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Linking cellular zinc status to body weight and fat mass: mapping quantitative trait loci in *Znt7* knockout mice

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Abstract Zinc transporter 7 (*Znt7*, *Slc30a7*) knockout (KO) mice display abnormalities in body weight gain and body adiposity. Regulation of body weight and body fat accumulation is complex, involving multiple genetic and environmental factors. To understand how zinc homeostasis influences body weight and fat deposit and to identify quantitative trait loci (QTLs) that link zinc metabolism to growth and adiposity, we conducted a genome-wide mapping study using male F₂ *Znt7* KO mice and wild-type (WT) littermates with a mixed 129P1/ReJ and C57BL/6J genetic background. The mice were fed a semi-purified diet containing 30-mg Zn/kg diet at weaning. Body weights and fat pad weights including epididymal, retroperitoneal, and femoral subcutaneous fat pads were measured at 16 weeks of age. We detected two significant QTLs ($p < 0.05$) for body weight and fat deposit. One was in the F₂ *Znt7* KO population and the other in the F₂ WT population. In *Znt7* KO mice, the body weight and fat deposit was significantly linked to a locus on chromosome 7 ranging from 64.3 to 78.3 Mb. In WT mice, a significant linkage of retroperitoneal fat mass was found on chromosome 8 between 14.5 and 63.5 Mb. In addition, several other suggestive QTLs

($p < 0.63$) for body weight and fat accumulation were detected in *Znt7* KO and WT mice. In conclusion, the QTLs identified in this study may provide new hints to uncover the genes linking cellular zinc status to growth and body fat accumulation.

Introduction

Zinc is an essential trace element for the human body (Prasad 1988), where it is required for many fundamental biological and biochemical processes. For instance, zinc as a structural element is required by many zinc-finger containing transcription factors for DNA binding to regulate promoter activity leading to changes in gene expression (Coleman 1992). Zinc is also an important catalytic cofactor for many metalloenzymes crucial for many biochemical reactions (McCall et al. 2000). Importantly, the enzymatic activities of zinc-containing metalloenzymes can be regulated by dietary zinc content (Lukaski 2005). Restriction in dietary zinc intake in humans and animals causes stunted growth, decreased immune function, impaired wound healing, hair loss, diarrhea, and delayed sexual maturation (Halsted and Prasad 1963).

Cellular zinc contents are dependent on dietary zinc intake and activities of zinc transporter proteins. Zinc transporter proteins are composed of ZnTs (zinc transporters, *Slc30a1-10*) (Huang and Tapaamorndech 2013) and Zips (Zrt- and Irt-like proteins, *Slc39a1-14*) (Jeong and Eide 2013). Members of ZnTs and Zips function coordinately to mobilize zinc across the cell or organelle membrane to keep free zinc in tight control in a given cell (Huang and Tapaamorndech 2013). In general, ZnT proteins are involved in sequestration of zinc into intracellular

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compartments or export of zinc out of the cell under the condition of zinc being replete, whereas Zip proteins function to bring zinc into the cell or to release zinc from intracellular stores during zinc depletion (Huang and Tapaamorndech 2013; Jeong and Eide 2013). Null mutations in *ZnTs* or *Zips* in animal models result in altered zinc homeostasis leading to embryonic lethality (Dufner-Beattie et al. 2007; Palmiter and Findley 1995), stunted growth (Hojyo et al. 2011; Huang et al. 2007; Inoue et al. 2002), low body adiposity (Huang et al. 2007), impaired glucose and insulin metabolism (Huang et al. 2012; Wijesekara et al. 2010), abnormality in bone development (Fukada et al. 2008; Hojyo et al. 2011; Inoue et al. 2002), skin rash and hair loss (Huang and Gitschier 1997), or heart failure (Inoue et al. 2002).

Zinc deficiency as a risk factor for developing diabetes has been revealed recently in *Znt7* and *Znt8* knockout (KO) mice. Null mutations in the mouse *Znt7* and *Znt8* genes disrupt insulin synthesis and insulin crystallization, respectively, leading to a defect in insulin secretion in pancreatic β -cells (Hardy et al. 2012; Huang et al. 2010, 2012; Wijesekara et al. 2010). Consequently, *Znt7* and *Znt8* KO mice have abnormalities in glucose metabolism. The null mutation in *Znt7* also influences growth and body fat accumulation in mice (Huang et al. 2007). Dietary zinc deficiency affecting lipid metabolism in mice has been reported. For example, Reeves and O'Dell showed that zinc depletion reduced the conversion of glucose to fatty acids in epididymal (EF) fat (Reeves and O'Dell 1983). On the other hand, dietary zinc supplementation stimulates the conversion of glucose to lipid in adipocytes (Chen et al. 1998; Shisheva et al. 1992). Nevertheless, the mechanism underlying low body adiposity in *Znt7* KO mice is not fully understood.

The C57BL/6 (B6) and 129 mouse strains have been widely used for quantitative trait locus (QTL) mapping of obesity-related traits as B6 mice are prone while 129 mice are resistant to development of obesity and diabetes (Almind and Kahn 2004; Reed et al. 2003, 2006; Su et al. 2008). QTL mapping using F_2 C57BL/6ByJ (B6By) and 129P3/J (129P3) mice identified a group of QTLs that influences fat deposits in the body (Reed et al. 2006). Some QTLs are specifically associated with the amount of fat deposited in a particular location of the body in mice (Reed et al. 2006). It is known that zinc deficiency affects growth and body adiposity in mice (Huang et al. 2007; Inoue et al. 2002). However, no attempt has been made to study the effects of zinc status on body weight gain and body adiposity using genetic and genomic approaches. In order to compare phenotypic differences between *Znt7* KO and wild-type (WT) control mice in a homogeneous genetic background, we placed the *Znt7*-null mutation (originally on a mixed B6 and 129P2 genetic background) into the B6

strain by continuous backcross of heterozygous animals to B6 (Huang et al. 2007). Although the phenotypic differences in body weight gain and body fat mass persist between the congenic *Znt7* KO and WT B6 strains after the completion of the mutation replacement, the phenotypes (body weight and fat mass) in congenic *Znt7* KO mice were less severe than that observed in *Znt7* KO mice with a 129P2 $\times$ B6 mixed genetic background (unpublished data). The findings suggested that the *Znt7* mutant phenotypes in body weight gain and body fat accumulation might be influenced by the 129P2 genetic background. We hypothesized that one or more chromosomal regions in the 129P2 strain might underlie the increased severity of the phenotypes (less body weight and fat gains) found in *Znt7* KO mice with a mixed B6 and 129P2 genetic background. We, therefore, carried out a genome-wide mapping study to identify QTLs linking zinc status to body weight and fat pad mass including EF, retroperitoneal (RF), and femoral subcutaneous fat (SF) in male F_2 *Znt7* KO mice and WT littermates with a mixed B6 and 129P1/ReJ (129P1) genetic background, a closest relative to the 129P2 strain that is available in the United States from the Jackson Laboratory.

We identified a QTL of body weight and fat pad mass on chromosome 7 in male F_2 *Znt7* KO mice using 100 informative single nucleotide polymorphisms (SNPs) that covered the whole mouse genome. This QTL covers a range from 64.3 to 78.3 Mb on chromosome 7. We also uncovered a QTL of RF mass on chromosome 8 ranging from 14.5 to 63.5 Mb in male F_2 WT control mice. The QTLs identified in this study may provide novel insight into the genes that link body zinc homeostasis to growth and adiposity in humans and animals.

Materials and methods

Mouse stains, husbandry, and diets

Mouse *Znt7* KO strain on a B6 genetic background was described previously (Huang et al. 2007). 129P1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Male *Znt7* KO mice were mated to female 129P1 mice. The resulting F_1 heterozygous *Znt7* KO mice were intercrossed to generate F_2 mice. Only male F_2 mice were genotyped to identify *Znt7* KO and WT control mice (Huang et al. 2007). Mice with desired genotypes were weaned at 3 weeks of age and fed a semi-purified diet containing 30-mg Zn/kg diet (Research Diets, New Brunswick, NJ, USA) ad libitum until 16 weeks of age (Huang et al. 2007). All mice were housed in a temperature-controlled room at 22–24 °C with a 12-h light:dark cycle. Breeding mice were fed a standard laboratory chow

Table 1 SNP markers genotyped in F₂ *Znt7* KO and WT mice

Chr.	SNP ID	Genomic location	Chr.	SNP ID	Genomic location	Chr.	SNP ID	Genomic location
1	rs13475714	7970074	7	rs13459100	34215767	13	rs3722313	41442786
1	rs13471817	63151632	7	rs45883751	43659827	13	rs13481848	63513762
1	rs13476053	112049292	7	rs13479241	50763185	13	rs13481893	77562077
1	rs13476182	152103329	7	rs32166708	64268702	13	rs13481948	93811116
1	rs13476281	184322353	7	rs13479335	73507603	13	rs4230100	113217999
2	rs13476334	7375358	7	rs4226708	79091488	14	rs3701221	7781233
2	rs13476451	40457363	7	rs13479436	102670048	14	rs13482128	32951642
2	rs6241463	74314791	8	rs13479605	10521755	14	rs13482227	65767514
2	rs13476739	121366748	8	rs13479704	36675087	14	rs13482388	116561320
2	rs13476765	128474600	8	rs6332474	68898283	15	rs13459145	7167980
2	rs3692409	160811177	8	rs13479939	97595535	15	rs31624713	46638460
3	rs13476956	5370727	8	rs6296597	119621901	15	rs3666576	59167636
3	rs6379433	65083426	9	rs13480100	21450142	15	rs13482660	78381619
3	rs6395350	101866655	9	rs13480122	31156626	15	rs13482709	91674168
3	rs13477373	120695698	9	rs6210248	56219054	16	rs4165069	17663364
3	rs13477480	147960948	9	rs13480361	97689986	16	rs4172689	35282625
4	rs13477544	7035110	9	rs13480461	123247445	16	rs4199410	70194759
4	rs6232550	41025574	10	rs13480464	3484885	16	rs4219897	92789987
4	rs13477745	65386538	10	rs13480575	33653023	17	rs13482889	15187862
4	rs13477871	101048090	10	rs13480643	70562348	17	rs13482999	44504322
4	rs13474356	125068158	10	rs13480709	92776199	17	rs13483055	60319945
4	rs6362333	151380224	10	rs13480819	127003427	17	rs13483156	88583915
5	rs6245801	7461340	11	rs13480837	3886943	18	rs6382472	11318728
5	rs13478320	70742061	11	rs13481006	45477053	18	rs13483296	35206506
5	rs13478424	101569177	11	rs6344645	59110478	18	rs13483393	61809181
5	rs13478496	122233595	11	rs13481137	86489141	19	rs13483548	17770192
5	rs13478522	128472892	11	rs13481232	111494046	19	rs3705022	36377749
6	rs13478619	8784161	12	rs13481285	7014383	19	rs13459158	60867542
6	rs13478699	33076836	12	rs13481403	39488993	X	rs13483724	36008085
6	rs13478834	76824061	12	rs6344105	67759222	X	rs13483737	47876770
6	rs13478934	102078015	12	rs13481600	96809203	X	rs13483829	75211246
6	rs6185163	121508338	13	rs13481702	14756099	X	rs6387944	130474732
6	rs13479099	147199226	13	rs6235989	23728949	X	rs13484074	153647196
7	rs13479104	4350873						

Chr. chromosome; Genomic positions of SNP markers (GRCm38) were obtained from the SNP database. (<http://www.ncbi.nlm.nih.gov/snp/>)

diet (Laboratory Rodent Diet 5001, LabDiet, Brentwood, MO, USA) and double-distilled water ad libitum. All animal experiments were conducted in accordance with National Institutes of Health guidelines for the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee of the University of California at Davis.

Genotyping F₂ mice

Tail clips were collected from male F₂ mice at 2 weeks of age. Genomic DNA was isolated from these tail clips using a DNeasy Tissue kit (Qiagen, Valencia, CA, USA). PCR

genotyping to identify *Znt7* KO and WT mice was performed as described previously (Huang et al. 2007).

Genome-wide SNP genotyping

Genomic DNA was purified from tail clips from F₂ mice using a DNeasy Tissue kit (Qiagen). A hundred polymorphic SNP markers that are informative between B6 and 129P1 strains were used for genotyping (TaqMan[®] SNP Genotyping Assays, Life Technologies, Carlsbad, CA, USA). All SNP markers used in this study and their genomic positions (based on the GRCm38.p1 assembly) are listed in Table 1. TaqMan[®] genotyping reactions were

performed on a PRISM® ABI 7900HT Sequence Detection System according to the manufacturer's instructions, and allelic discriminations were performed using SDS 2.2.1 software (Life Technologies). All ambiguous genotypes were repeated in independent PCR experiments.

Phenotyping F₂ mice

Only male F₂ mice were genotyped and studied as the body weight difference between *Znt7* KO and WT control mice was more noticeable in the male population than the female population (Huang et al. 2007, 2012). Body weights of the experimental mice were measured in the morning (~07:00, light phase of the day) when they were 16 weeks old $\pm$ 2 days except for one mouse in the *Znt7* KO group and one mouse in the WT group that were at +3 and -3 days, respectively (Supplemental Fig. 1). Mice were then fasted for 4 h, weighed again, and killed (Huang et al. 2012). Fat pads including EF, RF, and SF were isolated, weighed, snap-frozen, and stored at -80 °C. Liver and skeletal muscle were also harvested, snap-frozen, and stored at -80 °C. A linear regression study of ages at the necropsy time on body weights of the experimental mice indicated that 1–3 days in age difference at the dissection time did not post apparent effect on body weight in both *Znt7* KO ($R^2 = 0.024$) and WT ($R^2 = 0.005$) groups; ($p > 0.05$) (Supplemental Fig. 1).

Linkage analysis of body weight and individual fat pad mass

Phenotypic data (wet weights of EF, RF, and SF) were converted to logarithms to obtain normal distributions for analysis. Linkage analyses in the male F₂ populations (both *Znt7* KO and WT control mice) were performed using R/qtl software (Broman et al. 2003). Single QTL genome scans were performed using single marker regression and interval mapping with EM algorithm and Haley–Knott regression. Results of linkage analysis were expressed as logarithm of the odds ratio (LOD). Significance thresholds were derived from permutation tests (1,000 permutations) to determine suggestive ($p > 0.63$), significant ($p < 0.05$), and highly significant ($p < 0.01$) QTLs. Pairwise QTL genome scans were also performed to search for interacting QTLs. We defined the confidence interval for the identified QTLs as the 1.0-LOD dropoff on either side of the peak. This interval was expressed in physical map position (Mb, GRCm38.p1) by using the genotyped SNPs that were at or beyond the 1.0-LOD interval.

SNP analysis

Nonsynonymous coding SNPs within the identified QTL region that differ between B6 and 129P2 were obtained

from the Mouse Genomes Project—SNPs at Wellcome Trust Sanger Institute (Cambridge, UK; <http://www.sanger.ac.uk>). We used the genome sequence of the 129P2 strain from the database for our nonsynonymous coding SNP (cSNP) analysis instead of the 129P1 strain that we used for the current study because (1) the whole-genome sequence of the 129P1 strain is not available and (2) the 129P2 strain is the closest relative of the 129P1 strain among the 129 substrains (Simpson et al. 1997). However, the cSNPs including Pro196Leu and Pro208Ser in Adamts17 as well as Ile31Val and Phe53Leu in Mboat4 were verified in the 129P1 mouse genome by sequencing in our laboratory (data not shown).

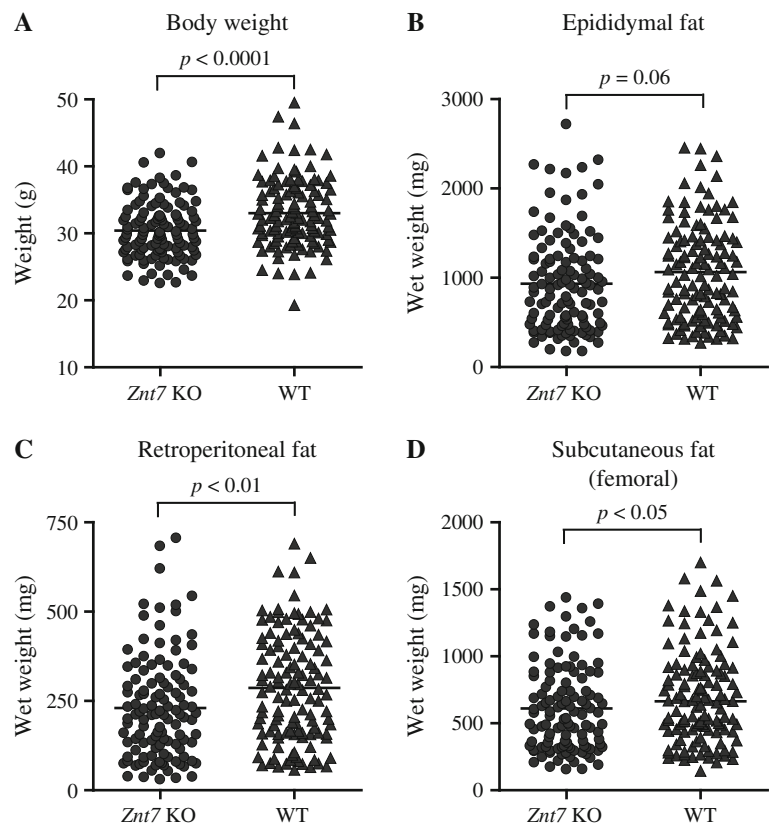
Total RNA isolation, cDNA synthesis, and quantitative RT-PCR analysis

100 mg frozen tissues ($n = 5$ –6 for WT or *Znt7* KO for each tissue type) were lysed in 1 ml TRIzol (Life Technologies). Total RNA was isolated according to the manufacturer's instructions. 1 μ g total RNA was converted into cDNA using an iScript cDNA synthesis kit (BioRad). For quantitative RT-PCR analysis, synthesized cDNAs were diluted tenfold with double-distilled water, and 2 μ l was used in a SYBR green-based PCR using SsoAdvanced™ SYBR® Green supermix (BioRad). Quantitative PCR was performed on a CFX384 Real-Time System (BioRad). *Rgma* primers were designed by Quantprime (<http://www.quantprime.de>) as follows: forward primer, 5'-TTCACAGACCACTTCCA GACATGC-3'; reverse primer, 5'-TCTTGAAGATGATG GTGAGCTTGC-3'. Melting temperature analysis for the reference (*Actb*) (Huang et al. 2012) and target (*Rgma*) genes revealed a single sharp peak at a typical melting temperature for each gene. Quantitative PCR was run in triplicate, and the average crossing point value was used for relative expression calculations ($-2^{\Delta\Delta C_T}$ method) (Livak and Schmittgen 2001). The expression of *Rgma* was normalized to the expression of *Actb*.

Data and statistical analysis

Phenotypic results are presented as the mean $\pm$ S.E. Student's *t* test was used in comparisons of two test groups with a two-tailed distribution for body weight and fat mass measurements. Differences were considered to be significant at $p < 0.05$ and highly significant at $p < 0.01$. In determination of the significance of an identified QTL, the likelihood ratios of 95 and 99 % calculated by the permutation test using R/qtl software represent significant ($p < 0.05$) and highly significant ($p < 0.01$), respectively. Suggestive QTLs were determined by $p < 0.63$.

Fig. 1 Body weights and fat pad weights in male F_2 *Znt7* KO and WT control mice at 16 weeks of age. Mice were fed a semi-purified diet containing 30 mg Zn/kg diet at weaning (3 weeks of age) and culled at 16 weeks of age after 4 h fasting. Body weights presented here were measured before fasting. Fat pads including epididymal, retroperitoneal, and femoral subcutaneous fat were excised and weighed. Weights in grams (g, body weight) and milligrams (mg, fat pads) are plotted for each F_2 mouse separated by genotypes ($n = 118$, *Znt7* KO; $n = 122$, WT littermates). Horizontal lines in the plotting area are the means for the respective group of the mice. p values are also indicated in each panel



Results

Phenotypic analysis of F_2 *Znt7* KO and WT mice

A total of 118 male F_2 *Znt7* KO and 122 F_2 WT littermates on a mixed B6 $\times$ 129P1 genetic background were collected for the QTL mapping study. To control dietary zinc intake, we fed F_2 *Znt7* KO and WT mice a semi-purified diet containing 30-mg Zn/kg diet (a zinc adequate diet) at weaning. At 16 weeks of age, body weights were recorded. Mice were then fasted for 4 h and killed. Fat pads (EF, RF, and femoral SF) were excised and weighed. On average, *Znt7* KO mice weighed 30.41 g (S.E. = 0.38, $n = 118$), while the WT mice weighed 33.03 g (S.E. = 0.45, $n = 122$). The difference in body weight (8 %) between *Znt7* KO and WT mice was statistically significant ($p < 0.0001$) (Fig. 1a). As expected, the *Znt7*-null mutation decreased fat gain in mice (Fig. 1b–d) (Huang et al. 2007). The EF, RF, and femoral SF isolated from *Znt7* KO mice ($n = 118$) weighed on averages of 932.76 mg (S.E. = 50.19 mg), 230.12 mg (S.E. = 13.40 mg), and 610.59 mg (S.E. = 29.78 mg), respectively, whereas the same fat pads isolated from WT mice ($n = 122$) weighed on averages of 1065.89 mg (S.E. = 48.76 mg), 286.52 mg (S.E. = 13.61 g), and 698.48 mg (S.E. = 31.57 mg), respectively. Compared to the fat pad masses in WT mice,

the average % reductions in EF, RF, and femoral SF masses in *Znt7* KO mice were 13, 20, and 13, respectively.

Linkage analysis of body weight and fat pad mass in F_2 *Znt7* KO mice

Single-locus QTL analysis revealed one significant QTL ($p < 0.05$) for body weight in male F_2 *Znt7* KO mice on chromosome 7 (Fig. 2a; Table 2). The peak of this QTL was at 73.5 Mb with a 1.0-LOD support range between 64.3 and 84.3 Mb in the mouse genome GRCm38.p1 assembly (Fig. 3a). The allele conferring less body weight was the 129P1 allele at this peak of the QTL (Fig. 3c). In addition, overlapping QTLs ($p < 0.01$) for EF, RF, and femoral SF masses were detected on chromosome 7 in male F_2 *Znt7* KO mice (Fig. 2b–d). The peaks of the QTLs were at 70.3, 70.3, and 71.3 Mb for EF, RF, and femoral SF masses, respectively (Fig. 3b and data not shown). The 1.0-LOD support ranges for EF, RF, and femoral SF masses were between 41.3 and 78.3, 40.3 and 78.3, and 42.3 and 81.3 Mb, respectively. Similar to the allelic effect of the QTL on the body weight, the allele conferring less fat accumulation was the 129P1 allele (Fig. 3d and data not shown). Furthermore, a similar QTL on chromosome 7 was detected for body fatness using adiposity index (sum of fat pad weights from EF, RF, and SF normalized to body weight) (Rogers and Webb 1980) in *Znt7* KO mice

Table 2 LOD scores at the SNP markers with at least suggestive significance ($p < 0.63$) associated with body weight and fat in F_2 *Znt7* KO and WT mice

Phenotype	<i>Znt7</i> KO				WT			
	Chr.	SNP ID	Genomic location	LOD score	Chr.	SNP ID	Genomic location	LOD score
BW	2	rs3692409	160811177	2.10 [‡]	4	rs13477745	65386538	3.19 [‡]
	7	rs45883751	43659827	2.29 [‡]				
	7	rs13479241	50763185	2.29 [‡]				
	7	rs32166708	64268702	2.50 [‡]				
	7	rs13479335	73507603	4.02*				
	7	rs4226708	79091488	2.89 [‡]				
	15	rs13459145	7167980	2.97 [‡]				
EF	7	rs45883751	43659827	5.98**	4	rs13477745	65386538	2.64 [‡]
	7	rs13479241	50763185	6.19**	7	rs45883751	43659827	2.13 [‡]
	7	rs32166708	64268702	5.93**	7	rs13479241	50763185	2.97 [‡]
	7	rs13479335	73507603	6.49**	7	rs13479335	73507603	2.12 [‡]
	7	rs4226708	79091488	4.86**	8	rs13479704	36675087	2.57 [‡]
	9	rs6210248	56219054	2.24 [‡]				
RF	7	rs45883751	43659827	5.34**	7	rs13479241	50763185	2.33 [‡]
	7	rs13479241	50763185	5.97**	8	rs13479704	36675087	3.83*
	7	rs32166708	64268702	5.57**	9	rs13480461	123247445	2.28 [‡]
	7	rs13479335	73507603	6.01**	14	rs13482128	32951642	2.15 [‡]
	7	rs4226708	79091488	4.34**				
SF	8	rs13479605	10521755	2.28 [‡]				
	7	rs45883751	43659827	3.88*	1	rs13476281	184322353	2.29 [‡]
	7	rs13479241	50763185	4.43**	7	rs13479241	50763185	2.13 [‡]
	7	rs32166708	64268702	4.26*	8	rs13479704	36675087	3.22 [‡]
	7	rs13479335	73507603	5.10**				
Adiposity index	7	rs4226708	79091488	4.01*				
	7	rs45883751	43659827	5.23**	7	rs45883751	43659827	2.25 [‡]
	7	rs13479241	50763185	5.64**	7	rs13479241	50763185	2.78 [‡]
	7	rs32166708	64268702	5.30**	7	rs13479335	73507603	2.12 [‡]
	7	rs13479335	73507603	5.90**	8	rs13479704	36675087	3.41 [‡]
	7	rs4226708	79091488	4.59**				

SNP markers are shown in sequential map order within a chromosome. Genomic positions of SNP markers (GRCm38) were obtained from the SNP database (<http://www.ncbi.nlm.nih.gov/snp/>). LOD scores were calculated by a marker regression method using R/qtl software

Chr. chromosome, *KO* knockout, *WT* wild type, *SNP* single nucleotide polymorphism, *BW* body weight, *EF* epididymal fat, *RF* retroperitoneal fat, *SF* subcutaneous femoral fat

** $p < 0.01$; * $p < 0.05$;

[‡] $p < 0.63$

(Table 2). The peak of this QTL was at 73.5 Mb (Table 2) with a 1.0-LOD support range between 40.3 and 79.0 Mb. The allele conferring less body weight was the 129P1 allele at this peak of the QTL (data not shown).

We also detected four weaker QTLs that exceeded suggestive thresholds ($p < 0.63$) in F_2 *Znt7* KO mice for body weight on chromosomes 2 (peaked at 160.8 Mb) and 15 (peaked at 7.1 Mb) and for EF and RF fat pads on chromosome 9 (peaked at 61.4 Mb) and 8 (peaked at 10.5 Mb), respectively. The LOD scores for these suggestive QTLs were ranging from 2.10 to 2.97 (Table 2). In the two QTLs for body weight, the 129P1 allele conferred low weight gain in the QTL on chromosome 2 while the 129P1/B6 heterozygous allele conferred high weight gain in the QTL on chromosome 15. In the two QTLs for fat accumulation, the B6 allele conferred low fat depot. These

suggestive QTLs provided initial evidence for the presence of other potential QTLs linking zinc status to growth and body fat accumulation besides the QTL on chromosome 7 described above. However, these QTLs need to be verified and confirmed by independent studies.

To determine whether the effect of the QTL on chromosome 7 for body weight and fat accumulation in *Znt7* KO mice depended on the genotype at other loci in the genome, we performed pairwise genome scans using full interactive (T_f), conditional-interactive (T_{fv1}), interactive-additive (T_i), full additive (T_a), and conditional-additive (T_{av1}) models. Significant thresholds were set at T_f , T_{fv1} , T_i , T_a , $T_{av1} = 9.1$, 7.1, 6.3, 6.3, 3.3 for a mouse intercross (Broman and Sen 2009). When these thresholds were applied, no significant epistatic interactions were detected in the genome of F_2 *Znt7* KO mice.

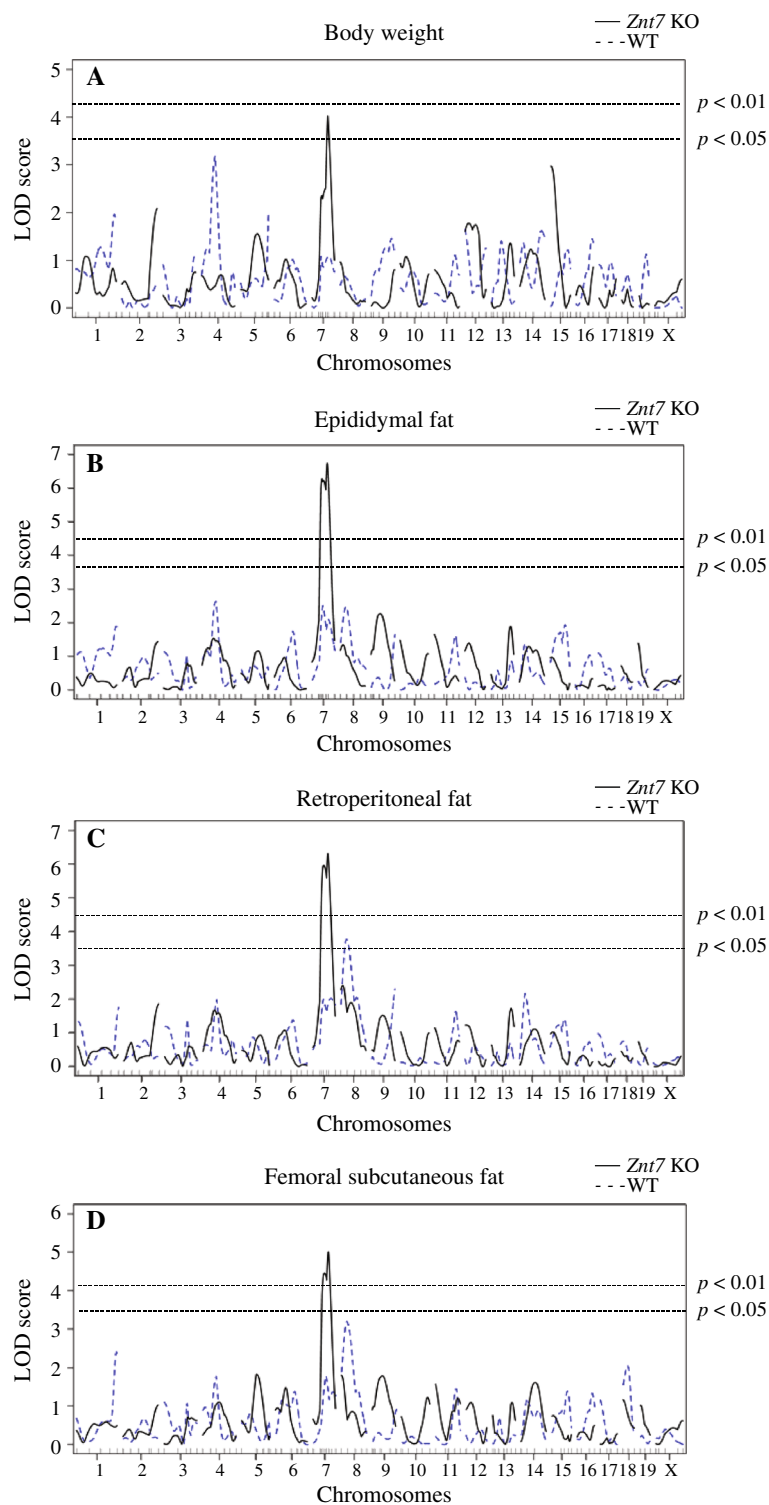


Fig. 2 Genome-wide LOD score distributions in male *F2* *Znt7* KO and WT mice for body weight and fat pad weights (epididymal, retroperitoneal, and femoral subcutaneous fat). The horizontal dotted

lines represent the permutation-derived significance (determined by 1,000 permutation tests; $p < 0.05$ and $p < 0.01$) thresholds

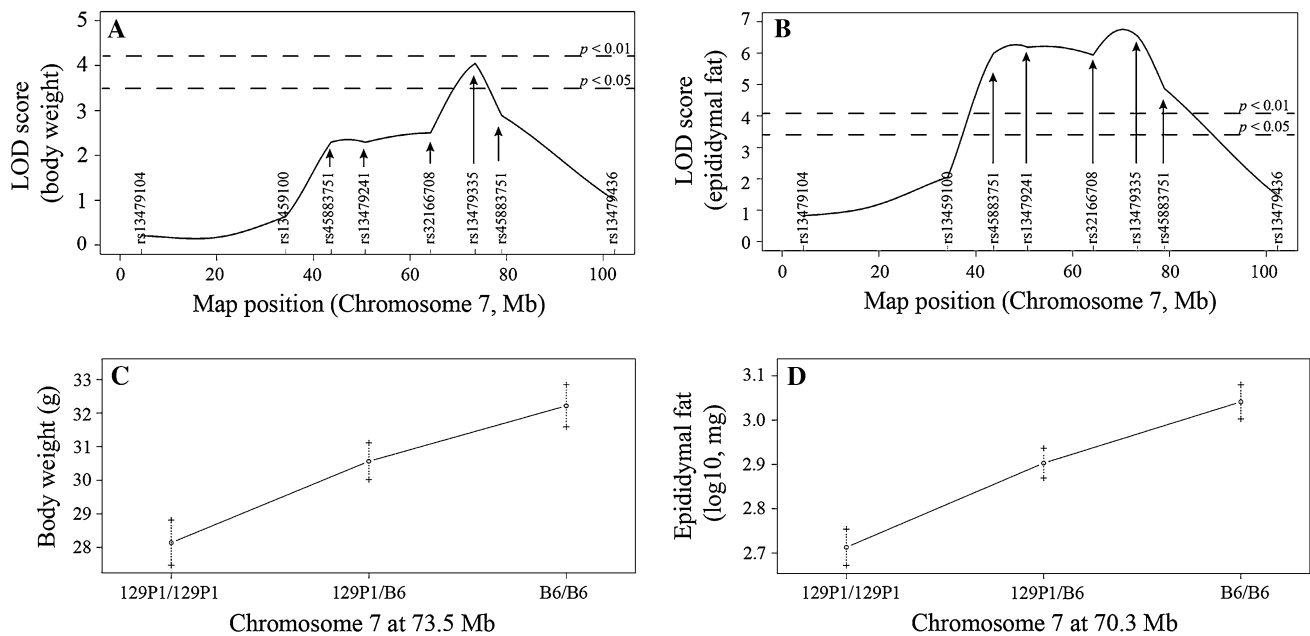


Fig. 3 LOD score plots for body weight (**a**) and epididymal fat mass (**b**) in male F_2 *Znt7* KO mice. Physical positions in Mb from the Mouse genome GRCm38.p1 assembly for each genotyped SNP marker (listed) on chromosome 7 are given on the x axis. LOD scores are displayed on the y axis. The horizontal dotted lines represent the permutation-derived significance (determined by 1,000 permutation tests; $p < 0.05$ and $p < 0.01$) thresholds. Arrows indicate the

positions of each genotyped SNP marker on the plots. The allelic effects of the strains on body weight (**c**) and epididymal fat mass (**d**) at the peaks on chromosome 7 in male F_2 *Znt7* KO mice. The peak locations of the QTLs in Mb are given for body weight and epididymal fat mass. 129P1/129P1 represents the homozygous 129P1 allele; B6/B6 represents the homozygous B6 allele; 129P1/B6 represents the heterozygous allele

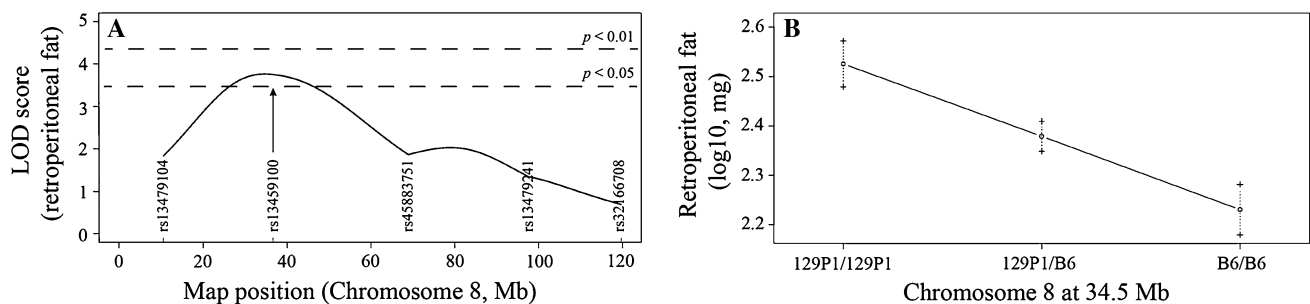


Fig. 4 A LOD score plot (**a**) and the allelic effect of the strains at the peak on chromosome 8 (**b**) for retroperitoneal fat in male F_2 WT mice. **a** Physical positions in Mb from the Mouse genome GRCm38.p1 assembly for each genotyped SNP marker (listed) on chromosome 8 are given on the x axis. LOD scores are displayed on the y axis. The horizontal dotted lines represent the permutation-derived significance (determined by 1,000 permutation tests; $p < 0.05$

and $p < 0.01$) thresholds. An arrow indicates the position of the rs13459100 SNP marker on the plot. **b** The allelic effects of the strains on retroperitoneal fat mass at the peak on chromosome 8 in male F_2 WT mice. 129P1/129P1 represents the homozygous 129P1 allele; B6/B6 represents the homozygous B6 allele; 129P1/B6 represents the heterozygous allele

Linkage analysis of body weight and fat pad mass in F_2 WT mice

Linkage analysis in F_2 WT mice of the B6 $\times$ 129P1 intercross was also performed to determine whether the identified QTL for body weight and fat pad mass in F_2 *Znt7* KO mice was specifically linked to the altered zinc homeostasis induced by the *Znt7*-null mutation. The linkage analysis in WT mice would also serve as a reference to

compare QTLs that we mapped in this study to the ones identified by others in B6 $\times$ 129 crosses (Almind and Kahn 2004; Reed et al. 2003, 2006; Su et al. 2008). As shown in Fig. 2c, we identified a QTL for the RF mass on chromosome 8 ($p < 0.05$) in F_2 WT mice. The peak of this QTL was at 34.5 Mb on chromosome 8 (Fig. 4a). The 1.0-LOD range was between 14.5 and 63.5 Mb (Table 2). The allele conferring heavy RF mass was the 129P1 allele (Fig. 4b).

We also detected one suggestive QTL ($p < 0.63$) for body weight on chromosome 4 (peaked at 65.3 Mb, the B6 allele conferring low body weight) and six other suggestive QTLs for fat mass and/or adiposity index on chromosomes 1 (peaked at 184 Mb, the B6 allele conferring low SF mass), 4 (peaked at 65.0 Mb, the B6 allele conferring low EF mass), 7 (peaked at 50.3 Mb, the 129P1 conferring low EF, RF, and SF masses as well as low adiposity index), 8 (peaked at 32.5 Mb, the B6 allele conferring low EF and SF masses), 9 (peaked at 123.2 Mb, the 129P1 allele conferring low RF mass), and 14 (peaked at 32.9 Mb, the 129P1 allele conferring low RF mass) in the WT population. The LOD scores for these suggestive QTLs were ranging from 2.12 to 3.41 (Table 2). The suggestive QTL on chromosome 8 for EF, SF or adiposity index overlapped with the candidate QTL for the RF mass on chromosome 8 in the WT population, indicating this QTL may contain gene(s) regulating general body fat accumulation not just RF mass in mice. It is worth to note that the suggestive QTLs for EF, RF, and SF masses on chromosome 7 in WT mice also overlapped with the candidate QTL for fat accumulation identified in the *Znt7* KO population. However, the peaks (50.7 Mb) of these suggestive QTLs were proximal to the candidate QTL (70.3 Mb) identified in *Znt7* KO mice. It is not clear whether the QTLs on chromosome 7 from both *Znt7* KO and WT mice correspond to the same gene(s) in the region or not. Nevertheless, the suggestive QTLs identified in the WT control mice need to be further verified.

Candidate genes in the QTL on chromosome 7

The QTL for body weight detected in *Znt7* KO mice ranged from 64.3 to 84.3 Mb on chromosome 7. A total of 192 genes including protein-coding genes, pseudogenes, and non-protein-coding genes are located in this genomic region according to the mouse genome GRCm38p.1 assembly (Table 3). Among them, 122 genes encode for proteins (Table 3; Fig. 5). The QTLs for body fat mass detected in *Znt7* KO mice were larger in sizes than the body weight QTL, covering more proximal region on chromosome 7 (Fig. 5). For example, the QTL for EF fat mass spans 37 Mb on chromosome 7 in which there are 272 protein-coding genes. The QTLs for RF and femoral SF contain 278 and 314 protein-coding genes, respectively (Table 3; Fig. 5). The QTL for adiposity index covered a similar region on chromosome 7 where 284 protein-coding genes are located (Table 3; Fig. 5). All the QTLs identified in *Znt7* KO mice overlapped each other on chromosome 7, highly suggestive of a common QTL on chromosome 7 for body weight and fat mass (Fig. 5). This assumption is supported by previous studies demonstrating that adipose deposits and body weight are highly correlated in mice

(Reed et al. 2006), especially in male *Znt7* KO mice (Huang et al. 2007). Moreover, it has been shown that the gonadal fat mass has the strongest correlation with body weight than the other fat deposits in both male and female mice (Reed et al. 2006). Therefore, by reducing the QTL to the interval in which both QTLs of body weight and EF fat mass overlapped, we could narrow the QTL down to a 14 Mb region (64.3–78.3 Mb) on chromosome 7, a 35 % reduction in the QTL size. This narrowed QTL region contains 43 protein-coding genes (Supplemental Table 1).

Variations in the genomic sequences of a defined QTL region could lead to a phenotypic difference between parental strains. Although a functional polymorphic change may occur in non-coding regions, often a SNP in the coding region of a gene (nonsynonymous change, cSNP) that changes an amino acid in the protein sequence is deleterious, resulting in a difference in protein expression or function of the protein. In addition, a SNP within a splicing donor or acceptor site of a gene that results in an alternative splicing variant or a SNP in the coding region of a gene that results in a stop codon gain or loss can have destructive effects on protein expression and function. We, therefore, examined these 43 genes listed in the narrowed QTL of body weight and fat mass for cSNPs including SNPs that resulted in a stop codon gain/loss or SNPs facilitating alternative RNA splicing events using the mouse phenome Database (MPD, <http://phenome.jax.org>) on a gene-by-gene basis between B6 and 129P2. The results are summarized in Table 4. Notably, we searched 129P2 genome for the polymorphisms instead of the 129P1 genome, a strain used in our mapping study, due to lack of a complete genomic sequence and a dense SNP mapping database for the 129P1 strain at the moment. Importantly, 129P2 strain was derived from the 129P1 strain in 1985 (Simpson et al. 1997). As such, it is genetically the closest relative of the 129P1 among the other 129 strains.

Out of the 43 protein-coding genes listed in the QTL, 19 genes have polymorphisms in the coding regions that change amino acids (Table 4), and one gene (*Tjp1*) has a SNP in the splice-site between 129P2 and B6. No SNP change that results in a stop codon gain or loss was detected among 43 genes. Among the 19 genes with cSNPs, ~20 % of them (*Agbl1*, *Fam189a1*, *Lysmd4*, and *Ttc23*) encodes for proteins of unknown function. The functions of the remaining genes in the list can be classified based on similarities of their encoded protein sequences to known functional proteins, phenotypes of KO mouse models, and/or symptoms of human genetic diseases. These include structural/adhesion proteins (*Synn* and *Tjp1*), enzymes (*Adamts17*, *Cers3*, *Fan1*, *Lrrk1*, *Pcsk6*, *Pgpep11*, and *Tarsl2*), adaptor/chaperone proteins (*Apha2* and *Akap13*), receptors (*Tm2d3*), selenoprotein (*Vimp*), preribosomal RNA processing (*Mphosph10*), and regulators of

Table 3 Genes in the candidate QTL regions on chromosomes 7 and 8

Chr.	Candidate QTL region	Genomic location ± 1.0 -LOD (Mb)	LOD peak (Mb)	No. of protein coding genes	No. of pseudogenes	No. of microRNA	No. of snoRNA	Other ^a
7	BW	64.3–84.3	73.5	122	44	4	0	22
7	EF	41.3–78.3	70.3	272	156	14	11	35
7	RF	40.3–78.3	70.3	278	156	14	11	35
7	Femoral SF	42.3–81.3	71.3	314	152	17	11	40
7	Adipo Index	40.3–79.0	70.3	284	157	15	11	36
8	RF	14.5–63.5	34.5	280	155	5	0	50

Genomic locations are listed based on the mouse genome GRCm38 assembly

Chr. chromosome, BW body weight, EF epididymal fat, RF retroperitoneal fat, SF subcutaneous fat, Adipo adiposity

^a including both tRNAs and miscRNAs

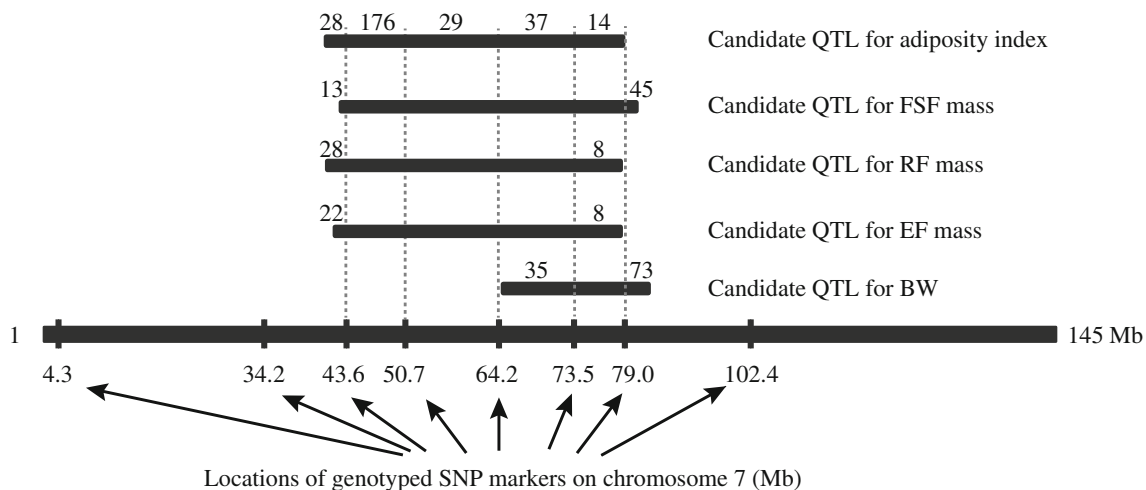


Fig. 5 Summary of the genomic intervals captured by QTLs for body weight and fat mass in male F_2 *Znt7* KO mice. The genotyped SNP markers and their physical locations in Mb on chromosome 7 are indicated. The ± 1.0 -LOD confidence intervals of the identified QTLs

and the number of genes with nonsynonymous coding changes, stop codon gain/loss, and/or splice-site variations in the QTLs are given. FSF femoral subcutaneous fat

transcription (*Lins*) (Table 4). The above-mentioned genes have been implicated in many biological pathways, such as signaling transduction, cell proliferation, apoptosis, and DNA repair during embryogenesis, early development, and growth in mice (Table 4).

Candidate genes in the QTL on chromosome 8

The significant QTL for the RF mass detected in WT mice ranges from 14.5 to 63.5 Mb on chromosome 8. A total of 490 genes including protein coding genes, pseudogenes, and non-protein coding genes are located in this genomic region (Table 3). Among them, 280 genes encode for proteins (Supplemental Table 1) in which 77 genes have cSNP in the coding regions (Table 5) between 129P2 and B6. No SNP change leading to splicing variants or a stop codon gain/loss was detected among 77 genes. The biological functions of 14 genes with cSNPs are currently

unknown. The functions of the remaining genes with cSNPs are completely or partially classified (Table 5). Twenty-eight genes are enzymes or cofactors for enzymes. Among the others, five are structural/adhesion proteins, two transporters, seven receptors or regulators of signal transduction, four regulators of DNA replication/recombination/repair, four regulators of transcription, two regulators of growth or apoptosis, three regulators of protein trafficking or cell dynamics, and eight secretory peptides. These genes have been implicated in many biological pathways during embryogenesis, early development, and growth in mice (Table 5).

Discussion

Genetic compositions have strong influence on body weight gain and body fat accumulation in mice and

Table 4 Genes with cSNPs and/or SNPs in the splicing regions between B6 and 129P2 strains in the candidate QTL on Chr. 7 between 64.3 and 78.3 Mb

Genes	Gene name	Function	Phenotypes associated with mutations
Fan1	Fancd2/Fanci-associated nuclease 1	DNA repair	ND
Mphosph10	M-phase phosphoprotein 10	Mitosis, pre-rRNA processing	ND
Apba2	Amyloid beta (A4) precursor protein-binding, family A, member 2	Synaptic vesicle exocytosis	Selective deficit in motivated approach behavior, but not in motivated avoidance behavior [†]
Fam189a1	Family with sequence similarity 189, member A1	Unknown	ND
Tjp1	Tight junction protein 1	Tight junction assembly	ND
Tarsl2	Threonyl-tRNA synthetase-like 2	Threonyl-tRNA aminoacylation	ND
Tm2d3	TM2 domain containing 3	Modulation of cell proliferation or death	ND
Pcsk6	Proprotein convertase subtilisin/kexin type 6	Precursor protein processing, tumor progression	Partial lethality by E15.5, embryos develop situs ambiguus with left pulmonary isomerism or craniofacial malformations including cyclopia, or both [†]
Vimp	VCP-interacting membrane protein	Cytokine production	ND
Lrrk1	Leucine-rich repeat kinase 1	GDP/GTP-binding, modulation of endosomal trafficking of EGFR	Prewaning lethality*, [†]
Lins	Lines homolog (<i>Drosophila</i>)	Modulation of Wnt target genes	ND
Cers3	Ceramide synthase 3	Ultra-long-chain ceramide synthesis	Complete loss of ceramides with ultra-long-chain acyl moieties ($\geq C26$), lack of continuous extracellular lipid lamellae and a non-functional cornified lipid envelope [†]
Adamts17	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 17	Zinc-dependent endopeptidase activity	A subtype of Weill–Marchesani syndrome with lenticular myopia, ectopia lentis, glaucoma, spherophakia, and short stature [‡]
Lysmd4	LysM, putative peptidoglycan-binding, domain containing 4	Unknown	ND
Ttc23	Tetratricopeptide repeat domain 23	Unknown	ND
Synm	Synemin	Cytoskeleton	ND
Pgpep1 1	Pyroglutamyl-peptidase I-like	Unknown	ND
Akap13	A kinase (PRKA) anchor protein 13	Modulation of signal transduction pathways involved in PKA	Embryonic lethality, arrested heart development, and forebrain hypoplasia. Small spleen, impaired lymphocyte response to osmotic stress, and decreased response to glucocorticoid observed in heterozygotes [†]
Agbl1	ATP/GTP binding protein-like 1	Unknown	ND

cSNP Data were obtained from the Sanger1 dataset in the MPD (Mouse Phenome Database). Genes are listed according to their genomic locations on chromosome 7 based on the mouse genome GRCm38p.1 assembly. Data for protein functions and null-mutation phenotypes were from the Mouse Genome Informatics (MGI) and National Center Biotechnology Information (NCBI)

ND not determined, *Chr.* chromosome

* Data were directly submitted to the Jackson Laboratory

[†] In mice

[‡] In humans

humans. Multiple QTLs for body weight and fatness in the F₂ B6 and 129 crosses have been described, suggesting a complex nature in regulation of body weight and fatness (Reed et al. 2003, 2006; Su et al. 2008). In the current study, we identified a QTL for body weight and fat pad mass in male *Znt7* KO mice on chromosome 7 ranging from 64.3 to 78.3 Mb using a genome-wide scan method.

Our data demonstrated that *Znt7* KO mice with homozygous 129P1 alleles in this QTL region were more prone to low body weight and low fat deposit at various locations in the body. Most importantly, we detected this QTL ($p < 0.01$) only in the F₂ *Znt7* population using ~50 % fewer numbers of animals typically used in QTL-mapping studies (Miles and Wayne 2008; Su et al. 2008), indicating

Table 5 Genes with cSNPs and/or splicing regions between B6 and 129P2 strains in the candidate QTL on Chr. 8 between 14.5 and 63.5 Mb

Gene	Gene name	Function	Phenotypes associated with mutations
Arhgef10	Rho guanine nucleotide exchange factor 10	Rho-dependent signal transduction	ND
Kbtbd11	Kbtbd11 kelch repeat and BTB (POZ) domain containing 11	Unknown	ND
Myom2	Myomesin 2	Structural constituent of cytoskeleton	ND
Csmd1	CUB and Sushi multiple domains 1	Tumor suppressor	No abnormal phenotype observed [†]
McpH1	Microcephalin 1	DNA repair	Primary microcephaly [†] Microcephaly 1; premature chromosome condensation syndrome [‡]
Xkr5	Kell blood group complex subunit-related family, member 5	Unknown	ND
Defa24, Defb5, 8, 40, and 46	Defensin alpha 24, beta 5, 8, 40, and 46,	Antimicrobial peptides	ND
Spag11b	Sperm associated antigen 11B	Sperm maturation	ND
Gm15284	Predicted gene 15284	Unknown	ND
AY761184	cDNA sequence AY761184	Unknown	ND
C86695	Expressed sequence C86695	Unknown	ND
Nek3	NIMA-related kinase 3	Serine/threonine protein kinase; regulates microtubule acetylation	ND
Tpte	Transmembrane phosphatase with tensin homology	Tyrosine phosphatase; testis-specific	ND
Slc20a2	Solute carrier family 20, member 2	Phosphate uptake	Decreased body weight gain; abnormal bone development [†]
Sfrp1	Secreted frizzled-related protein 1	Modulator of Wnt signaling, embryonic patterning	No abnormal phenotype with the Sfrp1-null mutation; severe shortening of the thoracic region in embryos with Sfrp1 and Sfrp2 double null mutations [†]
Adam3, 9, 18, 20, 24, 25, 29, and 39	A disintegrin and metalloproteinase 3, 9, 18, 20, 24, 25, 29, and 39	Zinc-dependent endopeptidase activity	Adam3-null, male infertile; Adam9-null, no observed phenotypes; Adam18-null, improved ability to execute integrated movements of muscle; Adam24-null, increased polyspermic embryos [†]
Ddhd2	DDHD domain containing 2	Phospholipase	Hereditary spastic paraplegia [‡]
Hgsnat	Heparan-alpha-glucosaminide <i>N</i> -acetyltransferase	Degradation of heparin sulfate in lysosomes.	Sanfilippo syndrome C [‡]
Pomk	Protein- <i>O</i> -mannose kinase	Dystroglycan	Hydrocephaly, cerebellar dysplasia, learning defects, impaired motor strength, and decreased sensitivity to pain [†]
Thap1	THAP domain containing, apoptosis associated protein 1	Proapoptosis	Walker-Warburg syndrome [‡] Blepharospasm [‡]
Tti2	TELO2 interacting protein 2	DNA repair, regulator of phosphoinositide-3-kinase-related protein kinase (PIKK)	ND

Table 5 continued

Gene	Gene name	Function	Phenotypes associated with mutations
Fut10	FUT10 fucosyltransferase 10	Alpha1,3-fucosyltransferase activity	ND
Wrn	Werner syndrome, RecQ helicase-like	DNA repair, DNA helicase activity	Elevated frequency of somatic reversion of the pink-eyed dilution unstable mutation [†] Werner syndrome [‡]
Purg	Purine-rich element binding protein G	DNA replication and transcription	ND
Tex15	Testis expressed 15	DNA repair	Male infertile [†]
Ubxn8	UBX domain protein 8	Modulator of ER-associated degradation of misfolded proteins	ND
Gsr	Glutathione reductase	Antioxidant	Decreased retinal artery-to-vein ratio [†] Hereditary glutathione reductase deficiency [‡]
Gtf2e2	General transcription factor IIE, polypeptide 2, beta 34 kDa	Transcription initiation	ND
Mboat4	Membrane bound <i>O</i> - acyl-transferase domain containing 4	Ghrelin activator by attaching octanoate to proghrelin	Absence of acylated ghrelin in circulation, abnormal glucose metabolism during calorie restriction [†]
Tmem66	Transmembrane protein 66	Negative regulator of Store operated calcium entry in the ER	ND
Gm4889	Predicted gene 4889	Unknown	ND
Ppp1r3b	Protein phosphatase 1, regulatory subunit 3B	Serine/threonine phosphatase activity, regulating glycogen synthesis	Improved glucose tolerance, increased weight loss and serum glucose level in response to starvation [†]
Eri1	Exoribonuclease 1	Exonuclease activity, DNA replication	Postnatal lethality, decreased body size beginning at E15.5, and decreased proliferation of mouse embryonic fibroblasts [†]
Gm19410	Predicted gene, 19410	Unknown	ND
D8Erd82e	DNA segment, Chr. 8, ERATO Doi 82, expressed	Modulator of the RhoA pathway	ND
Lonrf1	LON peptidase <i>N</i> - terminal domain and ring finger 1	ATP-dependent peptidase activity	ND
6430573F11Rik	RIKEN cDNA 6430573F11 gene	Putative methyltransferase activity	ND
Dlc1	Deleted in liver cancer 1	Regulator of small GTP-binding proteins involved in cytoskeletal dynamics, tumor suppressor	Embryonic lethality [†]
AI429214	Expressed sequence AI429214	Unknown	ND
Sgcz	Sarcoglycan, zeta	Structure protein that bridges the inner cytoskeleton and the extra-cellular matrix	ND
Msr1	Macrophage scavenger receptor 1	Mediating the endocytosis of acetyl-LDLs (low dense lipoproteins) in macrophages	Abnormal uptake and degradation of acetyl-LDLs in macrophages, increased interleukin-12 secretion in response to CpG oligo- deoxynucleotide administration, and increased bacterial and viral infection [†]
Fgf20	Fibroblast growth factor 20	Secreted neurotrophic factor involved in central nervous system development and function	Impaired cochlea lateral compartment differentiation and deafness without loss of vestibular function [†]
Zdhhc2	Zinc finger, DHHC-type containing 2	Palmitoyl transferase activity	ND
Slc7a2	Solute carrier family 7, member 2	Cationic amino acid transporter for uptake of arginine, lysine and ornithine	Reduction in nitric oxide production by cytokine- activated macrophages [†]

Table 5 continued

Gene	Gene name	Function	Phenotypes associated with mutations
<i>Pdgfr1</i>	Platelet-derived growth factor receptor-like	Tumor suppressor	Increased sporadic hepatocellular carcinomas, colorectal cancers, and non-small cell lung cancers [‡]
<i>Mtus1</i>	Microtubule associated tumor suppressor 1	Tumor suppressor involved in angiotensin II signaling pathways	ND
<i>Mtus1</i>	Microtubule associated tumor suppressor 1	Tumor suppressor involved in angiotensin II signaling pathways	ND
<i>Fgl1</i>	Fibrinogen-like 1	Liver regeneration and lipid metabolism	Increased body weight gain, increased gluconeogenesis resulting in fasting hyperglycemia, decreased free fatty acids and cholesterol in plasma, abnormalities in the morphology of white and brown fat tissues [†]
<i>Triml1</i> , <i>Triml2</i>	Tripartite motif family-like 1 and 2	Unknown	ND
<i>Zfp42</i>	Zinc-finger protein 42	Modulating differentiation	Premature age-related male germ cell loss, abnormal sperm morphology, and mild testicular atrophy [†]
<i>Pdlim3</i>	PDZ and LIM domain 3	Cytoskeletal assembly	Strain-dependent partial prenatal lethality, embryonic right ventricular (RV) dilation and dysplasia, defective trabeculation, and RV cardiomyopathy [†]
<i>Ccdc110</i>	Coiled-coil domain containing 110	Unknown	ND
1700029J07Rik	RIKEN cDNA 1700029J07 gene	Unknown	ND
<i>Acs11</i>	Acyl-CoA synthetase long-chain family member 1	Lipid biosynthesis and fatty acid degradation	Decreased triacylglycerol synthesis, decreased beta-oxidation, and altered fatty acid compositions in liver-specific knockout animals [†]
<i>Irf2</i>	Interferon regulatory factor 2	Transcription activator	Defect in thymocyte development, inhibited hematopoiesis and B lymphopoiesis in bone marrow [†]
<i>Trappc11</i>	Trafficking protein particle complex 11	Intracellular vesicle trafficking between reticulum and Golgi vesicle transport	ND
<i>Ing2</i>	Inhibitor of growth family, member 2	DNA repair, apoptosis	Impaired spermatogenesis and male infertility [†]
<i>Cldn22</i>	Claudin 22	Structure protein of tight junction	ND
<i>Wwc2</i>	WW and C2 domain containing 2	Unknown	ND
<i>Tenm3</i>	Teneurin transmembrane protein 3	Eye development	Microphthalmia [‡] Abnormal ipsilateral retinal ganglion cell projections, impaired performance in visually mediated behavioral tasks [†]
<i>Aga</i>	Aspartylglucosaminidase	Lysosomal breakdown of glycoproteins	Aspartylglucosaminuria [‡] , [†]
<i>Wdr17</i>	WD repeat domain 17	Unknown	ND
<i>Cep44</i>	Centrosomal protein 44 kDa	Unknown	ND
<i>Hmgb2</i>	High mobility group box 2	DNA repair and recombination	Reduced male fertility [†]
<i>Nek1</i>	NIMA-related kinase 1	Serine/threonine kinase activity, DNA repair, Cell cycle regulation	Premature death, facial dysmorphism, dwarfism, male sterility, anemia, cystic choroid plexus, a late-onset slowly progressive polycystic kidney disease [†]

Table 5 continued

Gene	Gene name	Function	Phenotypes associated with mutations
Sh3rf1	SH3 domain containing ring finger 1	Ubiquitin ligase activity involved in protein sorting at the trans-Golgi network, down-regulation of the JNK signaling pathway	ND

cSNP Data were obtained from the Sanger1 dataset in the MPD (Mouse Phenome Database). Genes are listed according to their genomic locations on chromosome 8 based on the mouse genome GRCh38p.1 assembly. Data for protein functions and null-mutation phenotypes were from the MGI (Mouse Genome Informatics) and NCBI (National Center Biotechnology Information)

ND not determined, *Chr.* chromosome

* Data were directly submitted to the Jackson Laboratory

† In mice

‡ In humans

that low body zinc status greatly increased expressivity of this QTL on chromosome 7. It is worth to note that we also detected a suggestive QTL ($p < 0.63$, Table 2) for the fat pad mass on chromosome 7 in the WT mice that overlapped with the candidate QTL for the fat accumulation in the *Znt7* KO mice. The overlapping QTLs could correspond to the same gene responsible for the QTL effect on the local fat deposit with a different magnitude between *Znt7* KO and WT mice. This is to say, if the same gene affects local fat deposit in both *Znt7* KO and WT populations, then the *Znt7*-null mutation may increase the magnitude of the QTL effect on fat accumulation. This could explain why we were able to detect a significant QTL with 50 % fewer numbers of *Znt7* KO mice in our study. Nevertheless, the peak of the suggestive QTL (50.7 Mb) for the fat pad mass found in the WT mice was proximal to the peak of the candidate QTL (70.3 Mb) identified in the *Znt7* KO mice. This may suggest that the corresponding genes underlying the QTL effects on fat accumulation observed in *Znt7* KO as well as in WT mice may actually be two different genes but closely located on chromosome 7.

In addition, we detected a QTL ($p < 0.05$) of RF in male B6 control mice on chromosome 8 between 14.5 and 63.5 Mb (LOD peak at 34.5 Mb). WT mice homozygous for the 129P1 alleles at this locus had high RF deposit. Interestingly, we also detected a suggestive QTL for the RF on chromosome 8 in *Znt7* KO mice which overlapped with the one found in WT mice. The peak of this QTL was at 18.5 Mb on chromosome 8, 16 Mb proximal to the peak of the QTL identified in WT mice. Nevertheless, it is currently not clear whether there are one or two genes underlying the QTL effects observed in WT and *Znt7* KO mice.

Both QTLs for body weight and local fat deposit on chromosomes 7 and 8 were reported using other mouse crosses (Diamant and Warden 2004; Reed et al. 2003, 2006; Su et al. 2008). For instance, Reed et al. reported a QTL for local fat deposits (gonadal and RF) on chromosome 7 between 32 and 45 cm in the F₂ C57BL/6ByJ and 129P3 cross fed a chow diet

(4.4 % fat) and the C57BL/6ByJ allele in this QTL conferred higher body weight and fat deposit (Reed et al. 2006). The results from our current study are consistent with the findings reported by Reed et al. Moreover, Reed et al. showed that the QTL for fat deposit on chromosome 7 was only found in male, not female mice (Reed et al. 2006) which is consistent with our findings that the *Znt7*-null mutation affects body adiposity more obviously in male than female mice (Huang et al. 2007, 2012). The findings in this study together with the results from previous studies strongly suggest that the responsive gene or genes in the QTL region on chromosome 7 influence body weight and fatness, and the activity of the candidate gene(s) is also affected by body zinc status. To the best of our knowledge, this is the first report that directly links zinc status to body weight and fatness on a genetic basis.

In the current study, we also detected a candidate QTL for RF mass on chromosome 8 (14.5–63.5 Mb) in the WT control mice. Interestingly, a QTL on chromosome 8 for RF weight ranging from 39 to 52 cM was also reported in the study conducted by Reed et al. (Reed et al. 2006). However, the QTL found in this study is proximal to and not overlapped with the one reported by Reed et al. The discrepancy may lay in two reasons: first, different mouse strains were used in our mapping study; second, our QTL on chromosome 8 was broad and volatile due to a limited number of animals involved in the study. Or, they are simply different QTLs altogether.

The genomes of the B6 and 129P2 strains were sequenced, and the physical locations of the genes on mouse chromosome 7 are known. According to the NCBI and Ensembl mouse genome databases, a total of 54 functionally expressed genes are located in the QTL region of body weight and fatness (chromosome 7 between 64.3 and 78.3 Mb) which includes 43 protein-coding genes and 11 miscRNA genes. Since the protein-coding region of a given gene is more conserved than untranslated regions, cSNPs between the B6 and 129P2 strains may result in changes in protein expression and/or function. Based on this assumption, we reduced the candidate genes in the QTL on chromosome 7 from 43 to 19

(Table 4). Among the 19 genes with cSNPs and known protein functions or structures, the strongest candidate is *Adamts17* (ADAM metalloproteinase with thrombospondin type 1 motif, 17).

Members of the ADAMTS family (a disintegrin and metalloproteinase with thrombospondin motifs) have been shown to be associated with body weight and fat tissue development (Moncada-Pazos et al. 2012; Morales et al. 2009). The mouse *Adamts17* gene encodes a zinc-dependent endopeptidase with 1122 amino acids. In mice, *Adamts17* contains two amino acid changes between the B6 and 129P1 strains, Pro196Leu and Pro208Ser (B6, 129P1) and three amino acid changes between the B6 and 129P2 strains, Pro196Leu, Pro208Ser, and Pro1116Leu (B6, 129P2). A majority of mouse strains listed in the Sanger1 data set within the MPD database (<http://phenome.jax.org>) have the same amino acid variants with the B6 strain. The two residues (Pro196 and Pro208) are highly conserved among the *Adamts17* orthologues from mammals to non-mammals (Fig. 6). Substitution of leucine for proline at the position 196 in 129P1 *Adamts17* was predicted to be deleterious to the *Adamts17* function by SIFT (Sorting Tolerant from Intolerant; <http://sift.jcvi.org/>) while the Pro208Ser change was predicted to be tolerated (Ng and Henikoff 2003). Nevertheless, cSNPs in these three amino acid codons in mouse *Adamts17* have not been reported in human ADAMTS17 although nonsynonymous SNPs are found close to these three amino acids (Ensembl Transcript: ADAMTS17-001 ENST00000268070).

Adamts proteins undergo maturation in the cell before secretion by removing the signal peptide and prodomain at the N-terminal end of the protein. The cSNPs (Pro196Leu and Pro208Ser) in *Adamts17* locate in the vicinity of a conserved putative “Cys-switch” domain (Cys200) (Cal et al. 2002), a domain required for propeptide cleavage between prodomain and metalloproteinase domain during enzyme maturation (Fig. 6). The “Cys-switch” domain is one of the most conserved domains among the *Adamts17* orthologues. Non-conservative proline-to-leucine substitutions in the vicinity of the “Cys-switch” domain in *Adamts17* may cause destructive effect on protein maturation and secretion. In fact, the Pro196Leu change was predicted to be deleterious by SIFT. Additionally, inhibition of propeptide cleavage due to these cSNP changes may affect zinc ions to be incorporated into the metalloproteinase domain in *Adamts17* during enzyme maturation. Moreover, reduced zinc availability in the secretory pathway due to the *Znt7*-null mutation may stage the deteriorating effect induced by the cSNPs in *Adamts17*. As the result, the amount and/or enzymatic activity of *Adamts17* in the extracellular matrix may be low in *Znt7* KO mice with a 129P1 or 129P2 genetic background compared to that with a B6 genetic background leading to low body weight and low fat accumulation.

Several lines of evidence from functional studies of other members of the *Adamts* family support the candidacy of *Adamts17* as a modifier gene for body weight gain and fatness in *Znt7* KO mice. For example, mice deficient for *Adamts1* gain much less body weight (30 % less than their WT or heterozygous littermates by 9 months old) and have less body fat among the other phenotypes (Shindo et al. 2000). Histological examination indicates that the morphology of the adipose tissue from *Adamts1* KO mice is normal but the size of individual fat cell is small. These phenotypes present in *Adamts1* KO mice are similar to those observed in *Znt7* KO mice (unpublished data) (Huang et al. 2012). Mice deficient for *Adamts12* increase weight loss during colitis induction (Moncada-Pazos et al. 2012). Moreover, *Adamts12* KO mice are prone to tumor growth due to inhibited apoptosis and increased angiogenesis (El Hour et al. 2010) similar to those observed in *Znt7* KO mice (Tapaamorndech et al. 2011).

The second candidate gene in the QTL on chromosome 7 is *Rgma* (repulsive guidance molecule domain family, member A). *Rgma* acts as a co-receptor for bone morphogenetic proteins (Bmps) including *Bmp2* and *Bmp4* to enhance *Bmp* signals in bone differentiation (Babitt et al. 2005). *Bmp2* and *Bmp4* also stimulate adipogenesis in adipose tissue (Tseng and He 2007). *Bmp4* was reported to play a role in mediating brown fat-like changes in white adipose tissue (Qian et al. 2013). Although no cSNP was identified in the coding region of *Rgma* between the B6 and 129P1 or 129P2 allele, SNPs that occur in regulatory regions including the promoter region, UTR (untranslated region), and introns between the B6 and 129P1 or 129P2 strain are found. These SNPs may produce differential gene expression of *Rgma* in white fat tissue in mice. In fact, differential expression of *Rgma* was documented in gonadal fat tissues of congenic obese mice (Sarahan et al. 2011). Our quantitative gene expression analysis in key metabolic tissues provided further and supportive evidence that *Rgma* expression was QTL allele-dependent (chromosome 7) between the B6 and 129P1 strains in EF (Fig. 7a). However, this differential expression of *Rgma* was not observed in skeletal muscle (Fig. 7b). *Rgma* mRNA expression in WT mice with the 129P1 allele in the candidate QTL region on chromosome 7 was ~3.5-fold higher than those with the B6 allele in the QTL region. This augmentation in gene expression was partially inhibited (~50 %) by the *Znt7*-null mutation in EF tissue (Fig. 7a). In addition, transcripts for *Rgma* were barely detectable in the liver of both *Znt7* KO and WT mice in our quantitative analysis (data not shown). Together, these data suggest that *Rgma* may be the target gene in the QTL for fat mass on chromosome 7.

The QTL for RF on chromosome 8 covers a large region at the proximal region of the chromosome. In this QTL region, seventy-seven genes contain cSNPs between B6 and 129P2 strains. Among them, the highest priority

	Adamts17	196	208	
Rodents	<i>Mus musculus</i>	GREHLIRRKWASTPSPATEVEG PRQR C KVVTEKK PR KDHLSDWRERRNAIR	225	
	<i>Rattus norvegicus</i>	RREHLIRRKWSSSTGLATKVEG PRQH C KVVTEKK PR KGLSDWRERRNAIR	225	
	<i>Cricetulus griseus</i>	GREHLIRRKWSSASSQSTSVEE PRQH C KVVTEKK PK KDKPSRDWRERRNAIR	101	
	<i>Octodon degus</i>	GREHLVRRKWSLKPSPPADTQVP QGQL C EVLTEKK PR KARPSRVWRERRNAIR	225	
	<i>Heterocephalus glaber</i>	GREHLIRRKWSLKPSPSAKTQVP QGQL C KVLTEKK PR KARPSRVWRERRNAIR	225	
	<i>Jaculus jaculus</i>	RQEHLIRRKGSFTSSPSAETKES QGQL C KVVTEKK PR KGKPLQDLRERRNAIR	202	
Primates	<i>Homo sapiens</i>	GREHLIRRKWSLTSPSAAEQ PEQL C KVLTEKK PT WGRPSRDWRERRNAIR	225	
	<i>Nomascus leucogenys</i>	GREHLIRRKWSLTSPSAAEQ PGQL C KVLTEKK PR RGRPSRDWRERRNAIR	225	
	<i>Pan troglodytes</i>	GREHLIRRKWSLTSPSAAEQ PEQL C KFLTEKK PT RGRPSRDWRERRNAIR	225	
	<i>Pan paniscus</i>	GREHLIRRKWSLTSPSAAEQ PEQL C KFLTEKK PT RGRPSRDWRERRNAIR	225	
	<i>Saimiri boliviensis</i>	GQEHLIRRRWSSTSPSAAEQ PGQL C TVLTEKK PG SRPSQDWRERRNAIQ	225	
	<i>Callithrix jacchus</i>	GREHLIRRKWSLTSPSAAEQ PEHL C KVLTEKK PR QGRPSQDWRERRNAIQ	225	
	<i>Macaca fascicularis</i>	GREHLIRRKWSLTSPSAAEQ SGQL C KVLTEKK PR RGRPSRDWRERRNAIR	225	
	<i>Macaca mulatta</i>	GREHLIRRKWSLTSPSAAEQ SGQL C KVLTEKK PR RGRPSRDWRERRNAIR	156	
	<i>Papio anubis</i>	GREHLIRRKWSLTSPSAAEQ SGQL C KVLTEKK PR RGRPSRDWRERRNAIQ	225	
	<i>Otolemur garnettii</i>	GREHLIRRKWSLTSPSAAEQ TGQF C KVLTEKK KL RKGKSQDWRERRNAIQ	225	
Small animals	<i>Condylura cristata</i>	GREHLIRRKWSLAPSPAEEAQT PGQP C EVLTDKK PS RGRPVGDLRERRNAIR	208	
	<i>Mustela putorius furo</i>	VREHLVRRKWSAPSSTEAQ PGQL C QVLTEKK PR DRASQDWRERRNAIQ	225	
	<i>Felis catus</i>	GREHLVRRKWSLTSPSPTEA IPGQL C KVLTEKK PR RGRPSQDWRERRNAIQ	225	
	<i>Canis lupus familiaris</i>	RGEHLVRRKWSLTSPSPTEA IPGQL C QVLTEKK PR KGRQSDWRERRNAIQ	225	
	<i>Monodelphis domestica</i>	GREHLVRRKRSSSGDHAKSWI PDEL C TVITEKK PK KNTSDWRERRNAIR	222	
	<i>Oryctolagus cuniculus</i>	GREHLIRRTWS-----TGAQ APGRF C KVLTEK QL NGSRPARDWRERRHAIQ	217	
Large animals	<i>Echinops telfairi</i>	GREHLARRTWSPAPRPSAAEQ VSGQP C QVLTEKK SR DRSSSDWRERRNAIR	225	
	<i>Equus caballus</i>	GQEHLIRRKWSLTSPSTDTQV PGRL C KVLTEKK PR RGRPSRDWRERRNAIQ	218	
	<i>Bos taurus</i>	GQEHLVRRKWSLTSPSPKPQ IPGQL C KVLTEKK PR KGRQSRDGRQRRNAIR	221	
	<i>Bos mutus</i>	GQEHLVRRKWSLTSPSPKAQ IPGQL C KVLTKKK PR KGRQSRDGRQRRNAIR	221	
	<i>Ovis aries</i>	GQEHLVRRKWSLTSPSPPEAQ IPGQR C KVLTEKK PR RGQASQDGRQRRSAIR	221	
	<i>Ailuropoda melanoleuca</i>	AQEHLVRRKWSLTSPSPTEAQV PGQL C QVLTEKK PR RGRPSQDWRERRNAIQ	220	
	<i>Ceratotherium simum</i>	GHEHLIRRKWSLTSPASTDAQ IPGQL C KVLTEKK PR RGKPLRDWRERRNAIR	215	
	<i>Loxodonta africana</i>	GREHLVRRKWSSTPGPSAEVQV PGQR C KVLTHKK SR KDRPSRDWRERRNAIR	225	
	<i>Odobenus rosmarus divergens</i>	AREHLVRRKWSLAPSPTEAQV PGQL C QVLTEKK PR KGRPSQDWRERRNAIQ	225	
	<i>Orcinus orca</i>	GQEHLIRRRWSLTSPSPAETQV SGQL C KVLTEKK PR RGWPSRDWRERRNAIR	221	
Non-mammals	<i>Myotis davidii</i>	GREHLIRRKWSLAPSPAQAQ APGPI C QVLTEKK PG KGGPSLDWRERRSAIR	225	
	<i>Columba livia</i>	GKEHFIRRRRSTKSSHPVKSRV PDEH C KVVAEKKR HK MMKSSDWRERRNAIR	225	
	<i>Meleagris gallopavo</i>	GKEHFIRRRRSTKSSSHLSK SHIPDEH C KVVAEKKR QK MMKSSDWRERRNAIR	225	
	<i>Anas platyrhynchos</i>	GKEHFIRRRRSTKPSHPMK SHIPDEH C KVVAEKKR QK MMKSSDWRERRNAIR	225	
	<i>Taeniopygia guttata</i>	GKEHFIRRRRSTKPGHPSWPQ GPEEH C KVVAEKKR QK KKSTSDWRERRNAIR	225	
	<i>Gallus gallus</i>	GKEHFIRRRRSTKSSSHLSK SHIPDEH C KVVAEKKR QK MMKSSDWRERRNAIR	215	
Non-mammals	<i>Anolis carolinensis</i>	GKEHFLRRKRSARSTRPARGV GEH C KVVAEKKR QK KKLTDDWRERRNAIR	221	

Fig. 6 Amino acid sequence alignment of the orthologues of Adamts17. The Adamts17 amino acid sequences from the mouse and human proteins are *boxed*. The protein alignment was obtained by BLAST hits with the mouse Adamts17 protein sequence using SIFT Blink (<http://sift.jcvi.org/>) (Ng and Henikoff 2003). The key amino acid residue (cysteine, C) in the “Cys-switch” domain is

shown in *bold* and *boxed*. The proline residues (P) in the orthologues that align with the amino acids at positions 196 and 208 in the mouse Adamts17 protein (B6) are shaded in *gray*. The amino acids residues (L196 and S208) that are conserved between 129P1 and other Adamts17 orthologues are *circled*

candidate is *Mboat4*, a gene that locates at 34.1 Mb nearby the QTL peak at 34.5 Mb. The *Mboat4* gene encodes a ghrelin *O*-acyl transferase (Goat). *Mboat4* contains two amino acid changes in the protein between B6 and 129P2, Ile31Val and Phe53Leu (B6, 129P1 or 129P2). Two additional SNPs were found in the U5 (5' untranslated) region and three others found in the U3 (3' untranslated) region of the *Mboat4* gene. Goat is known to activate ghrelin

resulting in increased food intake and adiposity (Tschop et al. 2000). Mice deficient for *Mboat4* are resistant to high fat-induced body weight gain and adiposity (Kirchner et al. 2009). *Mboat4* and *Znt7* KO mice present similar metabolic phenotypes in response to high fat-induced body weight gain and adiposity which make *Mboat4* a good candidate gene linking body weight and adiposity to zinc homeostasis.

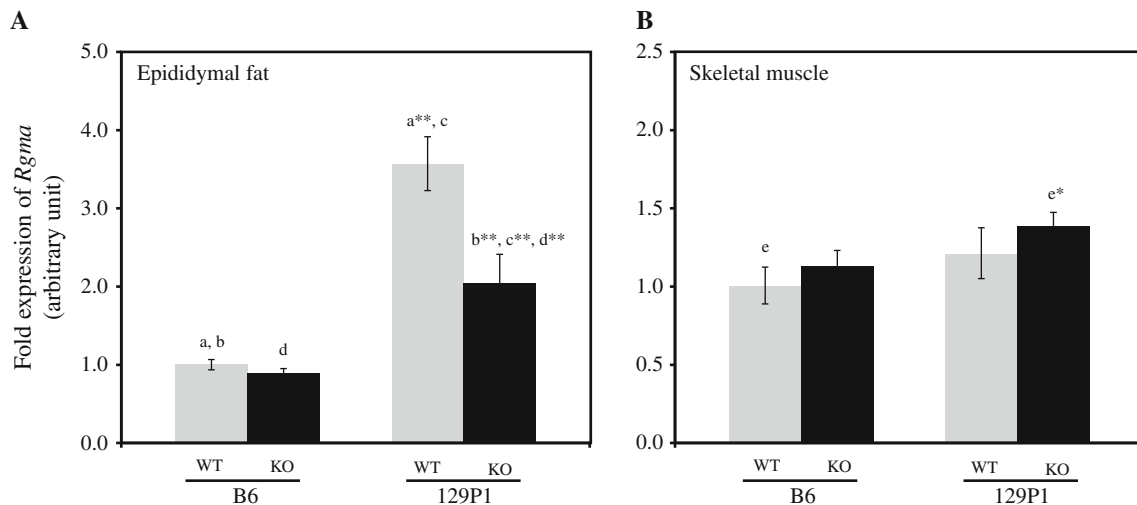


Fig. 7 mRNA expression of *Rgma* in epididymal fat and skeletal muscle. Total RNAs were purified from epididymal fat and skeletal muscle from F₂ *Znt7* KO ($n = 5-6$) and WT ($n = 5-6$) mice and subjected to quantitative RT-PCR (see “Materials and methods” section). The amount of *Rgma* transcripts was measured by a SYBR-based quantitative PCR method and normalized to the level of β -actin

mRNA expression. The *Rgma* expression (both in fat and muscle) in WT mice with the B6 *Rgma* allele was used as controls for calculation of relative expression (using the $\Delta\Delta C_T$ method) (Livak and Schmittgen 2001). Values are the mean $\pm$ S.E. Means with a common letter differ, * $p < 0.05$ and ** $p < 0.01$. WT wild type, KO *Znt7* knockout

In conclusion, we have identified QTLs on chromosomes 7 and 8 that may link body weight gain and/or fat accumulation to cellular zinc status. The QTL for body weight and fatness on chromosome 7 found in *Znt7* KO mice may uncover clues to understand how zinc metabolism affects growth and body adiposity. It will assist in positional cloning of the responsive gene or genes and understanding of the function of the responsive gene(s) at the crossroad between growth/adiposity and zinc metabolism. This report is the first to describe a genetic link between zinc status to growth and body fat accumulation.

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