

Supplementary information

Obesity resistance and increased hepatic expression of catabolism-related mRNAs in *Cnot3*^{+/-} mice

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Supplementary materials and methods

Generation of *Cnot3*-deficient mice

To delete the *Cnot3* gene in mice, we used a 13.1-kb DNA sequence containing exons 1-9 of the mouse gene (Lambda FixII Library; Stratagene). A 9.2-kb genomic fragment, a 2.4-kb genomic fragment, and a 1.5-kb genomic fragment from the mouse phage clone were subcloned for the 5'homology arm, conditional knockout region, and 3' homology arm, respectively (Figure S3). A PGK-*neo* cassette flanked by two loxP sites was inserted in the intron preceding exon 2 and used as a positive selection marker and a thymidine kinase (TK) expression cassette was included at end of the 3' arm for negative selection.

E14 mouse embryonic stem (ES) cells were cultured under standard conditions on a feeder layer of mitotic inactivated G418-resistant primary embryonic fibroblasts. The targeting vector was linearized using the single NotI site and electroporated into 1.0×10^7 E14 ES cells. The cells were cultured in the presence of G418 and 192 ES clones were selected for primary Southern blot analysis using a 0.5 kb 3' external probe and HindIII digestion of genomic ES DNA. Positive clones were identified by the presence of hybridization bands of both 12.8 kb and 5.2 kb. The hybridization band of 5.2 kb, corresponding to the homologous recombination allele, was detected in nine clones. Additional Southern blot analysis using a 0.5 kb 5' external probe confirmed the correct targeting events. With the 5' probe, positive clones were identified by the presence of hybridization bands of both 10.3. kb and 12.5 kb for NheI digested genomic DNA. Two correctly targeted ES clones were used for microinjection. C57BL/6 female mice were used as blastocyst donors for microinjection. The highest percentage male chimeras were bred with C57BL/6J female mice (CLEA Japan inc.) to produce germ line-transmitted heterozygous mice. The F1 *Cnot3*^{+/*neo*} mice carrying the targeted allele were bred with cytomegalovirus promoter-induced *cre*-transgenic mice

(CMV-*Cre*). From this mating we obtained mice with *Cnot3* alleles that had lost both the *neo* cassette and exons 2-9 but retained a single loxP site. *Cnot3*^{+/-} mice were identified by Southern Blot analysis of genomic DNA using the 5' probe. *Cnot3*^{+/-} F2 mice were backcrossed to the C57BL/6J background for at least seven generations.

Supplementary figure legends

Supplementary Figure S1 Generation of the *Cnot3* haplodeficient mice and reduced tissue weights of almost all organs in *Cnot3*^{+/-} mice. **(A)** Scheme of the targeting construct design and simplified restriction map of the *Cnot3* locus. *loxP* sequences are indicated by triangles. Negative (*HSV-TK*) and positive (*PGK-neo*) selection markers are indicated by gray boxes (*TK*, thymidine kinase; *neo*, neomycin). The numbered boxes represent exons in the *Cnot3* gene. Black boxes indicate ORFs and white boxes indicate UTRs. The 5' and 3' probes used in the Southern blot analysis are also indicated. A mouse genomic clone containing exons 1-10 was used to construct the targeting vector. Homologous recombination produced the *Cnot3 neo* allele. Mating with female *CAG-Cre* mice produced the *Cnot3 null* allele. **(B)** Southern blot analysis of genomic DNA from mice with the indicated *Cnot3* genotypes. **(C)** PCR genotyping of embryos. The positions of the oligonucleotide primers are indicated by arrows (Supplementary Figure S1A).

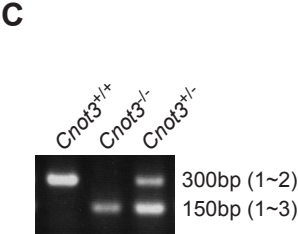
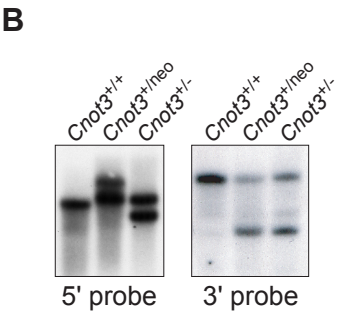
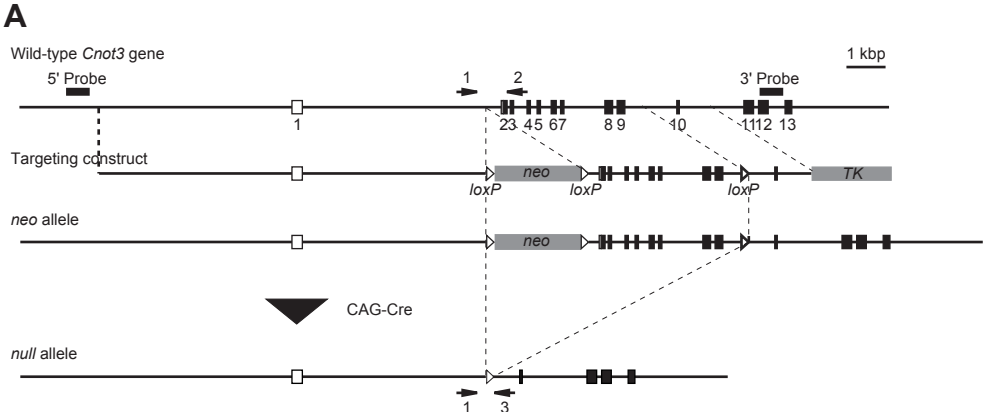
Supplementary Figure S2 Reduced weights of all organs in *Cnot3*^{+/-} mice. Tissue weights of the indicated organs were measured. *n* = 8-10. All values represent means ± s.e.m. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Open columns indicate wild-type mice and filled columns indicate *Cnot3*^{+/-} mice.

Supplementary Figure S3 The expression level of *Cnot3* mRNA is little altered under fasting conditions. **(A)** mRNA levels of *Cnot3*, *Cnot6L*, and *Cnot7* were measured by real-time PCR of the liver from *ad libitum*-fed, 24-hr fasted, and 24-hr refed mice. All values represent means ± SEM. **(B)** Representative northern blots of *Cnot3*, *Cnot6L*, and *Cnot7* mRNA in the liver of *ad libitum*-fed and 24-hr fasted mice. Ethidium bromide staining of the

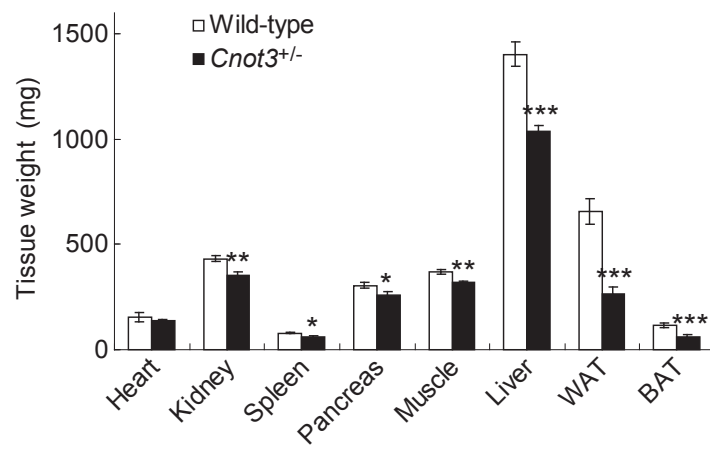
RNA was shown as a loading control.

Supplementary Figure S4 Western blot of IGFBP1 and α -Tubulin proteins from the livers of wild-type and Cnot3^{+/-} mice under ad libitum feeding or 24-hr fasting conditions. It may be worthy to note that transcriptional upregulation of IGFBP1 upon fasting has been reported.

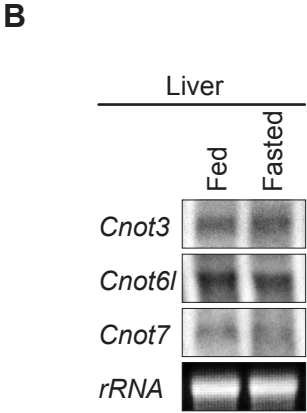
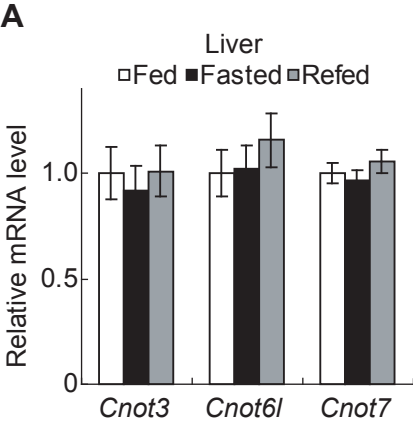
Supplementary Figure S1. Morita et al.



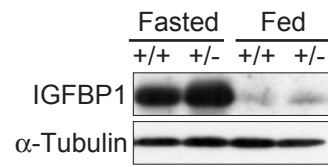
Supplementary Figure S2. Morita et al.



Supplementary Figure S3. Morita et al.



Supplementary Figure S4. Morita et al.



Supplementary tables

Supplementary Table S1 *Cnot3*^{-/-} mice died at an early embryonic day. Genotyping of embryos and offspring was performed by PCR. Results obtained for the *Cnot3*^{+/-} mouse crosses are shown.

Supplementary Table S2 List of up- or down-regulated genes in *Cnot3*^{+/-} mice. For microarray analysis, RNAs were isolated from livers of 12-week-old wild-type and *Cnot3*^{+/-} mice. The genes whose expression is altered more than 1.5-fold were listed. *n* = 2 for each genotype.

Supplementary Table S3 List of overlapping genes up-regulated in *Cnot3*^{+/-} mice and up-regulated in fasted mice. For microarray analysis, RNAs were isolated from livers of 8-week-old fed wild-type, fasted wild-type, and fasted *Cnot3*^{+/-} mice. The genes whose expression is altered more than 1.5-fold were listed. *n* = 2 for each condition.

Supplementary Table S4 List of the rich sequences in the 3'UTR among identified 68 CNOT3-regulated mRNAs. To find the consensus sequences among 68 mRNAs listed in Table S3, The 3'UTR regions of 68 mRNAs were computationally extracted and the number of each sequence were counted and listed.

Supplementary Table S5 Primer sequences used for real-time PCR.

Supplementary Table S1 *Cnot3*^{-/-} mice died at an early embryonic day

| Stage | <i>Cnot3</i> ^{+/+} | <i>Cnot3</i> ^{+/-} | <i>Cnot3</i> ^{-/-} | Total |
|------------------------|-----------------------------|-----------------------------|-----------------------------|-------|
| Postnatal (3-week-old) | 25 | 41 | 0 | 66 |
| Embryo (E9.5) | 6 | 13 | 0(1) | 20 |
| Embryo (E8.5) | 4 | 10 | 0(4) | 18 |

(Growth was retarded)

Supplementary Table S3 List of overlapping genes up-regulated in *Cnot3*^{+/-} mice and up-regulated in fasted mice.

| Probe Set ID | Symbol | Name | Gene ID | Hetero fasted vs | WT fed vs WT fasted |
|--------------|---------------|--|---------|------------------|---------------------|
| 1415996_at | Txnip | thioredoxin interacting protein | 56338 | 1.69 | -3.35 |
| 1416250_at | Btg2 | B-cell translocation gene 2, anti-proliferative | 12227 | 1.91 | -2.93 |
| 1416332_at | Cirbp | cold inducible RNA binding protein | 12696 | 1.84 | -1.51 |
| 1417017_at | Cyp17a1 | cytochrome P450, family 17, subfamily a, polypeptide 1 | 13074 | 1.75 | -4.78 |
| 1417065_at | Egr1 | early growth response 1 | 13653 | 2.08 | -8.26 |
| 1417268_at | Cd14 | CD14 antigen | 12475 | 1.57 | -1.76 |
| 1417273_at | Pdk4 | pyruvate dehydrogenase kinase, isoenzyme 4 | 27273 | 1.63 | -2.60 |
| 1417851_at | Cxcl13 | chemokine (C-X-C motif) ligand 13 | 55985 | 2.20 | -5.23 |
| 1417852_x_at | Clca1 | chloride channel calcium activated 1 | 12722 | 1.82 | -2.07 |
| 1418328_at | Cpt1b | carnitine palmitoyltransferase 1b, muscle | 12895 | 1.59 | -1.97 |
| 1418826_at | Ms4a6b | membrane-spanning 4-domains, subfamily A, member 6B | 69774 | 2.13 | -1.55 |
| 1418918_at | Igfbp1 | insulin-like growth factor binding protein 1 | 16006 | 1.85 | -3.90 |
| 1419144_at | Cd163 | CD163 antigen | 93671 | 2.09 | -1.63 |
| 1419665_a_at | Nupr1 | nuclear protein 1 | 56312 | 1.65 | -1.50 |
| 1420249_s_at | Ccl6 | chemokine (C-C motif) ligand 6 | 20305 | 1.93 | -1.78 |
| 1420447_at | Sult1e1 | sulfotransferase family 1E, member 1 | 20860 | 6.99 | -2.01 |
| 1420697_at | Slc15a3 | solute carrier family 15, member 3 | 65221 | 1.66 | -1.55 |
| 1420965_a_at | Enc1 | ectodermal-neural cortex 1 | 13803 | 1.74 | -3.85 |
| 1421349_x_at | Cend1 | cell cycle exit and neuronal differentiation 1 | 57754 | 3.00 | -1.87 |
| 1422557_s_at | Mt1 | metallothionein 1 | 17748 | 1.91 | -5.94 |
| 1422804_at | Serpib6b | serine (or cysteine) peptidase inhibitor, clade B, member 6b | 20708 | 1.64 | -1.81 |
| 1425225_at | Fcgr4 | Fc receptor, IgG, low affinity IV | 246256 | 1.60 | -1.62 |
| 1425644_at | Lepr | leptin receptor | 16847 | 2.34 | -2.66 |
| 1425875_a_at | Lepr | leptin receptor | 16847 | 1.74 | -2.26 |
| 1425979_a_at | Fbf1 | Fas (TNFRSF6) binding factor 1 | 217335 | 1.75 | -1.75 |
| 1426037_a_at | Rgs16 | regulator of G-protein signaling 16 | 19734 | 1.64 | -3.56 |
| 1426787_at | Sfi1 | Sfi1 homolog, spindle assembly associated (yeast) | 78887 | 1.57 | -1.62 |
| 1427104_at | Zfp612 | zinc finger protein 612 | 234725 | 1.62 | -2.17 |
| 1428024_at | Map1c3b | CDNA clone IMAGE:5149237 | 67443 | 1.60 | -1.76 |
| 1428083_at | 2310043N10Rik | RIKEN cDNA 2310043N10 gene | 66961 | 1.81 | -6.04 |
| 1428306_at | Ddit4 | DNA-damage-inducible transcript 4 | 74747 | 2.75 | -2.16 |
| 1428942_at | Mt2 | metallothionein 2 | 17750 | 3.42 | -4.28 |

| | | | | | |
|--------------|---------------|---|--------|------|-------|
| 1429157_at | Hhipl2 | hedgehog interacting protein-like 2 | 78772 | 1.50 | -1.93 |
| 1430377_at | 1300015D01Rik | RIKEN cDNA 1300015D01 gene | 74161 | 1.92 | -4.10 |
| 1430964_at | 2310034O05Rik | RIKEN cDNA 2310034O05 gene | 69571 | 2.51 | -2.97 |
| 1433073_at | 4933425E08Rik | RIKEN cDNA 4933425E08 gene | 71127 | 1.55 | -2.12 |
| 1433837_at | 8430408G22Rik | RIKEN cDNA 8430408G22 gene | 213393 | 1.55 | -2.25 |
| 1435525_at | Kctd17 | potassium channel tetramerisation domain containing 17 | 72844 | 1.93 | -1.98 |
| 1436544_at | Atp10d | ATPase, class V, type 10D | 231287 | 1.96 | -1.62 |
| 1437021_at | Arl13b | ADP-ribosylation factor-like 13B | 68146 | 1.76 | -1.73 |
| 1437751_at | Ppargc1a | peroxisome proliferative activated receptor, gamma, coactivator 1 alpha | 19017 | 1.58 | -1.81 |
| 1438194_at | 2900019G14Rik | RIKEN cDNA 2900019G14 gene | 72932 | 1.97 | -1.57 |
| 1439342_at | Clpx | ClpX protein | 270166 | 1.79 | -1.76 |
| 1440443_at | E030016H06Rik | RIKEN cDNA E030016H06 gene | 402722 | 4.14 | -1.54 |
| 1442406_at | 9230104K21Rik | RIKEN cDNA 9230104K21 gene | 77688 | 1.63 | -1.71 |
| 1443969_at | Irs2 | insulin receptor substrate 2 | 384783 | 1.88 | -1.57 |
| 1446316_at | Lpin2 | Lipin 2, mRNA (cDNA clone MGC:46819 IMAGE:5101211) | 64898 | 3.45 | -1.95 |
| 1447670_at | Psmc9 | proteasome (prosome, macropain) 26S subunit, non-ATPas | 67151 | 2.43 | -1.72 |
| 1448162_at | Vcam1 | vascular cell adhesion molecule 1 | 22329 | 1.88 | -2.71 |
| 1448272_at | Btg2 | B-cell translocation gene 2, anti-proliferative | 12227 | 1.64 | -2.78 |
| 1449106_at | Gpx3 | glutathione peroxidase 3 | 14778 | 1.82 | -2.11 |
| 1449498_at | Marco | macrophage receptor with collagenous structure | 17167 | 1.58 | -2.02 |
| 1449846_at | Ear2 | eosinophil-associated, ribonuclease A family, member 2 | 13587 | 1.62 | -1.56 |
| 1449901_a_at | Map3k6 | mitogen-activated protein kinase kinase kinase 6 | 53608 | 1.51 | -2.16 |
| 1449907_at | Bcmo1 | beta-carotene 15,15'-monooxygenase | 63857 | 1.61 | -1.91 |
| 1450018_s_at | Slc25a30 | solute carrier family 25, member 30 | 67554 | 1.69 | -2.17 |
| 1450063_at | Fmn2 | formin 2 | 1E+08 | 2.10 | -1.55 |
| 1450505_a_at | Fam134b | family with sequence similarity 134, member B | 66270 | 2.20 | -1.70 |
| 1450790_at | Tg | thyroglobulin | 21819 | 2.45 | -1.64 |
| 1451798_at | Il1rn | interleukin 1 receptor antagonist | 16181 | 1.88 | -6.37 |
| 1453707_at | 4930480G23Rik | RIKEN cDNA 4930480G23 gene | 74988 | 1.72 | -2.12 |
| 1453851_a_at | Gadd45g | growth arrest and DNA-damage-inducible 45 gamma | 23882 | 2.06 | -3.44 |
| 1454078_a_at | Gal3st1 | galactose-3-O-sulfotransferase 1 | 53897 | 1.69 | -1.73 |
| 1455434_a_at | Ktn1 | kinectin 1 | 16709 | 1.52 | -2.01 |
| 1456156_at | Lepr | leptin receptor | 16847 | 2.57 | -2.34 |
| 1456395_at | Ppargc1a | peroxisome proliferative activated receptor, gamma, coactivator 1 alpha | 19017 | 1.95 | -1.57 |

| | | | | | |
|--------------|------------|--|--------|------|-------|
| 1458469_at | Cblb | Casitas B-lineage lymphoma b | 208650 | 2.85 | -1.52 |
| 1458701_at | Prei4 | preimplantation protein 4 | 74182 | 4.83 | -1.77 |
| 1459424_at | D3Ertd108e | DNA segment, Chr 3, ERATO Doi 108, expressed | 51914 | 1.74 | -1.85 |
| 1460259_s_at | Clca1 | chloride channel calcium activated 1 | 12722 | 1.85 | -1.53 |

Supplementary Table S5 Primer sequences used for real-time PCR

| | Forward primer | Reverse primer |
|---------|--------------------------|---------------------------|
| Pdk4 | TTTTGCATTGTAGATGTTGTCCTT | TCAACCAATGTGGGAGTCAA |
| Lepr | GTTCCAAACCCCAAGAATTG | GACTTCAAAGAGTGTCCGTTCTC |
| Irs2 | GTCCAGGCACTGGAGCTTT | GCGCTTCACTCTTTCACGA |
| Cpt1b | GCCCATGTGCTCCTACCA | CTCTGAGAGGTGCTGTAGCAAG |
| Hsd17b6 | CAGTAAGCCTGTCCTGAGAGTTC | GTCTGTCCTGGAAAGCCTTG |
| Aldh1b1 | CGGAGAACGCTGATACTAGAATTT | AGTCGGGCAGTCAGCATC |
| Ucp2 | AGCCTGAGACCTCAAAGCAG | CCTTCAATCGGCAAGACG |
| Pgc1a | GAAAGGGCCAAACAGAGAGA | GTAATCACACGGCGCTCTT |
| Soat1 | CGCTATGCAGTTTTTACAGGTG | GGGCACAGAGTCTCTCAAAGAT |
| Igf1bp1 | TTCAGCTCCCAGCATGAAG | GGGCTCTCAGAAAGCTCATC |
| Hprt | TCCTCCTCAGACCGCTTTT | CCTGGTTCATCATCGCTAATC |
| Cnot3 | AGAGGCCGATCTACAGATAGTGA | GACAGGCTTGGAGCCATTT |
| Cnot6l | TCATGCTTGACAATCTTGCAAG | GGTCTCGTTCTTTTAATGTAATCCA |
| Cnot7 | CCTCATGAAGAGCTGCAAAA | GAGTCAGATCCTGCCTGGTG |