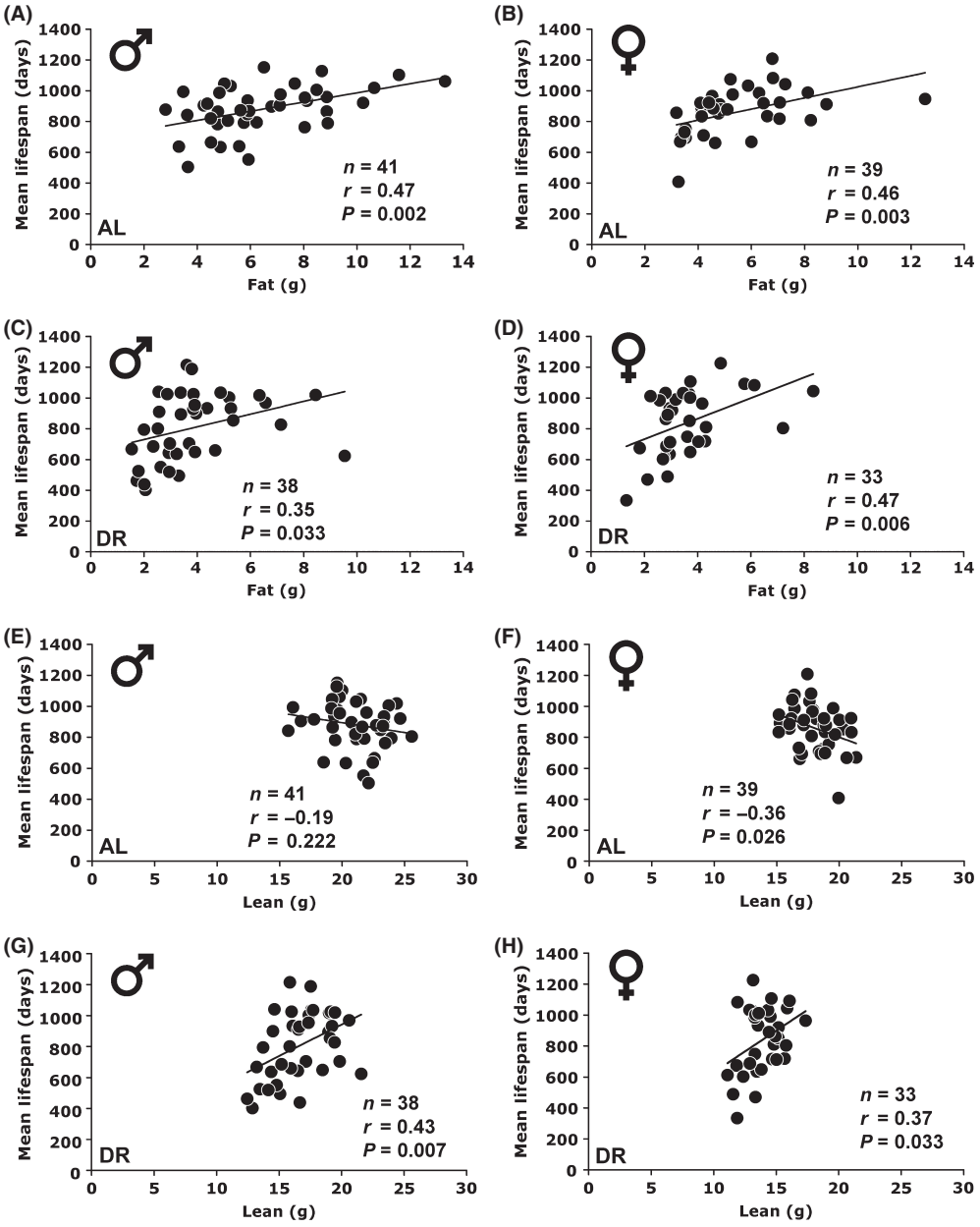

Fig. 3 Scattergram showing the correlation between absolute fat mass, lean mass, and lifespan in *ad libitum* (AL) and 40% dietary restriction (DR) diets for mice aged 15–17 months. Mean values of each ILSXISS recombinant inbred (RI) strain (A, C, E, G: males; B, D, F, H: females) are shown. Mean lifespan was derived from Liao *et al.* (2010a). The lines represent linear regression. The *P* values of the Pearson correlation coefficients (*r*) are all from two-tailed tests. *n,* number of strains.

lifespan (Table 1; Fig. 3); lean mass and fat mass also covaried positively with each other (Table S3). Interestingly, fat mass and lifespan were also positively correlated in mice under AL feeding (Table 1; Fig. 3A,B). However, BW was not correlated with lifespan in AL mice because of a negative relationship between lean mass and lifespan (Table 1; Fig. 3E, F). All of these correlations persisted at 20–22 months of age (Table S4).

To address directly the relationship between fat reduction by DR and lifespan extension, we defined fat reduction as the difference score between AL fat mass and DR fat mass and compared this construct to the difference score defining lifespan extension (DR lifespan–AL lifespan) across all strains (Fig. 4A,B). Fat reduction correlated inversely with lifespan extension in both males ($r = -0.41$, $P = 0.01$) and females ($r = -0.63$, $P = 0.0001$). Thus, the strains with the least reduction of fat were more likely to have lifespan extension, and those with the greatest reduction of fat were more likely to have lifespan shortening (Fig. 4A,B). The same negative correlation was also found at 20–22 months of age (males, $r = -0.60$, $P < 0.0001$; females, $r = -0.60$, $P = 0.0003$). In fact, of the ten strains with significantly increased lifespan under

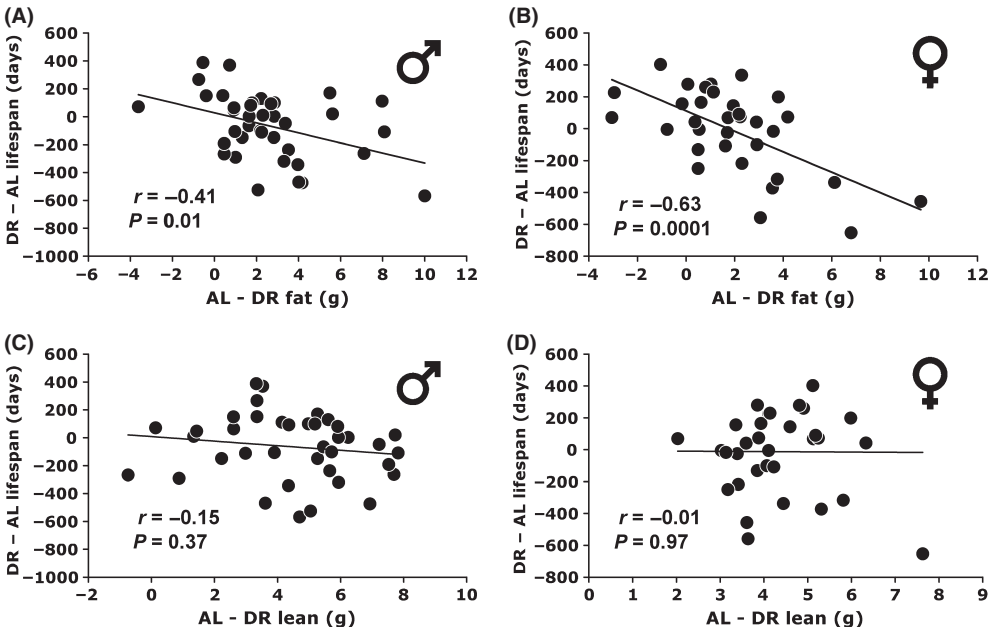

Fig. 4 Correlation between fat reduction and lifespan modulation under 40% dietary restriction (DR). *X*-axis: fat (or lean) reduction was measured by subtracting fat (or lean) mass under DR from fat (or lean) mass under AL feeding for each strain. *Y*-axis: lifespan modulation was measured by subtracting mean lifespan under AL from mean lifespan under DR (Liao *et al.*, 2010a). Fat and lean mass were derived from 15–17 months of age. Reduction in lean body mass was not correlated with differential lifespan (C: males; D: females). The lines represent linear regression. The *P* values of the Pearson correlation coefficients (*r*) are all from two-tailed tests.

DR (Liao *et al.*, 2010a), none had a significant reduction in fat (Fig. 1C,D). We also compared the reduction in lean mass, calculated as lean mass under AL minus lean mass under DR, to the extension of longevity. The reduction in lean mass did not correlate with lifespan extension in either males or females (Fig. 4C,D). The same result was obtained at 20–22 months of age (males, $r = -0.26$; females, $r = -0.13$). The result differs from the positive correlation between absolute lean mass and lifespan under DR because the difference scores incorporate AL lean mass and AL lifespan and their negative correlation.

QTL analysis

We next sought to identify chromosomal regions that modulate these genetic traits. To identify these loci, we conducted QTL mapping. The DR strain means for fat mass, lean mass, and lifespan were regressed on their respective AL means (Rikke *et al.*, 2010). The regression corrects for the strain differences in the absence of DR without making any assumptions about whether a difference score or percent change is more appropriate (Kaiser, 1989).

In males, we identified two significant QTLs on chromosomes 7 and 8 and one suggestive QTL on chromosome 18 affecting DR fat mass (Fig. 5A; Table 2). We designated the significant QTLs as fat response to DR, QTL1 (*Fdr1*), and *Fdr2*. The inbred short-sleep (ISS) allele of the chromosome 7 QTL [peak logarithm of odds (LOD) at marker D7Mit91] was associated with greater fat mass; the ISS allele of the chromosome 8 QTL (peak D8Mit200) was associated with lower fat mass (Table 2). These alleles also appeared to affect lean body mass in the same direction, and *Fdr1* coincided with a suggestive QTL for lean mass (Fig. 5B; Table 2).

For the lifespan response to DR in males, a suggestive QTL was found near the distal end of chromosome 7 (Fig. 5C; Table 2). This QTL did not overlap with *Fdr1*. At *Fdr1*, there was a small QTL peak (single-marker $P = 0.08$) in which the ISS allele was associated with greater lifespan consistent with the positive genetic correlation that we observed between DR fat and lifespan.

In females, we identified four suggestive QTLs affecting the body fat response (on chrs 8, 9, 15, 16; Fig. 5D; Table 2). The locus on chromosome 8 overlaps with *Fdr2*, and the ISS allele is associated with reduced fat as it is in males. The suggestive QTLs on chromosome is overlap with and provide further validation of the QTL that we identified previously as affecting fuel efficiency in response to DR (Rikke *et al.*, 2010), the inbred long-sleep (ILS) alleles of both QTLs are associated with greater BW, growth, and fat. A suggestive QTL affecting lean body mass was found on chromosome 10 (Fig. 5F; Table 2). Two suggestive QTLs affecting the female lifespan response to DR were found on chromosomes 7 and 14 (Fig. 5G; Table 2).

For AL mice, we identified a significant QTL on chromosome 1 (LOD peak at D1Mit135; 59.7 cM) affecting female lifespan, which we have designated *LS3* (Fig. S4; genome-wide $P = 0.038$; 95% CI of 56.8–62.7 cM; effect size ~18%). No significant QTLs were identified by mapping on the difference scores for fat mass, lean mass, or lifespan. This indicates that using difference scores reduced the power to detect QTLs.

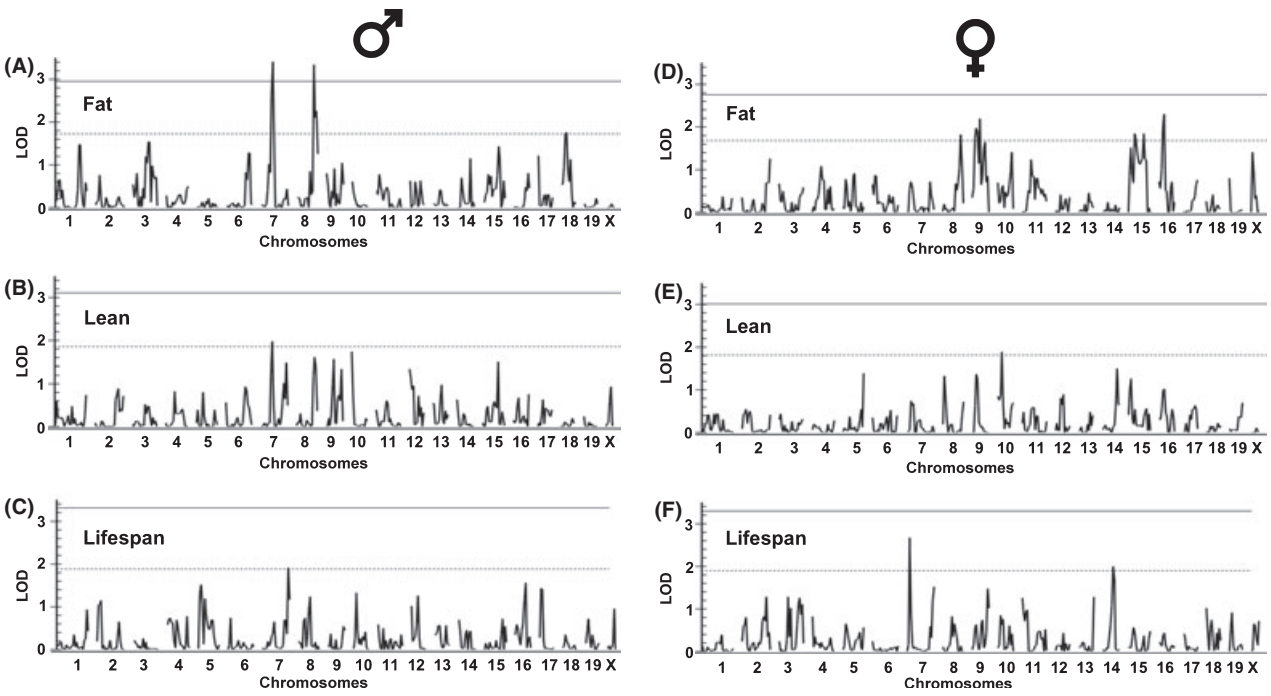

Fig. 5 Quantitative trait loci (QTL) mapping for specific dietary restriction (DR) effects. QTL mapping for fat (A, D), lean (B, E), and lifespan (C, F) response to DR was screened across the genome for the ILSXISS recombinant inbred (RI) strains aged 15–17 months after they were under AL or DR diet since 2–5 months of age. Chromosome location is on the *x*-axis, and logarithm of odds (LOD) score is on the *y*-axis. Solid lines and dash lines indicate the genome-wide significant ($P < 0.05$) and suggestive ($P < 0.63$) threshold of LOD, respectively, as determined by permutation tests using 10,000 permutations.

Discussion

Our results indicate that reduction in total fat stores correlates inversely with life extension by DR: strains with the least reduction in fat were significantly more likely to show life extension. Strikingly, none of the strains showing lifespan extension exhibited a significant reduction in adiposity. Absolute fat mass under DR, which was uncorrelated with absolute fat mass under AL feeding, was also positively correlated with DR lifespan. Both ways of examining the data suggest that the maintenance, not reduction, of adiposity under DR or factors associated therewith are important to DR's life-extending effect.

A limitation of this study is that our measures of body composition were conducted (for logistical reasons) relatively late in life: from 15 to 17 months onward. Therefore, the genetic relationship between fat mass and lifespan under DR could be different at younger ages. The following argument makes this possibility unlikely. Although we have no data for adiposity at ages earlier than 15 months, BW reduction, which correlates with fat reduction at 15–17 months of age ($r = 0.87$ for females and $r = 0.88$ males; $Ps < 0.0001$), was inversely correlated with DR life extension at younger ages. In males, BW reduction was significantly correlated with life extension at 8 and 12 months of age ($r = -0.31$, $P = 0.047$; $r = -0.45$, $P = 0.004$, respectively) and at 12 months of age in females ($r = -0.39$, $P = 0.02$). Therefore, assuming that body fat and BW reduction were correlated at these younger ages as they were at 15–17 months and older, it seems unlikely that the genetic relationship between lifespan and body fat measured at older ages was appreciably different at younger ages. Another issue concerns the relative importance of body compositional changes to the longevity effect of DR. The correlation coefficients between fat reduction, absolute fat mass, and lean body mass with lifespan range between 0.35 and –0.63, thus only accounting for a fraction of the variance in lifespan. The large amount of variation unexplained by these factors implicates other factors in the lifespan modulation by DR – a result consistent with the view that DR acts through multiple physiological processes and biochemical pathways (Masoro, 2005).

Why is maintenance of adiposity associated with lifespan extension under DR, and conversely, loss of adiposity with lifespan shortening under DR? Insight may be gained by considering the major compensatory adaptations employed to reduce energy expenditure and preserve fat mass during nutrient deprivation, namely reductions in body temperature, metabolic rate, and motor activity (Waterlow, 1986) – especially because the first two responses have been implicated as potential anti-aging mechanisms in DR (Rikke & Johnson, 2004, 2007; Conti *et al.*, 2006; Ferguson *et al.*, 2008) (reviewed by Masoro, 2005).

We examined the relationship between the responses of fat mass and body temperature to DR by comparing our data for fat to the temperature data obtained by Rikke & Johnson (2007) in different cohorts of the same strains. In contrast to the expectation that lower body temperature would be associated with energy conservation and thus the preservation of fat, we found that fat mass and body temperature were *positively* correlated

Table 2 Quantitative trait loci (QTLs) for response of fat mass, lean mass, and lifespan to dietary restriction (DR) for the ILSXISS recombinant inbred (RI) strains

Trait	Sex	Chr	Locus	LOD	*P* value	Marker position (cM)	Effect size (%) and direction (+/−)
Fat	Male	7	D7Mit85	2.6	0.0005	26.5	+27
		7	**D7Mit91**	**3.4**	**0.00007** (**0.015**)*	**28.1** (**16.5–39.7**)†	**+34** (**+20**)‡
		8	**D8Mit200**	**3.3**	**0.00009** (**0.027**)*	**58**§	**−22**
		8	D8Mit120	2.1	0.00184	61	−15
		8	D8Mit148	2.3	0.00127	67	−16
		18	D18Mit200	1.7	0.00465	16	−8
		18	D18Mit94	1.8	0.00443	17	−8
	Female	8	D8Mit120	1.8	0.00371	61	−23
		9	D9Mit256	1.7	0.00475	27	−21
		9	D9Mit4	2.0	0.00261	29	−24
		9	D9Mit300	1.9	0.0031	31	−23
		9	D9Mit289	2.2	0.0015	38	−26
		15	D15Mit84	1.8	0.00352	21.1	−23
		15	D15Mit86	1.7	0.00539	22.2	−21
		15	D15Mit93	1.8	0.00354	43.7	−23
		16	D16Mit103	2.0	0.00255	22.2	+24
		16	D16Mit58	2.3	0.00113	23.1	+27
Lean	Male	7	D7Mit91	2.0	0.00257	28.1	+21
	Female	10	D10Mit106	1.9	0.0031	17	+23
Lifespan	Male	7	D7Mit292	1.9	0.00309	69	+19
	Female	7	D7Mit154	2.7	0.00046	4	+27
		14	D14Mit71	2.0	0.00244	44	−21

Significant QTLs (*Fdr1* and *Fdr2* on chr 7 and 8, respectively) are indicated in bold. All other QTLs are suggestive. Effect size (%): Percentage of phenotypic variance accounted for by QTL, which is overestimated because of low statistical power; Direction (+/−) for the effect size refers to the direction of the inbred short-slee (ISS) allele.

Chr, chromosome; LOD, logarithm of odds; *P* value, single marker.

*Genome-wide *P* value.

†95% confidence interval (CI) of *Fdr1* QTL.

‡Corrected effect size.

§95% CI is not available because of the estimated chromosome region is larger than chr 8.

($r = 0.56$, $P = 0.003$, $n = 27$ strains): strains with lower body temperature under DR had less fat (and BW). The strength of this relationship is remarkable given that the body temperature and body composition responses to DR were measured in separate cohorts and colonies, and surprising given that the body temperature and BW responses to DR were not correlated when measured in the same cohort (Rikke & Johnson, 2007). These results do not support a physiological trade-off between temperature reduction and fat maintenance.

Interestingly, reduction in body temperature was also a negative predictor of life extension in this study ($r = -0.47$, $P = 0.008$, $n = 31$ strains; females; males were not studied in the earlier report): strains with the greatest reduction in body temperature were at greatest risk for life shortening and those maintaining higher body temperature were more likely to have extended lifespan under DR. This relationship was dependent on the correlation between the body temperature and body fat responses, suggesting a coordinated genetic modulation of all three responses. Therefore, the results suggest that, if anything, the genetic profile associated with extended lifespan under DR in these strains minimizes losses in body temperature as well as body fat. These findings also contradict a simple model in which lower temperature *per se* (independent of lower metabolic rate) extends lifespan (Turturro & Hart, 1991; Koizumi *et al.*, 1992; Rikke & Johnson, 2004; Conti *et al.*, 2006; Conti, 2008).

Another energy-conserving response to reduced food availability is a reduction in lean body mass, which reduces whole animal metabolic rate (Hambly & Speakman, 2005; Li *et al.*, 2010). In contrast to the expectation that reduced lean mass as an energy-preserving response would be greater in strains that maintained adiposity, we found that it was *positively* correlated with the reduction in fat mass (males: $r = 0.54$, $P < 0.001$; females: $r = 0.33$, $P = 0.06$). Thus, the data do not support a significant role for reduced whole animal metabolic rate, to the extent that it is determined by reducing lean body mass, in either the maintenance of fat mass or life extension under DR. These results, however, do not exclude the possibility that reductions in resting metabolic rate *per unit lean mass* could be an adaptive response to preserve adiposity. DR rats have reduced plasma thyroid hormone (Herlihy *et al.*, 1990), a hormonal response that is consistent with reduced resting metabolic rate. However, several studies have reported that resting metabolic rate normalized to lean body mass is not decreased by DR after the initial period of body mass adjustment to reduced caloric intake (McCarter & Palmer, 1992; Hambly & Speakman, 2005).

Reduced motor activity is another adaptive response to conserve energy and preserve fat (Hambly & Speakman, 2005). The LSXSS RI strains (derived from the same parental stock as the ILSXISS strains used in this study) exhibit dramatically reduced runwheel activity, by about 50% when fed 60% AL, with only

one strain of 14 showing increased activity (Rikke *et al.*, 2003). However, the genetic variation in runwheel and home-cage activity was not correlated with the BW response in that study (Rikke *et al.*, 2006). Therefore, given the high correlation between BW and body fat in this study, it appears unlikely that the motor response to DR is having an appreciable effect on the fat response in these strains.

Despite the lack of support for body fat being genetically maintained as a consequence of reduced energy utilization by classic physiological responses, the observation that maintenance of adiposity is a predictor of lifespan extension by DR supports a fuel efficiency model (Rikke *et al.*, 2010). Fuel efficiency is a surrogate measure of metabolic efficiency defined in terms of maintaining higher BW and growth rate (Rikke *et al.*, 2010). We have previously shown that this construct predicts DR's coordinated genetic effect on lifespan and female fertility. This study further supports this model by showing that the maintenance of body fat correlates positively with BW and lifespan under DR. Furthermore, a QTL that we previously identified as affecting fuel efficiency, on chromosome 15, proved to be a suggestive QTL in this study, affecting the body fat response in the direction expected (ILS alleles associated with maintaining higher body fat and BW).

A related question raised by this study concerns the observation that absolute adiposity was positively correlated with lifespan not only in DR mice but also in mice under AL feeding. Although this seemingly runs counter to the evidence that obesity is a risk factor for early mortality in humans as well as rodents, strains in this study may not be obese under AL feeding. Obesity, defined operationally, would be a level of adiposity increasing risk for morbidity and mortality. Obese (*ob/ob*) mice, which develop diabetes and have short lifespan, have 67% fat (Harrison *et al.*, 1984). By contrast, most strains in our study have < 30% of fat at 15–17 months of age (Fig. S5). Epidemiologic studies show that both too much or too little fat are deleterious for lifespan (Flegal *et al.*, 2007; Orpana *et al.*, 2010) – a result that produces an inverted U-shaped curve for the relationship between adiposity and longevity. Although obesity is strongly associated with hyperglycemia and insulin resistance, lipoatrophy, a condition of depleted fat reserves, is also a risk factor – not only for insulin resistance (Reitman *et al.*, 1999) but also for elevated inflammatory tone (Herrero *et al.*, 2010). Thus, the RI strains in this study may not be in the range of excess adiposity that puts them at risk for premature death under AL feeding. Moreover, strains with the shortest lifespan and the lowest level of adiposity may be in the range associated with potentially life-shortening lipoatrophic sequelae (Fig. 1A,B). Such lipoatrophy may also play a role in the lifespan-shortening effect of DR observed in many of the RI strains, the surprising finding reported earlier (Liao *et al.*, 2010a,b; Rikke *et al.*, 2010).

Our data indicate that reduction in total fat mass below an as yet undefined threshold is a risk factor for lifespan shortening under DR; however, the data do not rule out the possibility that selective reduction in visceral adiposity may still play a role in lifespan extension in some or all strains (Muzumdar *et al.*, 2008). The beneficial effects of subcutaneous fat over visceral fat have been reported (Tran *et al.*, 2008). Interestingly, DR in C57BL/6J females redistributed fat stores in favor of subcutaneous depots over visceral (Varady *et al.*, 2010); a limitation of our study is that fat depots were not distinguishable by our measurement technique. Given the different effects of subcutaneous, visceral, and brown fat on metabolic function (Rosen & Spiegelman, 2006; Tran *et al.*, 2008), it will be informative to determine (i) whether the inverse relationship between fat and lifespan modulation holds for all or only selected fat depots and (ii) whether strains in which DR extended lifespan show a redistribution of fat stores favoring subcutaneous depots, despite minimal reduction in total fat stores. In addition, DR not only may redistribute fat mass, but it also may modify the secretary profiles of adipokines from fat, which have effects on systemic metabolism as well as inflammation (Rosen & Spiegelman, 2006). For example, DR increases the plasma level of adiponectin, which is secreted exclusively from adipose tissue and is involved in glucose homeostasis and insulin sensitivity (Berg *et al.*, 2001; Zhu *et al.*, 2004). DR also decreases tumor necrosis factor-α (TNF-α) from adipose tissue, which impairs insulin signaling and thereby increases insulin resistance (Barzilai & Gupta, 1999; Bordone & Guarente, 2005). It will be informative to measure the secretory profiles of adipose tissue from these mouse strains under DR. Strain variation of those traits may be also critical for animal survival under DR.

The inverse correlation between lifespan extension and fat reduction in response to DR demonstrates that these two traits share genetic pathways. However, the absence of significant QTLs affecting lifespan indicates that the QTL effect sizes for longevity are below our limit of reliable detection; in this study, a QTL would have to explain 43% of the genetic variation to be detected with 80% power (Belknap *et al.*, 1996). In our studies, power for detecting QTLs the size of *Fdr1* and *Fdr2* was < 50%, which greatly limited our ability to establish whether these QTLs also affected lifespan. Nonetheless, we did find evidence that the ISS allele on *Fdr1* in males was associated with positive effect on the fat mass and lifespan response to DR, and the ISS allele at *Fdr2* was associated with a negative effect on both traits. Additional studies using more powerful statistical analyses and genetic resources will be needed to validate these results.

In summary, this study demonstrates remarkable strain variation in fat reduction by DR in RI mouse strains and shows that factors associated with maintaining, rather than reducing fat, are important to the mechanism of survival and life extension under DR. These results also provide further support for the metabolic efficiency model underlying the prolongevity effect of DR.

Experimental procedures

Mouse strains and husbandry

This study used 41 ILSXISS RI mouse strains (Williams *et al.*, 2004) (formerly called LXS) originally developed to analyze genetic variation in alcohol sensitivity (Bennett *et al.*, 2006).

Briefly, ILSXISS RI strains were derived from an F_1 cross between two progenitor inbred strains, ILS and ISS strains. The F_1s were then crossed to produce heterozygous F_2s. The F_2s were inbred using 20 or more consecutive generations of brother/sister matting to generate up to 75 strains of ILSXISS lines (Williams *et al.*, 2004). As each RI strain is inbred, any given genetic locus has either an ISS or ILS allele. These strains have distinct strain distribution patterns of their ISS and ILS alleles that provide a powerful means of associating genetic variation with causal genetic loci (Williams *et al.*, 2004; Rikke & Johnson, 2007).

The animal husbandry was described in a previous report (Liao *et al.*, 2010a). Briefly, mice were typically maintained 5/cage in a specific pathogen-free vivarium dedicated to murine aging research. Males (38 strains) and females (33 strains) were separately housed in the same room with a 10:14-hour light/dark cycle (lights out at 5:00 PM). DR was implemented at 2–5 months of age immediately at 60% of AL intake calculated for each strain on the basis of AL food intake measured weekly and adjusted for wastage. The rations were given daily just before lights out. At 12 months of age, the DR rations were fixed to avoid tracking the reduction in food intake that can occur with aging. The diet was Harian-Teklad 7912, which is an irradiated, nonpurified mouse chow (> 19% crude protein, > 5% crude fat, < 5% crude fiber).

Measurements of body composition and body weight

The whole-body-composition analysis was conducted using QMR machine (Echo Medical System, Houston, TX, USA) (Tinsley *et al.*, 2004), the AL and DR mice being analyzed over the same time period. This machine utilizes nuclear magnetic resonance (NMR) to reliably and accurately analyze the physical state of the tissue (Taicher *et al.*, 2003; Tinsley *et al.*, 2004), which provides an estimate of total body fat, lean mass, and free water. The procedure involved immobilizing the mice in plastic restrainer tubes (no sedation) placed in the QMR machine. Scanning takes < 2 min per mouse. BW was also recorded at the same time and every 3 weeks from weaning to death.

Statistical analysis

The effect of strain on adiposity and lean mass was calculated by one-way analysis of variance (ANOVA). AL and DR groups in each strain were compared using unpaired *t*-tests. Correlations were assessed using Pearson product-moment analysis (two-tailed). Statistical tests were conducted using Statistical Package for Social Sciences 16.0 for Mac (SPSS® Inc., Chicago, IL, USA); *P* values < 0.05 were considered significant.

To determine whether fat and lean mass were heritable and thus suitable for linkage analysis, the heritability (h^2_{RI}, narrow sense) was calculated as previously described (Rikke *et al.*, 2004). Basically, heritability is estimated from the components of variance between and within strains calculated from ANOVA.

Regression

The fat response to DR is defined as the strain means for DR fat regressed on the AL means, which removes any correlation with the AL variation. Although DR fat was not correlated with AL fat, we regressed DR fat on AL fat for genetic mapping in both sexes to clarify the specific effects of DR on fat reduction. Such an adjustment removes the confound between variation under AL and variation imposed by DR, and enhanced statistical power for identifying QTL for DR response with this approach has been shown (Rikke *et al.*, 2006, 2010). For QTL mapping, the lean mass response and lifespan response to DR were defined similarly by regressing on their respective AL strain means.

QTL mapping

The phenotypic data (the mean of each strain and group) were subjected to a QTL mapping using Map Manager QTXb20 (Manly *et al.*, 2001). The program uses marker regression and an additive regression model to detect genetic loci influencing complex traits. The strain distribution pattern of 330 simple sequence length polymorphism (SSLP) markers was used in the QTL analysis. The positions of these microsatellite markers were updated using the Mouse Genome Database (http://www.informatics.jax.org/) as of January 2010. Briefly, the strength of the association between the genotypes and phenotypes is calculated as a LOD. The significant QTLs exceed the threshold of genome-wide $P < 0.05$, and suggestive QTLs exceed $P < 0.63$ (cutoff that permits one false positive) determined empirically by permutation testing (10 000 trials) (Churchill & Doerge, 1994).

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Scattergrams showing the correlations of fat in AL and DR mice measured at 15–17 and 20–22 months of age.

Fig. S2 Scattergram showing the correlations of lean in AL and DR mice measured at 15–17 and 20–22 months of age.

Fig. S3 Strain variation in body weight (BW) of ILSXISS recombinant inbred (RI) mice aged 15–17 months under *ad libitum* (AL) and 40% dietary restriction (DR) diets.

Fig. S4 Quantitative trait loci (QTL) mapping for lifespan in females under *ad libitum* (AL).

Fig. S5 Strain variation in % fat mass of ILSXISS recombinant inbred (RI) mice aged 15–17 months under *ad libitum* (AL) and 40% dietary restriction (DR) diets.

Table S1 Relationships of lifespan, fat mass, lean mass, and body weight (BW) at 15–17 months of age between *ad libitum* (AL) and 40% dietary restriction (DR) feedings.

Table S2 Relationships between males and females of lifespan, fat mass, lean mass, and body weight (BW) at 15–17 months of age under *ad libitum* (AL) and 40% dietary restriction (DR) feedings.

Table S3 Correlation coefficients among strain means between absolute fat mass, lean mass, and body weight (BW) at 15–17 months of age with mean lifespan in both sexes and diets.

Table S4 Correlation coefficients among strain means between absolute fat mass, lean mass, and body weight (BW) at 20–22 months of age with mean lifespan in both sexes and diets.

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