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Expression of carnitine palmitoyl-CoA transferase-1B is influenced by a cis-acting eQTL in two chicken lines selected for high and low body weight

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Ka S, Markljung E, Ring H, Albert FW, Harun-Or-Rashid M, Wahlberg P, Garcia-Roves PM, Zierath JR, Denbow DM, Pääbo S, Siegel PB, Andersson L, Hallböök F. Expression of carnitine palmitoyl-CoA transferase-1B is influenced by a cis-acting eQTL in two chicken lines selected for high and low body weight. *Physiol Genomics* 45: 367–376, 2013. First published March 19, 2013; doi:10.1152/physiolgenomics.00078.2012.—Carnitine palmitoyl-CoA transferase-1B is a mitochondrial enzyme in the fatty acid oxidation pathway. In a previous study, *CPT1B* was identified as differentially expressed in the hypothalamus of two lines of chickens established by long-term selection for high (HWS) or low (LWS) body weight. Mammals have three paralogs (*CPT1a, b* and *c*) while nonmammalian vertebrates only have two (*CPT1A, B*). *CPT1A* is expressed in liver and *CPT1B* in muscle. *CPT1c* is expressed in hypothalamus, where it regulates feeding and energy expenditure. We identified an intronic length polymorphism, fixed for different alleles in the two populations, and mapped the hitherto missing *CPT1B* locus in the chicken genome assembly, to the distal tip of chromosome 1p. Based on molecular phylogeny and gene synteny we suggest that chicken *CPT1B* is pro-orthologous of the mammalian *CPT1c*. Chicken *CPT1B* was differentially expressed in both muscle and hypothalamus but in opposite directions: higher levels in hypothalamus but lower levels in muscle in the HWS than in the LWS line. Using an advanced intercross population of the lines, we found *CPT1B* expression to be influenced by a cis-acting expression quantitative trait locus in muscle. The increased expression in hypothalamus and reduced expression in muscle is consistent with an increased food intake in the HWS line and at the same time reduced fatty acid oxidation in muscle yielding a net accumulation of energy intake and storage. The altered expression of *CPT1B* in hypothalamus and peripheral tissue is likely to be a mechanism contributing to the remarkable difference between lines.

body weight; chromosomal mapping; fatty acid; genotyping; hypothalamus; malonyl-CoA; skeletal muscle; lipid metabolism; mitochondria; mRNA expression; pectoral muscle

CARNITINE PALMITOYL-COENZYME A TRANSFERASE-1 (CPT1; palmitoyl-CoA: L-carnitine *O*-palmitoyltransferase, EC 2.3.1.21) is a key enzyme in the control of fatty acid oxidation, which catalyzes the rate-limiting step of β-oxidation by translocating fatty acids across the mitochondrial membranes. Malonyl-coenzyme A (CoA), an intermediate in fatty acid synthesis, inhibits CPT1 action, thus blocking the oxidation of fatty acids during times of fatty acid synthesis and energy surplus (26). There are three mammalian CPT1 paralogs. *CPT1a* is predominantly expressed in liver, *CPT1b* in muscle, while *CPT1c* is mainly expressed in the brain (30). Only two paralogs have been described in chicken, denoted *CPT1A* and *B,* but only *CPT1A* is present in the chicken genome assembly (WASHUC2, May 2006), whereas *CPT1B* is only known from cDNA sequences (36). Malonyl-CoA as an intermediate metabolite in the fatty acid metabolism has recently been shown to be involved in hypothalamic regulation of energy expenditure and food intake (23, 27, 32, 40) and serves as an indicator of the energy status in hypothalamic neurons. Experimental manipulation of the malonyl-CoA system alters feeding behaviors (10, 17, 34). Although *CPT1c* is structurally similar to its mammalian paralogs *CPT1a* and *b*, it does not seem to catalyze the prototypical fatty acid transport (40). *CPT1c* is expressed in the mammalian hypothalamus, and knockout mice exhibit a marked reduction in food intake, lower body weight, and less body-fat content than their wild-type littermates. This knockout phenotype is consistent with a role for *CPT1c* as an energy-sensing malonyl-CoA target (40), which regulates energy homeostasis via the hypothalamus (20).

We have studied the chicken *CPT1A* and *B* in two chicken lines established by divergent selection for body weight at 56 days of age for >50 generations from a founder population of White Plymouth Rock chickens (8, 25, 35). The two populations, the high-weight (HWS) and the low-weight (LWS) selection lines, now have more than a ninefold difference in body weight at selection age. Several behavioral, metabolic, immunological, and endocrine differences between lines have evolved during the course of the selection experiment (5, 7, 18, 19). Among the correlated responses to the selection for body weight are differences in feeding behavior and food consumption (8). The involvement of the hypothalamus in these phenotypic differences was implied after lesioning of the ventromedial hypothalamus, which affected the feeding behavior (4). Interestingly, only minor differences were seen between lines when we analyzed the hypothalamic neuropeptide mediators of appetite such as the melanocortin receptors (13, 15), indicating

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that the behavioral alteration cannot be explained solely by changes in the hypothalamic neuropeptide regulatory system. Analysis of global gene expression in hypothalamus of the two lines have identified multiple differentially expressed genes in networks and processes regulating neuronal plasticity and development, DNA metabolism and repair, and induction of apoptosis (13). Although *CPT1B*, which is mainly expressed in muscle, was identified as one of the most differentially expressed genes in hypothalamus by the expression analysis, its contribution to the phenotypes of these lines could not be pursued until now because its chromosomal location was not known.

In this work we follow up on the observation that *CPT1B* was differentially expressed in our earlier array analysis of hypothalamus of the two selected lines (13). We confirmed that *CPT1B*, but not *CPT1A*, has higher expression levels in hypothalamic tissue from the HWS than from the LWS line. Interestingly, expression change in the opposite direction was found for *CPT1B* in muscle. These results suggested the hypothesis that the regulation of the expression of *CPT1B* has been a target for the divergent selection with differential *CPT1B* expression in the two lines as the result. We tested this by identifying a genetic polymorphism in the *CPT1B* gene that was first used to determine the hitherto unknown position of *CPT1B* in the chicken linkage map. The *CPT1B* polymorphism was then genotyped in an advanced F_9 intercross between the two chicken lines. Using body weight and *CPT1B* muscle expression data in the F_9 animals, we found that *CPT1B* expression is affected by an expression quantitative trait locus (eQTL). The data expose one possible regulatory mechanism that underlies the genetics of variable behaviors to satisfy the quest for energy and food in vertebrates and shed light on the molecular evolution and establishment of novel functions after gene duplications in this important gene family.

MATERIALS AND METHODS

Animals and tissues. The LWS and HWS lines were established from a common founder population generated by crosses among seven inbred lines of White Plymouth Rock chicken. The lines have been maintained as closed populations by continuous selection for high or low body weight at 56 days of age. Descriptions of the selection program and various correlated responses to the selection within the lines are provided elsewhere (8, 11). For example, each selected generation of the parental lines is hatched annually the first Tuesday in March, and dietary formulation has remained constant throughout. Feed and water were supplied ad libitum. Animal handling and care were performed according to the National Research Council publication *Guide for Care and Use of Laboratory Animals* and were approved by the Virginia Tech Institutional Animal Care and Use Committee. The experiment adheres to American Physiological Society's Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training.

HWS and LWS chickens from generation 45 (G45) were used for the quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis in peripheral and brain tissues. Five males and five females at 56 days (selection age) and three males and seven females at 4 days of age from each selected line and four White Leghorn chickens were used for tissue dissections. Liver, pectoral muscle, adipose tissue, total brain, brain regions according to Fig. 1 including hypothalamus, and a region containing diencephalon, mesencephalon, pons, and medulla (DMPM) were dissected at 56 days after hatch, immediately frozen in liquid nitrogen, and stored at −70°C until used.

A reciprocal advanced intercross population between HWS and LWS chickens was produced with the main purpose of identifying genes that explain the large difference in body weight and growth between the parental lines. The intercross was initiated from G41 of

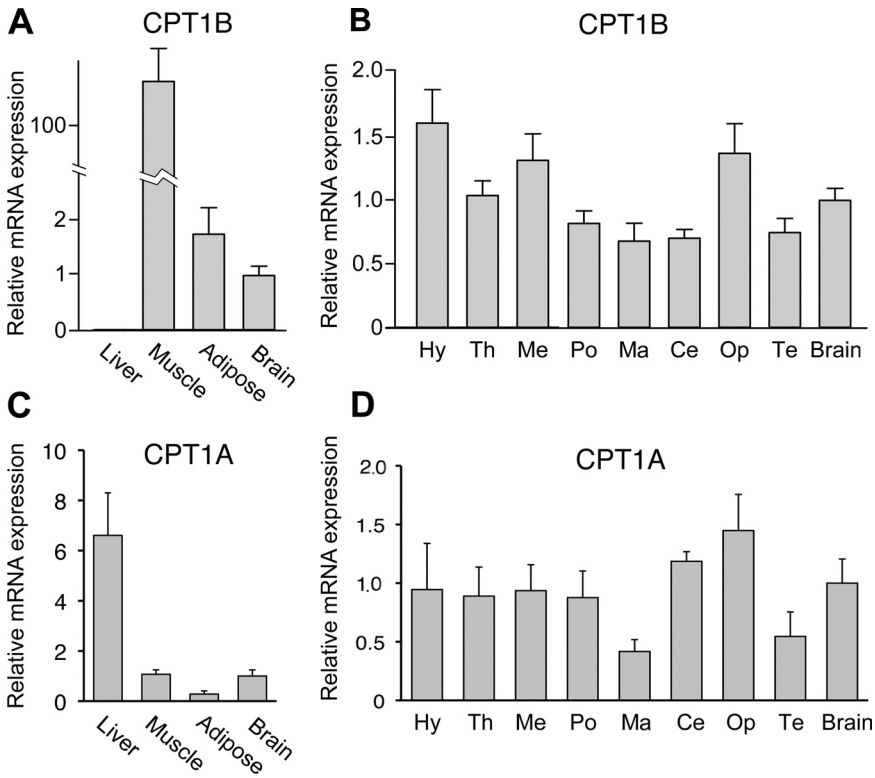

Fig. 1. Relative carnitine palmitoyl-coenzyme A transferase-1(*CPT1*)*A* and *CPT1B* mRNA levels. Analyses of relative mRNA levels in peripheral tissues and brain from White Leghorn chickens at 56 days of age by using qRT-PCR. Relative mRNA expression levels of *CPT1B* (*A, B*) and *CPT1A* (*C, D*) in liver, muscle, adipose tissues, whole brain, and in different brain regions. The average level in brain was arbitrarily set to 1 and used to relate the other samples to. Error bars ± SD, $n = 3$. Brain, total brain; Ce, cerebellum; Hy, hypothalamus; Ma, medulla; Me, mesencephalon; Op, optic tectum; Po, pons; Te, telencephalon; Th, thalamus.

the parental lines. Eight HWS males were mated to 22 LWS females, and 8 LWS males were mated to 19 HWS females to generate 150 F_1 progeny in 40 families. Thereafter 40 pair-matings with one male and one female from each family were done for each generation during nine generations (F_9 intercross) as in Ref. 29 to minimize genetic drift. The husbandry of the intercross was identical to that of Refs. 11, 28. Body weights at 56 days were recorded for all individuals, and 42 male and 38 female F_9 chickens from the advanced intercross were *CPT1B* genotyped. Pectoral muscle tissue from 28 females and 25 males was dissected for total RNA preparation and used for analysis of relative *CPT1B* mRNA levels.

RNA preparation and quantitative RT-PCR. Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA) purified with RNeasy mini kit (Qiagen, Valencia, CA), the quality was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and the RNA samples were frozen at −70°C until used. Complementary DNA synthesis from total RNA and two-step quantitative (q)PCR were performed as described (14). Random primers were used for first-strand cDNA synthesis (Superscript III RT, Invitrogen). Briefly, qRT-PCR was performed using the iQ SYBR Green supermix (Bio-Rad Laboratories) with MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). Primers were designed with Primer Express v2.0 (Applied Biosystems, Carlsbad, CA) with the default settings Tm 60°C, 50% G/C, amplicon size minimum 100 base pairs. Each primer sequence was BLASTed separately against GenBank and EMBL, and only primers with one perfect match within the target sequence and with the second best hit <75% identity were used. The primer pairs were checked for PCR efficiency, linear dynamic range, and specificity. To confirm identity of amplified PCR products, dissociation curve analysis and agarose gel electrophoresis were performed after analysis. The initial mRNA levels were first normalized to β-actin and TATA-box binding protein (TBP) mRNA levels to correct for variations in the cDNA syntheses and experimental procedure. We validated the use of β-actin and TBP for normalization purposes by testing for the most stable mRNA expressions of TBP, β-actin, β-2-microglobulin, and glyceraldehyde 3-phosphate dehydrogenase over the time points and tissues. The software geNorm (also included in Bio-Rad MyIQ software package) was used. Control reactions containing primers but without reverse transcriptase were analyzed in parallel. The data were analyzed by the $2^{-\Delta\Delta Ct}$ method (21). Analysis of variance (ANOVA) was used for the statistical analysis.

Phylogenetic and synteny analysis. Maximum likelihood phylogenetic trees were based on deduced amino acid sequences of all predicted and annotated *CPT1* family homologs available in Ensembl and constructed using the Ensemble on-line analysis software package Gene Tree. The reference tree is available on http://www.ensembl.org (GeneTree ENSGT00550000074345) (37). Syntenic regions in the chicken and human genome hosting CPT1 paralogs were identified by whole genome alignment using BlastZ-net/Lastz-net pairwise alignment analysis available in the Ensemble on-line analysis software package (16, 33).

Discovery of an intronic polymorphism and genotyping. A region from the end of exon 23 to the beginning of exon 24 (numbering based on human *CPT1B*) from two HWS and four LWS chickens was amplified and sequenced with primers CTP1ex23F and CPTex24R (Table 1), and an intronic polymorphism was identified. A genotyping assay for the two intronic sequence alleles was designed. We used a fluorescently labeled common forward primer located in exon sequence and two different reverse primers located in intronic sequence (Table 1), giving a four base-pair size difference between the two alleles (155 and 159 base pairs). The PCR reaction contained 0.2 μM of each primer and PCR Buffer II, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 U AmpliTaq GOLD polymerase (Applied Biosystems), and 40 ng of DNA. The PCR cycling conditions were 95°C for 5 min, 8 touchdown cycles 95°C 30 s, 65–58°C 30 s, 72°C 20 s, 40 cycles 95°C 15 s, 58°C 15 s, 72°C 20 s, and 72°C for 5 min. The difference in the fragment length was detected using a MegaBACE 1000 DNA Analysis System (Amersham Biosciences/GE Healthcare, Uppsala, Sweden) and analyzed with Genetic Profiler (GE Healthcare). We genotyped 47 parental animals (25 HWS and 22 LWS) and 10 F_1 animals to assess fixation of the alleles in the two chicken lines. We used this assay to genotype 80 individuals from the F_9 intercross, and a subset of F_2 individuals ($n = 108$) from a large intercross pedigree between the HWS and LWS lines (38) was used for linkage analysis to map *CPT1B* on the chicken linkage map. The analysis was carried out with the CRI-MAP software package as previously described (38). A genome-wide set of 432 markers previously scored in the pedigree was included in a two-point linkage analysis.

Western blotting. Pectoral muscle tissue was dissected, frozen on dry ice, and later homogenized in a lysis buffer containing 20 mM HEPES pH 7.4, 100 mM NaCl, 100 mM NaF, 1% NP-40, 1 mM Na_3VO_4, 5 mM EDTA, and 1× protease inhibitor cocktail (Halt Protease and phosphatase inhibitor cocktail, #78440; Thermo Scientific, Rockford, IL). The protein lysate was subjected to two cycles of freezing and thawing on dry ice to release the mitochondrial proteins. Protein concentrations were determined with the D_C Protein Assay

Table 1. *Antibodies and PCR primer sequences*

Antibody/Primer Name	Antigen, Species/Sequence	Catalog/Accession ID	Note
CPT1-M	carnitine palmitoyl transferase-1 muscle EC 2.3.1.21	CPT1M11-A Alpha Diagnostics	rabbit
NUO	NADH-ubiquinol oxidoreductase, (complex I) EC 1.6.5.3	A21344 Molecular Probes	mouse
SUO	succinate-ubiquinol oxidoreductase, (complex II) EC 1.3.5.1	A11142 Molecular Probes	mouse
Actin	β-isoform of actin	4970, Cell Signaling Technology	rabbit
chCPT1B.72F	TGCTGCTTCCAATTCGACG	DQ314726	qRT-PCR
chCPT1B.157R	TGCAGCGGCGATCTGAATG		
chCPT1A F	TCAACGAGTCAGACACCACAGC	NM_001012898	qRT-PCR
chCPT1A R	CCGTAACCATCATCAGCCACA		
β-Actin F	AGGTCATCACCATTGGCAATG	NM_205518	qRT-PCR
β-Actin R	CCCAAGAAAGATGGCTGGAA		
GAPDH F	GGGAAGCTTACTGGAATGGCT	NM_204305.1	qRT-PCR
GAPDH R	GGCAGGTCAGGTCAACAACA		
TBP F	TAGCCCGATGATGCCGTAT	NM_205103.1	qRT-PCR
TBP R	GTTCCCTGTGTCGCTTGC		
CPT1ex23F	TGATGACGTCGATTTCTGCT	DQ314726	intron amplification
CPT1ex24R	ACGCCTCGTAGGTCAGACAG		
F Fam	GATTTCTGCTGCTTCCAATTCGAC		genotyping
L_R	TACTCCATAGCCGCCCCATACTGT		genotyping
H_R	CCATAGCCGCCCCACACAGA		genotyping

F, forward; R, reverse primer.

(Bio-Rad). We separated 20 μg protein per sample on 10% denaturing SDS polyacrylamide-electrophoresis Mini-Protean TGX Precast gels (Bio-Rad) and electrophoretically transferred that to Immun-Blot PVDF membrane (Bio-Rad) with the recommended buffers. Nonspecific binding was blocked in 4% bovine serum albumin in TBST buffer (20 mM Tris, 140 mM NaCl, 0.05% Tween-20) for 1 h at room temperature. The membrane was incubated with primary antibody diluted in 1% bovine serum albumin in TBST at 4°C overnight. Primary antibodies are listed in Table 1. After washing the membrane, we incubated it with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) that were detected by chemiluminescence with the ChemiDoc MP Imaging System (Bio-Rad) and quantified with the software Image Lab (Bio-Rad) (9). Each sample was normalized to the actin level and related to the mean levels of the female LWS protein. The filter was stripped from primary antibodies using 100 mM glycine buffer, pH 2.5, overnight at 4°C and reprobed with a new primary antibody. The same filter was used for all four primary antibodies. ANOVA with Tukey's multiple-comparison post hoc test was used for the statistical analysis.

RESULTS

Organ-specific expression of CPT1B and CPT1A. Our earlier microarray analysis indicated that *CPT1B* is expressed in nervous tissue (13). Using qRT-PCR we re-examined the expression of *CPT1A* and *B* mRNA in peripheral and brain tissues from White Leghorn chickens. Expression of *CPT1B* mRNA in brain tissue was confirmed at levels similar to those in adipose tissue, but 100 times lower than in pectoral muscle. Background levels were seen in liver tissue (Fig. 1*A*). When brain regions from 1 wk old chicks were analyzed, the highest expression was found in hypothalamus, mesencephalon, and optic tectum (Fig. 1*B*). Levels of *CPT1A* mRNA were high in liver compared with the other analyzed tissues (Fig. 1*C*) and were not elevated in hypothalamus compared with the other brain regions (Fig. 1*D*).

Differential CPT1B mRNA expression between HWS and LWS chicken in muscle and hypothalamus. The overall relative *CPT1B* mRNA levels in muscle, liver, adipose, and brain tissue from the two selection lines were similar to those in White Leghorn chicken tissue. However, in a comparison of the two lines, the expression in muscle was significantly higher ($P < 0.01$) (adipose tissue borderline significantly higher, $P = 0.06$) in the LWS than HWS line (Fig. 2*A*). A differential hypothalamic *CPT1B* expression between the lines was initially suggested by one probe set in an Affymetrix array analysis (13)

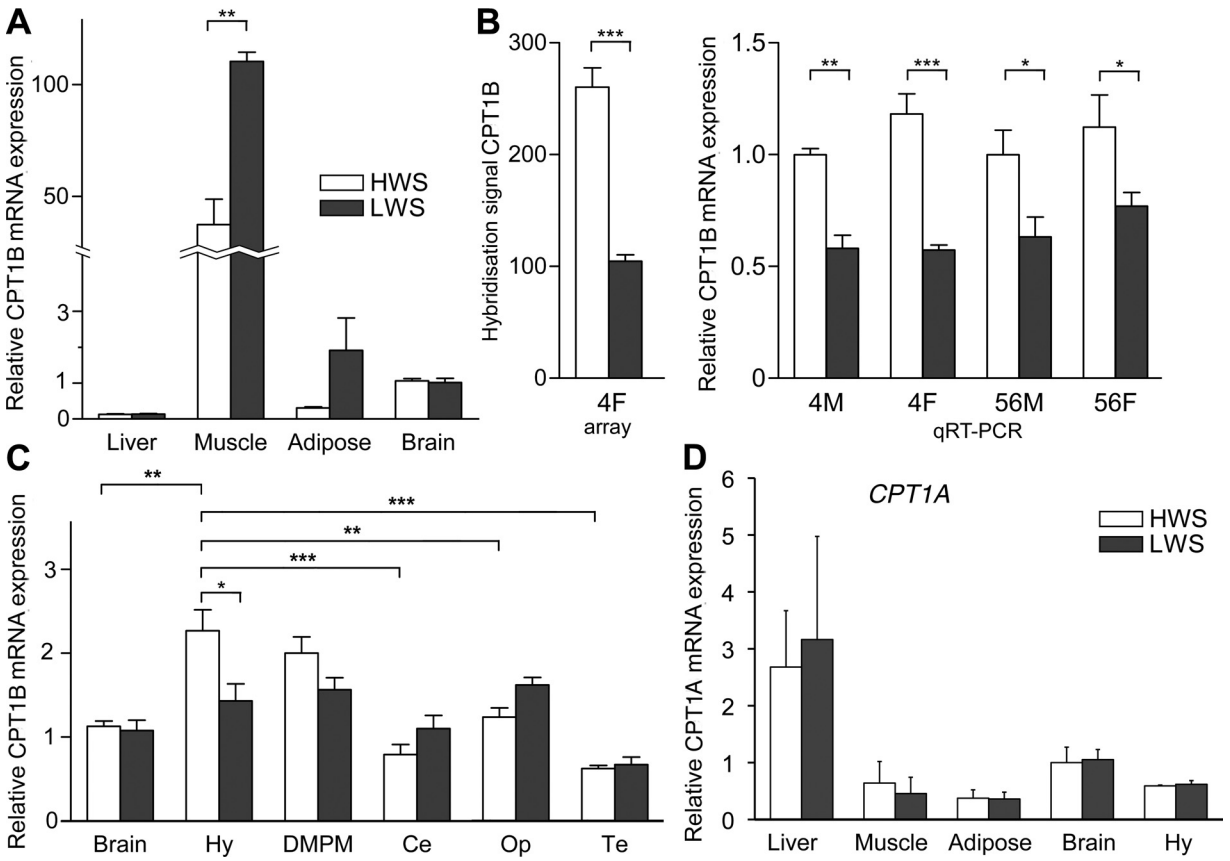

Fig. 2. Relative *CPT1B* and *CPT1A* mRNA levels in the high-weight selection (HWS) and low-weight selection (LWS) lines. Relative mRNA expression levels were analyzed in peripheral tissues and brain of HWS and LWS chickens. *CPT1B* mRNA expression in liver, muscle, adipose tissues and whole brain from 56-day-old individuals (*A*), in hypothalamus of 4 and 56 days of age individuals by using Affymetric array analysis or qRT-PCR analysis (*B*), and in different brain regions from HWS and LWS individuals at 56 days of age (*C*). *D*: *CPT1A* mRNA expression in liver, muscle, adipose tissues, whole brain, and hypothalamus from 56-day old male lines. The average level in brain (*A, C, D*) and in HWS hippocampus (*B*) was arbitrarily set to 1 and was used to relate other samples to. Error bars ± SD. One-way ANOVA with Tukey's multiple-comparison post hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 4M, 4-day-old males, $n = 3$ each line; 4F, 4-day-old females, $n = 7$ each line; 56M, 56-day-old males, $n = 5$ each line; 56F, 56-day-old females, $n = 5$ each line ($n = 4$ for each of the HWS and LWS individuals for the other experimental groups). DMPM, a brain region consisting of diencephalons, mesencephalon, pons, and medulla (including hypothalamus).

(probe ID: Gga.9299.1.S1_at, Reference sequence BU413970) having 99.9% sequence identity to the chicken *CPT1B* (accession number DQ314726) (Fig. 2*B*). This differential expression, with higher levels in the HWS than LWS line, was confirmed by qRT-PCR analysis in both hypothalamus from 4 and 56 days of age and from both sexes (Fig. 2*B*) but not in whole brain tissue, cerebellum, optic tectum, or in telencephalon (Fig. 2*C*). It is noteworthy that the differential *CPT1B* mRNA expression with high levels in the LWS line in muscle and adipose tissue was reversed in hypothalamus with significantly lower levels ($P < 0.05$) in the LWS than HWS line. Differential *CPT1A* expression was not seen (Fig. 2*D*).

Phylogeny and evolution of the vertebrate CPT1 gene family members. CPT1c has only been found in mammalian genomes. Using maximum likelihood molecular phylogeny analysis of all available CPT1 amino-acid sequences, we generated a tree in which CPT1c sequences formed a separate clade containing solely mammalian species. This clade was positioned inside the branch holding both mammalian and nonmammalian vertebrate CPT1B sequences. Both CPT1B- and CPT1A-like sequences were found in genomes throughout the vertebrate species (Fig. 3*A*).

Chicken HWS and LWS line-specific CPT1B DNA sequence polymorphism. The significant differential *CPT1B* expression between the lines implied that the expression level may have been targeted by the divergent selection for body weight and may be due to genetic changes at cis-acting and/or trans-acting regulatory elements. To distinguish between these possibilities, it is necessary to examine patterns of DNA polymorphism around the gene. However, the *CPT1B* gene has not been mapped, nor has the genomic sequence been retrieved in the chicken genome assembly. To find a polymorphism that could be used for genotyping and for linkage mapping of the chicken *CPT1B* locus we amplified intronic sequences. Primers based on the partial chicken *CPT1B* cDNA sequence located in adjacent exons deduced by the gene structure of orthologous genes were used, and only a few of several intron-spanning primer pairs gave a specific amplification product. We amplified a region from the end of deduced exon 23 to the beginning of exon 24 (numbering based on human *CPT1B*) from two HWS and four LWS chickens. Sequencing of the PCR product confirmed the identity of the sequence but revealed that the intronic sequence largely contained tandem repeats. Due to the highly repeated nature of the DNA sequence, only partial DNA sequence from the fragment could be obtained (data not shown). One sequence length polymorphism was found, and sequence of several individuals indicated that the HWS and LWS birds were homozygous for two different alleles. Using the MegaBACE 1000 DNA Analysis System we designed a genotyping assay that distinguished between the two alleles by PCR fragment length (155 and 159 base pairs) (Fig. 4*A*). Out of 47 tested, all HWS (25) and all LWS (22) birds were confirmed to be homozygous for different alleles, and 10 F_1

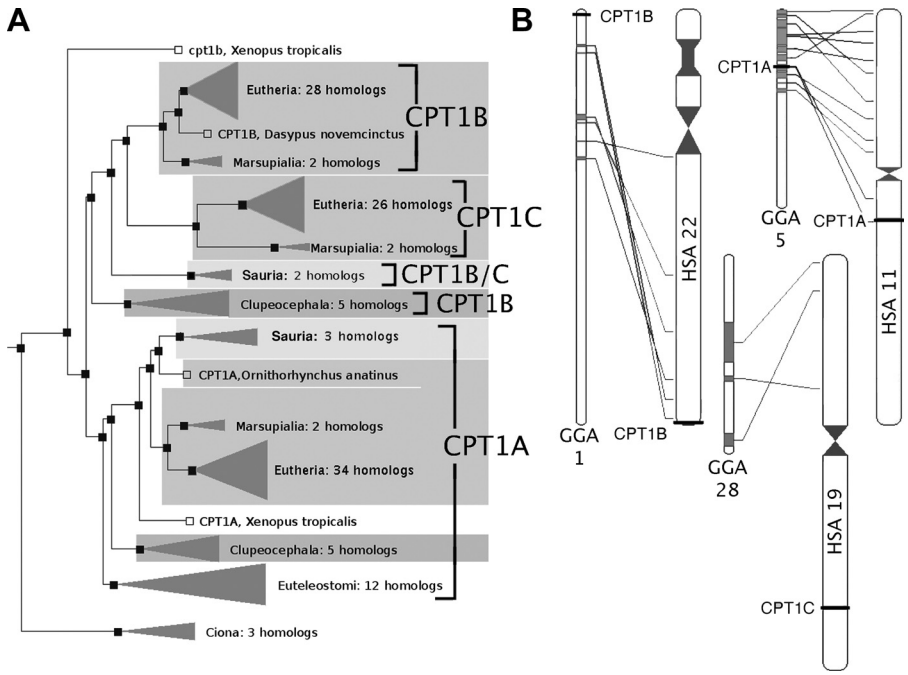

Fig. 3. Phylogeny and comparison of gene synteny in the chromosomal regions of human and chicken *CPT1* paralogs. *A*: schematic phylogenetic tree of *CPT1* homologs based on deduced amino acid sequences from the Ensembl database. The figure shows 1 branch within a large tree with all available homologs for enzymes catalyzing the reversible transfer of acyl groups from an acyl-CoA thioester to carnitine (GeneTree ENSGT00550000074345). The other gene families within this superfamily (*CROT, CRAT, ChAT*) form the out-group for this branch and are not shown. Clades are collapsed as indicated by dark triangles with relevant clade taxon name (http://www.ncbi.nlm.nih.gov/taxonomy) and the number of homologs. This maximum likelihood phylogenetic tree was generated with Gene Tree in the Ensemble on-line analysis software package (37). Mammals are divided in Eutheria and Marsupialia; Sauria contains birds and reptiles; Clupeocephalia and Euteleostei are bony fish. *B*: schematic diagram showing corresponding syntenic regions in the chicken and human genomes hosting *CPT1* paralogs. Orthology of some genes on chicken and human chromosomes is indicated by a connecting line. The regions were identified by whole genome alignment using BlastZ-net/Lastz-net pairwise alignment analysis, available in the Ensemble on-line analysis software package (16, 33). Note that there is no chicken homologous region of the region around the human *CPT1C*. Note also that the mapped chromosomal position of chicken *CPT1B* on GGA 1 is accordant with the predicted region based on conserved gene synteny. GGA, *Gallus gallus* chromosome; HSA, *Homo sapiens* chromosome.

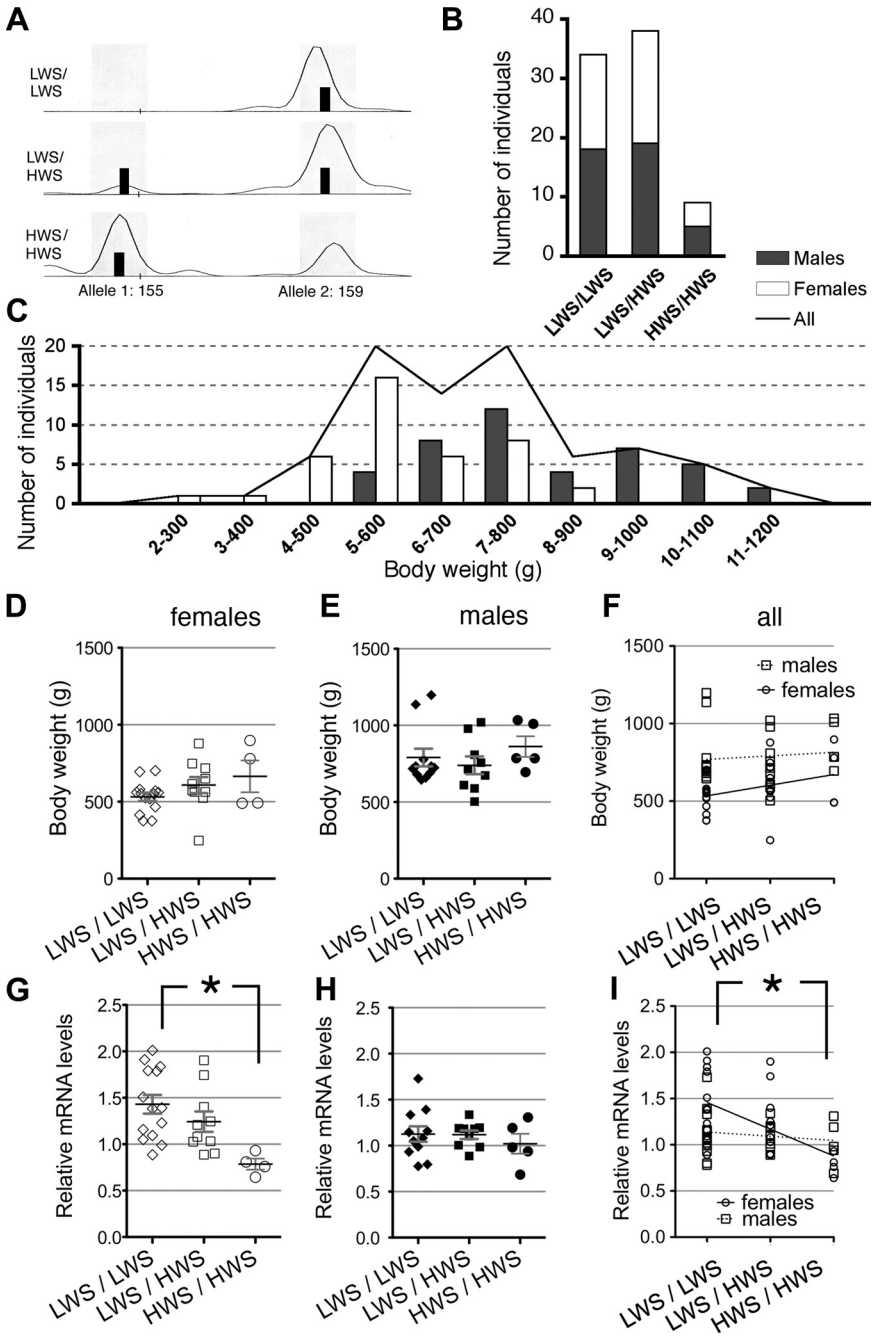

Fig. 4. *CPT1B* genotypes, body weight, and relative *CPT1B* mRNA muscle expression in F_9 intercross individuals. The F_9 chickens were genotyped using the MegaBACE 1000 DNA Analysis System, their body weight at 56 days of age was registered, and the relative *CPT1B* mRNA expression in pectoral muscle was analyzed. *A*: megabase chromatograms showing the 155 and 159 nucleotide length polymorphisms used in the genotyping assay. Examples of homozygous HWS, LWS, and heterozygous HWS/LWS individuals are shown. *B*: number of F_9 individual genotypes stratified for sex. *C*: the body weight distribution in the F_9 population stratified for sex. The individual body weight (*D–F*) and relative *CPT1B* mRNA expression (*G–I*) compared with the genotypes of the females (*D, G*), males (*E, H*), and all individuals (*F, I*). The average level in male homozygous HWS muscle (*H*) was arbitrarily set to 1 and used to relate other samples to. Means ± SE. One-way ANOVA with Tukey's post hoc test, $*P < 0.05$ (Table 3). Linear regression: slope is significantly nonzero in *F* (female body weight $P = 0.071$) and in *I* (female *CPT1B* expression $P = 0.004$).

animal were heterozygous, implying that the alleles were fixed or close to fixation in the two parental lines.

Assignment of CPT1B to the distal tip of chicken chromosome 1p. The genotyping assay was used for typing 108 individuals from a F_2 intercross pedigree between the HWS and LWS lines (38), and two-point linkage analysis revealed highly significant evidence for linkage between *CPT1B* and one marker *RBL438* (LOD score 28.0, recombination fraction 2.6%) located at the distal tip of chicken chromosome 1p at position 308,721 base pairs (Table 2). The order between *CPT1B* and *RBL438* in relation to the other markers could not be established due to the few recombination events between them and the long distance to the next markers.

The chromosomal position on the tip of chromosome *1p* was supported by comparison of gene synteny in the human and chicken genomes. Chicken and human orthologs of *CPT1B* are syntenic with several of the same genes on both chicken chromosome 1p and human chromosome 11 and *CPT1A* with genes on chicken chromosome 5 and human chromosome 22q. The human *CPT1c* gene is on chromosome 19 in a region without any clear homology to any region in the chicken genome (Fig. 3*B*).

Polymorphism and genotyping of the F_9 intercross. We genotyped 80 individuals of an F_9 advanced intercross of the HWS and LWS lines (Fig. 4*B*), previously developed for analyzing the contribution of gene regions to the phenotypes of

Table 2. *Linkage map for the distal tip of chicken chromosome 1p*

Locus	Position	
	Genome Assembly, bp	Linkage Map, cM
*CPT1B**		0.0
*RBL438**	308,721	2.6
rs14080307	681,331	41.1
rs13824562	2,371,934	47.5
rs13621730	6,050,972	54.2
rs14792587	15,878,686	58.2

*An inverted order of these markers was equally probable. LOD score is 28.03 for the distance between RBL438 and *CPT1b.*

the two lines. Body weight measurements and skeletal muscle tissue had been collected from each individual in the F_9 intercross. The body weight at 8 wk was 200–900 g in females and 600–1,200 g in males (Fig. 4*C*). Comparisons of HWS and LWS genotypes and body weights did not reveal any significant association even when stratified for sex (Fig. 4, *D–F*). A trend for association between lower body weight and the LWS genotype in females could be seen ($P = 0.06$, Fig. 4*F*). The relative *CPT1B* mRNA levels in pectoral muscle from the F_9 intercross individuals were analyzed by qRT-PCR and were compared with the genotypes of the individuals. *CPT1B* mRNA levels were significantly associated with genotype in females (Table 3). Although the association was significant when all individuals were taken into consideration, it was not when analyzed only for males (Fig. 4, *G–I*).

The association, after nine generations of intercrosses, between genotype (LWS *CPT1B* allele) and high mRNA levels in female pectoral muscle (Fig. 4*G*) showed that the *CPT1B* locus is linked to an eQTL for *CPT1B* expression in muscle in these lines. We could not analyze the expression in hypothalamus because hypothalamus was not sampled at the time when the F_9 intercross was collected. *CPT1A* mRNA expression was also analyzed, but no differential expression between genotypes in the F_9 intercross was seen (data not shown), in line with the observation of no difference between the lines (Fig. 2*D*). When we re-examined the QTL data from previous intercross analysis with respect to the markers on the tip of chromosome 1p with the *CPT1B* gene, there was no evidence for a significant QTL for growth that overlapped with the *RBL438* marker (11, 28, 38). This indicated that the *CPT1B* locus is not a major QTL for growth under the investigated experimental conditions.

Mitochondrial proteins in muscle of HWS and LWS lines. The differential expression of *CPT1B* mRNA in muscle suggested that mitochondria or mitochondrial biosynthesis may be different in the two lines and that differential mitochondrial function may be a contributing factor to the divergent line phenotypes. We analyzed the protein levels by Western blot of CPT1B, NADH-ubiquinol oxidoreductase, and succinate-ubiquinol oxidoreductase. NADH-ubiquinol oxidoreductase and succinate-ubiquinol oxidoreductase represent the mitochondrial oxidative chain complexes and are used to monitor the overall synthesis of mitochondrial proteins in a sample. No significant differences of the analyzed mitochondrial protein levels between genotypes were seen (Fig. 5, *A–D*), indicating that there are no gross differences in mitochondrial protein levels in pectoral muscle from the lines. However, borderline significantly differential *CPT1B* levels ($P = 0.054$) in female pectoral muscle supported the observed differential mRNA expression (Fig. 5, *A* and *D*).

DISCUSSION

The HWS and LWS lines are characterized by marked differences in growth, feeding behavior, food consumption, and a ninefold difference in body weight by 56 days. *CPT1B* was differentially expressed in the lines in both hypothalamus and muscle but with the opposite relation in the two tissues: lower levels in hypothalamus in the LWS line than HWS line, but higher in muscle in LWS than HWS line. The difference attracted our interest, and initially we hypothesized, based on the function of *CPT1B*, that expression differences could have direct contributions to the establishment of the phenotypes of the two chicken lines. *CPT1B* and *CPT1A* catalyze the rate-limiting step of β-oxidation by transporting fatty acids into mitochondria, a function that is essentially conserved throughout the vertebrate tree. The combination of low *CPT1B* expression in muscle that may reflect a low fatty acid β-oxidation, with high feed consumption both seen in the HWS line, could result in increased fat accumulation and a higher body weight. The reverse relationship, with high mitochondrial fatty acid metabolism and low appetite, would result in lean chickens with a low body weight as seen in the LWS chickens. The finding is fully consistent with a previous study showing that the LWS chickens have a higher rate of lipolysis than HWS chickens (5). The observation in this work that *CPT1B* is expressed in chicken hypothalamus and that the expression has been a subject of change in the LWS and HWS lines during selection for growth is intriguing. The *CPT1B* expression in chicken central nervous system (CNS) was not observed in previous studies (36). However, *CPT1B* expression has been recently reported in the brain of the sea bream, another non-mammalian vertebrate species (2).

Our results are consistent with reduced feeding in the *CPT1c* knockout mice and the proposed regulatory role for the mammalian brain-specific *CPT1c* gene in sensing energy status and regulating food intake (27, 30, 40). The function of *CPT1c* has been proposed to be a mammalian innovation and even to represent a gene neofunctionalization (30, 39). The observation that chicken *CPT1B* is expressed in both hypothalamus and muscle and that its expression is different between these lines implies that chicken *CPT1B* may have dual roles, the role both of mammalian *CPT1b* in muscle and that of *CPT1c* in hypothalamus. This would also imply that the proto-ortholog of *CPT1b* and *CPT1c* had roles in both CNS and muscle.

Table 3. *Least square mean analysis of CPT1B muscle expression in the F_9 intercross using a model with CPT1B genotype and sex*

Genotype	Sex	
	Males	Females
LWS/LWS	1.13 ± 0.07	1.43 ± 0.10*
LWS/HWS	1.11 ± 0.05	1.23 ± 0.11
HWS/HWS	1.02 ± 0.11	0.79 ± 0.06*

Means ± SE. *Female low weight selection (LWS)/LWS vs. female high weight selection (HWS)/HWS, $P = 0.012$. All other comparisons, not significant. One-way ANOVA, Tukey's post hoc test.

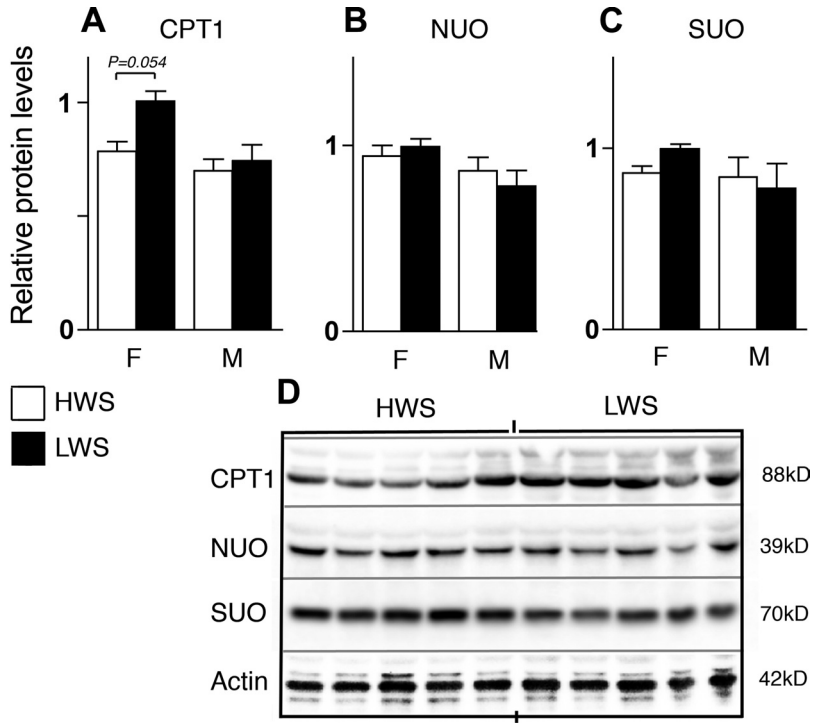

Fig. 5. Mitochondrial protein in muscle from HWS and LWS lines. Mitochondrial protein levels were analyzed by Western blot analysis of pectoral muscle tissues from HWS and LWS line female and males. Each sample was normalized to the actin level, and the levels relative to the mean levels of the female LWS protein for CPT1B (*A*), NADH-ubiquinol oxidoreductase (NUO, *B*), and succinate-ubiquinol oxidoreductase (SUO, *C*) are shown in the graphs stratified for sex. *D*: Western blot gel images for the female samples are shown. Means ± SE. One-way ANOVA with Tukey's post hoc test, $n = 5$. F, females; M, males; kD, kiloDalton.

Mammals have three *CPT1* paralogs, while nonmammalian vertebrates have two. Orthologs of *CPT1A* and *CPT1B* are found in genomes throughout the vertebrate tree, and phylogeny displays two monophyletic clades joined by a common root shared with agnathan and nonvertebrate *CPT1* homologs. *CPT1A* and *B* are both located in two well-characterized paralogons that were formed by the two total genome doublings at the base of the vertebrate tree (24). This strongly indicates that vertebrate *CPT1A* and *CPT1B* were generated by these genome doublings. All hitherto identified *CPT1c* orthologs form one clade within the branch with *CPT1B* sequences, separate from those of *CPT1A* (Fig. 3*A*), and since *CPT1c* only has been found in mammalian genomes, we conclude that *CPT1c* was formed by a duplication after the divergence of sauropsids including chicken but before the mammalian species radiation. Our data showing a resemblance between mammalian *CPT1c* and chicken *CPT1B* but not *CPT1A* in the mode of regulation also suggest that chicken *CPT1B* rather than *CPT1A* represents the proto-orthologous homolog to mammalian *CPT1c* (and *CPT1b*). Furthermore, when the expression of CPT1A and B was analyzed in hypothalamus, *CPT1B* but not *CPT1A* levels were elevated in hypothalamus compared with the other brain regions. We suggest that the gene function in the proto-orthologous *CPT1b/c* was divided on the mammalian *CPT1b* and *CPT1c* paralogs and is therefore an initial subfunctionalization event with a rapidly diverging *CPT1c* gene taking on the regulatory CNS functions but leaving the metabolic functions in muscle to the other paralog: *CPT1b*. We also suggest that the fixation of two such gene functions would have been accelerated by the presence of different regulatory genetic polymorphisms, similar to what have been suggested in this study.

The phenotypes of the HWS and LWS chicken are a result of selection at multiple loci across the genome. A recent genome-wide analysis of 60,000 single nucleotide polymorphisms in the lines identified >100 chromosomal regions that have been divergently selected for during the 50 generations after the start of the selection (12). The analysis was restricted to sequences present in the chicken genome assembly, and therefore the *CPT1B* region not analyzed. The distal portion of chicken chromosome 1p, which is present in the assembly and presumably is adjacent to the *CPT1B*-region, shows a small but significant elevation of the signal for divergent selection in the lines (12). This suggests that the marked allele frequency difference at the *CPT1B* locus between the lines is not a result of genetic drift during the development of the HWS and LWS lines. It was unexpected that the allele frequency of the *CPT1B HWS* allele had dropped from 0.5 in the F_1 generation to ~0.35 in the F_9 generation (Fig. 4*B*) as the design with 40 pair-matings in each intercross generation should reduce random genetic drift. However, at present we cannot exclude the possibility that this change in allele frequency is due to drift, but an alternative explanation would be that the HWS homozygotes have a lower viability or reduced fertility.

At least 13 QTL modifying growth have been detected (11, 28, 38). *CPT1B* was assigned by linkage analysis to the distal tip of chromosome 1p. The absence of a QTL for growth on the tip of chicken chromosome 1 shows that the *CPT1B* locus is not a major QTL for growth in these lines. However, there are several reasons why our QTL analysis may have failed to detect a QTL although it exists. First, it may be due to lack of power, although an F_2 generation of ~800 birds was used. Our previous studies have shown that a large number of QTL each with a rather small individual effect are underlying the dramatic phenotypic differences between the HWS and LWS lines. Second, epistatic interaction between QTL has contributed significantly to the selection response in these lines (6), making QTL detection even more challenging. Third, most F_2

individuals have an intermediate body weight, and it is possible that the *CPT1B* locus has a more pronounced effect at the more extreme body weights present in purebred HWS and/or LWS chickens. The preserved association between high *CPT1B* mRNA expression in pectoral muscle and the LWS genotype even after nine generations of intercrosses strongly suggests that the differential expression of *CPT1B* has a genetic component (Fig. 4*G*). We therefore conclude that the differential expression in muscle between lines most likely is caused by a cis-acting eQTL.

It is very unlikely that the line-specific intronic sequence polymorphism that was used for mapping and genotyping in this study is by itself the regulatory mutation. Repeated attempts were made to isolate and sequence other nonexonic regions of the gene but were not successful. The difficulties in amplifying and sequencing the intron sequence and the fact that the region containing *CPT1B* on the distal chromosome 1p is missing from the chicken genome assembly suggest that the region contains DNA with a repetitious nature that is difficult to sequence or even amplify. The poor coverage of this region may in fact have affected the result in the previously performed QTL analyses.

The gene structure of chicken *CPT1B* is not known, but other *CPT1* homologs all have a complex gene structures with >18 exons and alternative splicing in the 5′-end of the gene (42). Transcripts with different 5′-exons encoding different 5′-untranslated region (UTR) sequences indicate different transcriptional start sites and the existence of multiple regulatory elements that each separately may confer tissue-specific expression (http://www.ensembl.org; human *CPT1A* ENSG00000110090, *CPT1B* ENSG00000205560, *CPT1C* ENSG00000169169). The hypothalamic and muscle-specific chicken *CPT1B* expressions can therefore be regulated separately by unique regulatory elements that would allow for accumulation of cis- as well as trans-acting mutations with opposite effects in the two lines during the course of divergent selection. Such a mode of regulation is consistent with results showing that the human *CPT1c* 5′-UTR contains repressing sequences under the regulation of specific conditions of cellular energy availability (22).

Malonyl-CoA regulates lipid and glucose metabolism in skeletal muscle (3, 31). The levels of malonyl-CoA are physiologically regulated by fasting and feeding, and increasing amounts of evidence support a role for malonyl-CoA as a signaling mediator of energy balance in the CNS (10). Pharmacological inhibition of CPT1 in the CNS inhibits food intake, suggesting that malonyl-CoA may suppress food intake through a CNS-expressed CPT1 (27). A similar regulatory role in the chicken brain is suggested by the differential expression of *CPT1B* without a general change in mitochondrial biogenesis and regulation at 56 days as indicated by similar levels of NADH-ubiquinol oxidoreductase and succinate-ubiquinol oxidoreductase in muscle in the lines (Fig. 5). The AMP-activated protein kinase modulates the malonyl-CoA concentrations by regulating Acetyl-CoA carboxylases and is thereby linked via the malonyl-CoA signaling mechanism to the role of CPT1 (1). In a recent experiment, food intake was studied after stimulation or inhibition of the AMP-activated protein kinase in the chicken lines used in this study (41). The result showed differences between the lines in the responses after the activity of the AMP-activated protein kinase was manipulated. The differential *CPT1B* expression identified in this study may be one of the factors that contribute to these different responses.

We propose that altered regulation of *CPT1B* expression in both hypothalamus and peripheral tissue is one important mechanism underlying the remarkable differences in appetite, fat deposition, and growth between the HWS and LWS lines. This is supported by the findings that *1*) the two lines are fixed or nearly fixed for different *CPT1B* alleles and *2*) a cis-acting eQTL affects *CPT1B* expression at least in muscle. Our genetic data and expression data suggest that both cis-acting and trans-acting factors contribute to the differential *CPT1B* expression in these lines. The increased expression of *CPT1B* in hypothalamus and its reduced expression in muscle are consistent with increased appetite and food intake in the HWS line and at the same time reduced fatty acid oxidation in muscle, yielding a maximum net accumulation of energy intake and storage. This inverse regulation of *CPT1B* expression, uncovered by the divergent selection for growth, may constitute a mechanism that promotes fast accumulation of energy stores by combining large appetite and low fatty acid oxidation and metabolism in the peripheral tissues. In nature this kind of regulation may have been favorable for animals when efficient and fast accumulation of sufficient energy stores is required, for example during preparations for winter hibernation or long-distance migration that are performed by many birds every year. The normal satiety mechanisms should in such cases be set out of play, in order for the individual to collect sufficient reserves of energy. The results presented in this study thus uncover one link in the complex network that regulates energy homeostasis and appetite, which underlies the genetic foundation for our variable responses that drive behaviors to satisfy our quest for energy and food.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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