Supplemental Methods

Generation of constitutive *ET-2* null mice

A Lamda FIX II mouse 129/SV genomic library (Stratagene) was screened with a 0.56 kb fragment of the mouse *ET-2* cDNA. A 17.5 kb genomic clone containing all five exons of the ET-2 gene and about 4 kb of upstream genomic sequences was selected and was subcloned into either pUC119 or Stratagene KS+ vector. To construct the targeting vector, a universal neo-TK template plasmid vector that contained a neo and two tandem TK cassettes was used (1). A 1 kb Bst XI-EcoR1 genomic fragment containing a portion of exon 1 was blunt-end inserted into the unique Xho I site of the plasmid between the neo and TK cassette. A 11.5 kb BstXI-Sal I genomic fragment containing exon 3, 4 and 5 was then blunt-end ligated into the unique BamHI site of the plasmid. This arrangement deletes a portion of exon 1 and all of exon 2 of the ET-2 gene replacing it with the neo cassette. The construct was linearized at its unique Sal I site immediately 3' to the TK cassettes. The SM-1 mouse ES cell line was cultured on irradiated LIF-containing STO feeder layers as described (1). ES cell were electroporated with the linearized targeting vector and selected for double resistance to G418 and FIAU as described (2). Double-resistant ES cell clones were screened by PCR for correct homologous recombination and reconfirmed by Southern blot analysis for a single insertion event with a fragment of the Neo cassette and additionally with 5' ET-2 genomic fragments. Microinjection of blastocysts and production of

chimeric mice were performed as described (2). Global *ET-2* knockout mice were maintained on 129/SV genetic backgrounds.

Generation of conditional *ET-2* null mice

Floxed *ET-2* mice were obtained from Dr. Jeremy Nathans (Johns Hopkins University, Baltimore, MD). These mice contain *loxP* sites inserted 160 bp upstream and 850 bp downstream of exon 2, the exon coding for the mature ET-2 peptide and G418 resistance marker PGK-neo flanked by *Frt* sites at immediately downstream of this second *loxP* site for selection in ES cells and then eventual excision with Flp recombinase. Mice carrying two floxed *ET-2* alleles were born at the expected Mendelian frequency, viable, fertile and showed no pathological phenotype. *ET-2*$^{f/f}$ mice were crossed with transgenic mice carrying Cre recombinase to generate mice heterozygous for both alleles. The second cross between *ET-2*$^{f/f}$ mice and heterozygous mice from the first cross produced *ET-2*$^{f/f}$; promoter-Cre/0 mice, which were used for further experiments. For systemically inducible *ET-2* null mice, *ET-2*$^{f/f}$ mice were crossed with a transgenic mice carrying tamoxifen-inducible Cre recombinase under the control of the chicken β-actin promoter/enhancer (B6.Cg-Tg(cre/*Esr1*)5Amc, The Jackson Laboratory). To delete ET-2 at P0, tamoxifen (3mg/40g of body weight; Sigma) in sunflower oil (Sigma) was delivered by gavage feeding to the dam. To inactivate ET-2 in adulthood, mice were injected intraperitoneally with tamoxifen (3mg/40g of body weight) in sunflower oil into 6~8 week-old mice for 4 consecutive days. For intestine epithelium- or neuron-specific *ET-2* null mice, *ET-*

2$^{f/f}$ mice were crossed with a transgenic line expressing Cre recombinase under the control of the mouse villin 1 promoter (B6;SJL-Tg(*Vil-cre*)997Gum, The Jackson Laboratory) or carrying Cre recombinase under the control of the nestin promoter (kindly provided by Dr. Keith Parker at University of Texas Southwestern Medical Center, Dallas, TX). Conditional *ET-2* null mice were maintained on mixed genetic backgrounds (SVJ129 x C57BL6).

Genotyping

Tail genomic DNA was prepared by standard methods. All primers sequences used for PCR genotyping are available as supplemental material (Supplemental Table S3). PCR products were analyzed by 1.5% agarose gel electrophoresis.

Measurement of serum insulin and T3, and blood chemistry

Blood for analysis was collected through retrobulbar plexus or either by cardiac puncture or by decapitation. For serum, blood was transferred to Vacutainer® SST™ Tubes (BD Biosciences) and centrifuged (1500 x *g* for 15 min at 4°C), and serum was stored at -20°C until analysis. For plasma, blood was transferred to Vacutainer® K2 EDTA Tubes (BD Biosciences) and centrifuged (1000 x *g* for 15 min at 4°C), and plasma was frozen in liquid nitrogen and stored at -80°C. Serum insulin, glucocorticoid, and growth hormone were measured by Mouse Metabolic Phenotyping Core Facility of University of Texas Southwestern Medical Center. Thyroid hormones were analyzed by Pituitary and Antisera Center of Harbor-

UCLA Medical Center. Blood chemistry was analyzed by Pathology Laboratory in Dallas Children's Medical Center.

T3 supplementation

ET-2 null mice (3 week-old) were provided with T3 (3.5 ng/g body weight/day; (3)) by subcutaneous injection for 2 weeks.

Milk consumption

Milk was taken from the stomach of the wild type and mutant mice and weighed.

Antiacid supplementation

Sodium bicarbonate ($NaHCO_3$) was dissolved in water and provided to *ET-2* null mice at 20 days of age.

Quantitative RT-PCR analysis

Total RNA was extracted from tissues using STAT 60 (Tel-Test), treated with RNase-free DNase I (Roche Molecular Biochemicals), and reverse-transcribed into cDNA with random hexamers (Roche Molecular Biochemicals) and SuperScript II First-Strand Synthesis System (Invitrogen). Real-time PCR reactions contained 25 ng of cDNA, 150 nM of each primer, and 10 μl of SYBR Green PCR Master Mix (Applied Biosystems) in 20 μl of total volume and were performed with ABI Prism 7000 Sequence Detection System. Relative mRNA

levels were calculated with ddC_T method normalized to 18s rRNA level. Primer sequences were designed using Primer Express Software (PerkinElmer Life) and all sequences are available as supplemental material (Supplemental Table S2).

Histology

Brown adipose tissues were harvested and fixed in 4 % paraformaldehyde for paraffin embedding. 5 μm sections were stained with hematoxylin and eosin and analyzed by light microscopy.

Data analysis

Values are presented as means ± (SEM). Statistical significance was evaluated by conducting a two-tailed unpaired Student's *t*-test using Prism 5.0 (GraphPad Software). A *P* value of <0.05 was regarded as statistically significant.

Supplemental References

1. Ishibashi, S., Brown, M.S., Goldstein, J.L., Gerard, R.D., Hammer, R.E., and Herz, J. 1993. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest* 92:883-893.
2. Rosahl, T.W., Geppert, M., Spillane, D., Herz, J., Hammer, R.E., Malenka, R.C., and Sudhof, T.C. 1993. Short-term synaptic plasticity is altered in mice lacking synapsin I. *Cell* 75:661-670.

3. Trost, S.U., Swanson, E., Gloss, B., Wang-Iverson, D.B., Zhang, H., Volodarsky, T., Grover, G.J., Baxter, J.D., Chiellini, G., Scanlan, T.S., et al. 2000. The thyroid hormone receptor-beta-selective agonist GC-1 differentially affects plasma lipids and cardiac activity. *Endocrinology* 141:3057-3064.

Supplemental Figure Legends

Figure S1. ET-2 expression in embryonic tissues at E15. Quantitative RT-PCR analysis of *ET-2* mRNA expression in individual tissues from embryo at E15 (n=5). Values are presented as fold change relative to gene expression in whole embryo.

Figure S2. Generation of constitutive and conditional *ET-2* knockout mice. Exons are represented by boxes with the coding region filled and exon numbers marked above. (A) Schematic representation of the gene-targeting strategy to generate a constitutive ET-2 allele. Dashed lines show site-specific recombination events. tk, thymidine kinase; Neo, neomycin resistance cassette; E, EcoR I; BX, BstX I; V, EcoRV; X, Xba I. (B) Schematic representation of the gene-targeting strategy to generate a conditional ET-2 allele. The initial gene targeting product in which exon 2 is flanked by *loxP* sites (open triangles) and is followed by FRT (filled triangles)-flanked PGK-neo ($ET\text{-}2^{neo\text{-}loxP}$); the targeted allele following FRP-mediated excision of the PGK-neo cassette ($ET\text{-}2^{flox/flox}$); the *ET-2* null allele obtained by Cre-mediated excision of exon 2 ($ET\text{-}2^{-/-}$). Dashed

lines show site-specific recombination events. Position of the Southern blot probe is shown as probe. The predicted lengths of the EcoRI restriction fragments are shown above each of the alleles. PGK, phosphoglycerine kinase; E, EcoR I. (C) Southern blot hybridization of EcoR I digested genomic DNA showing hybridization patterns for the four alleles illustrated in panel B.

Figure S3. Normal milk intake of constitutive *ET-2* null mice. (A) A representative photograph displaying presence of milk (arrow) in wild type (left) and *ET-2* null (right) mice at 3 days of age. (B) Milk intake of *ET-2* null mice at 10 days of age (n=10). n.s., non-significant

Figure S4. Insulin and T3 levels of constitutive *ET-2* null mice. (A) Insulin was measured with serum taken from fed and fasted conditions (n=5). (B) T3 was assayed with serum from only fed condition (n=7). Six weeks of aged control and ET-2 null mice survived on the warm environment were used in these experiments.

Asterisks denote statistically significant differences between the compared values: $*P < 0.05$, n.s., non-significant

Figure S5. No detectable effect of T3 supplement on growth regulation and survival of constitutive *ET-2* null mice. (A and B) Body weight (A) and median

life span (B) of constitutive *ET-2* null mice treated with T3 at 3 weeks of age (n=10).

Figure S6. No significant change in the expression of genes responsible for nutrient uptake in intestine. (A, B and C) Quantitative RT-PCR analysis of genes are involved in the management of fat (A), carbohydrate (B) and protein (C) in intestine of constitutive *ET-2* null and littermate WT mice (n=8). Values are presented as fold change relative to gene expression in WT mice.

Figure S7. Intestine epithelium-specific recombination of floxed ET-2 allele. A representative picture shows PCR genotyping products. Genomic DNA extracted from stomach and each part of intestine of indicated genotypes was applied to PCR with primers for recombined floxed ET-2 allele**.** PCR products were visualized after electrophoresis through 1.5% agarose.

Figure S8. No compensatory increase of intestinal *ET-1* and *ET-3* mRNA level by *ET-2* deficiency. (A and B) Quantitative RT-PCR analysis of ET-1 and ET-3 in the small intestine of constitutive *ET-2* null (A) and *ET-2*$^{f/f}$; Vil-Cre (B), and their littermate WT mice (n=4). Values are presented as fold change relative to gene expression in WT mice.

Figure S9. No significant change in the expression of genes responsible for nutrient digestion in stomach. Quantitative RT-PCR analysis of genes responsible for acidification, protein digestion, and milk coagulation in stomach of constitutive *ET-2* null and littermate WT mice (n=8). Values are presented as fold change relative to gene expression in WT mice.

Figure S10. No beneficial effect of antacid diet. Life span of *ET-2* null mice was monitored from the time of sodium bicarbonate was provided (20 days of age; n=5).

Figure S11. No significant change in the expression of genes responsible for nutrient uptake or digestion in pancreas. Quantitative RT-PCR analysis of genes involved in the management of fat, carbohydrate and protein in pancreas of constitutive *ET-2* null and littermate WT mice (n=20). Values are presented as fold change relative to gene expression in WT mice.

Figure S12. Histological phenotypes of the brown adipose tissues of constitutive *ET-2* null and control mice. (A) Sections of brown adipose tissues from constitutive *ET-2* null and littermate WT mice were stained with haematoxylin and eosin (magnification: ×20). (B) Nuclei number per field was scored by manual counting (n=2 sections per mouse, 5 per group). $^{**}P < 0.01$

Figure S13. No growth defect in neuron-specific *ET-2* null mice.

Body weight of *ET-2*$^{f/f}$; Vil-Cre and their littermate control *ET-2*$^{f/f}$ mice at 2 weeks of age (n=10).

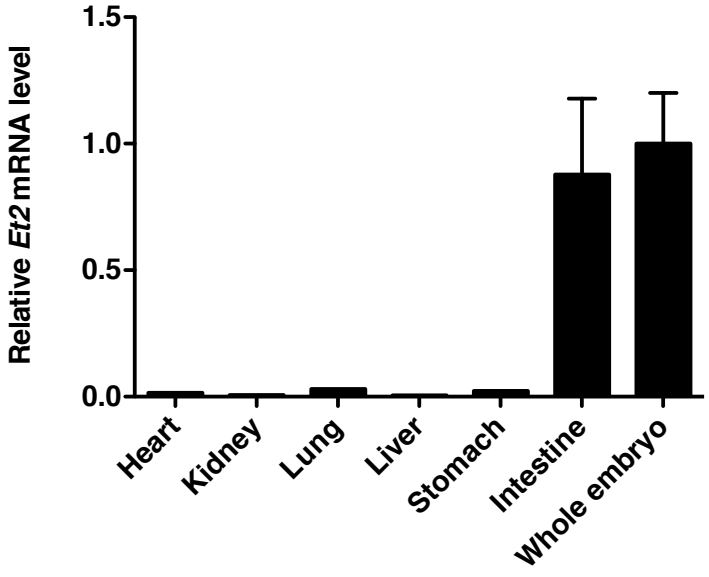
Figure S14. Paradoxical down-regulation of pancreatic lipase family in *ET-2* null mice.

Quantitative RT-PCR analysis of genes known to be induced by torpor in liver of constitutive *ET-2* null and littermate WT mice at 2 weeks of age in ambient environment (n=4). $^{**}P < 0.01$

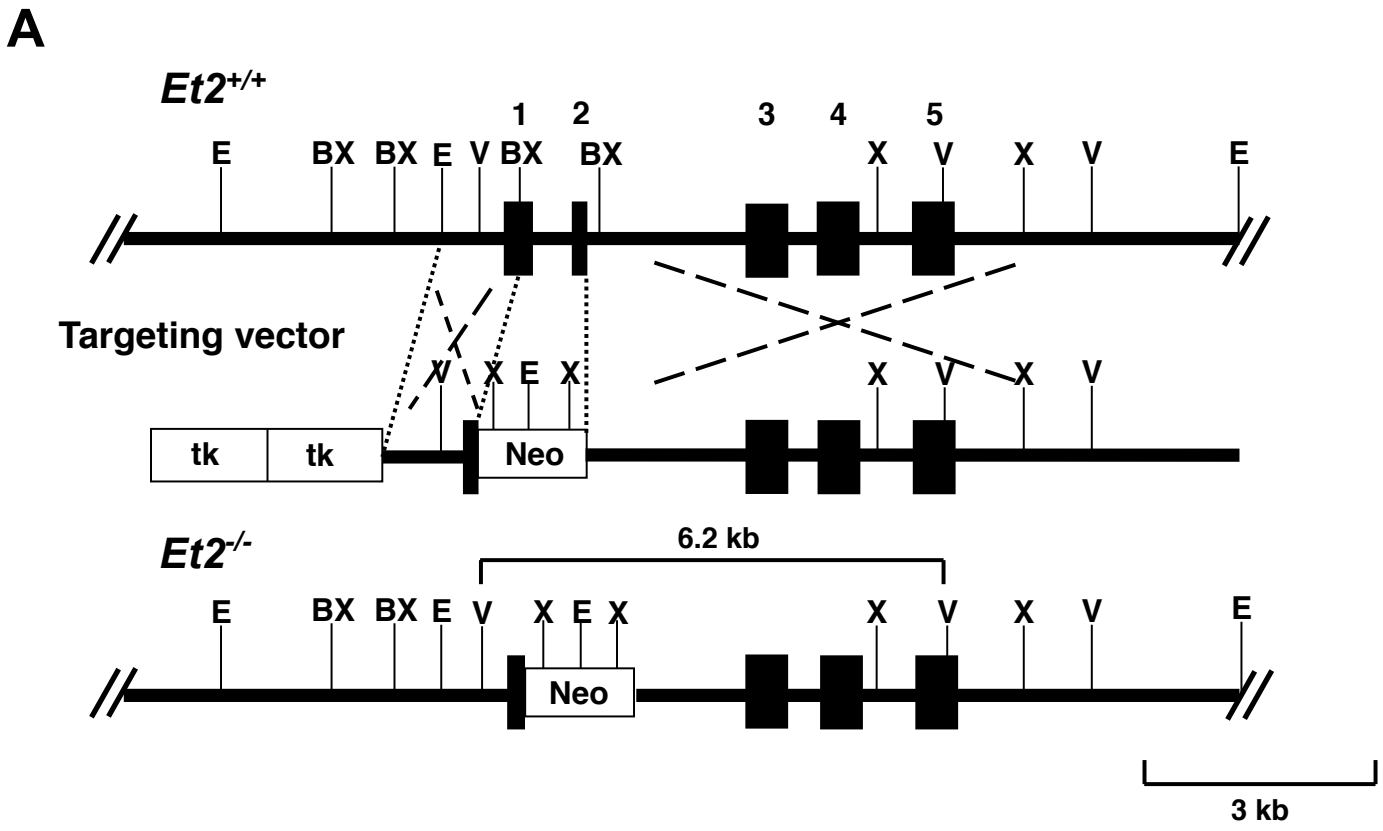
Figure S15. Time-dependent reduction of ET-2 expression in neonate *ET-2*$^{f/f}$; CAGGCre-ER^{TM} mice.

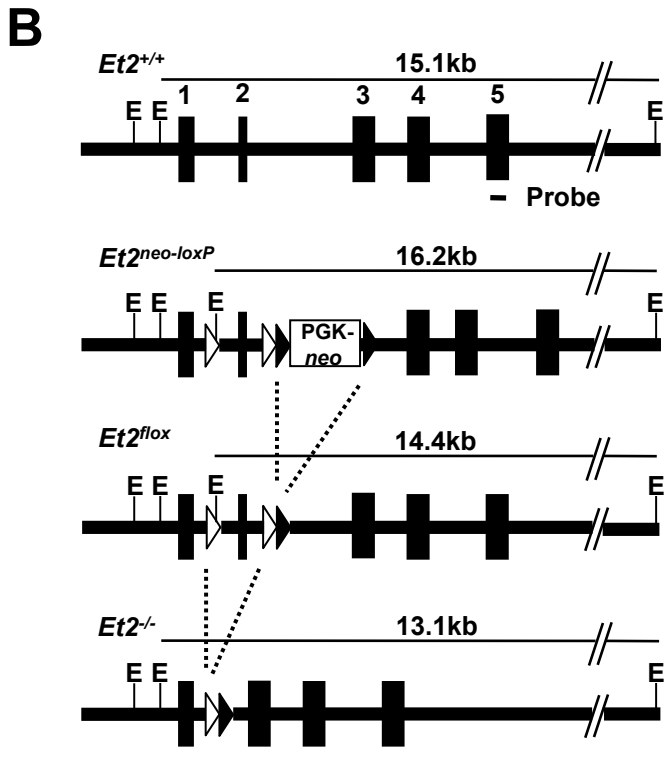
Quantitative RT-PCR analysis of ET-2 in the small intestine of neonate *ET-2*$^{f/f}$; CAGGCre-ER^{TM} mice at the indicated time after birth (n=10 per time point). Constitutive *ET-2* null mice was used as a positive control (*ET-2* KO).

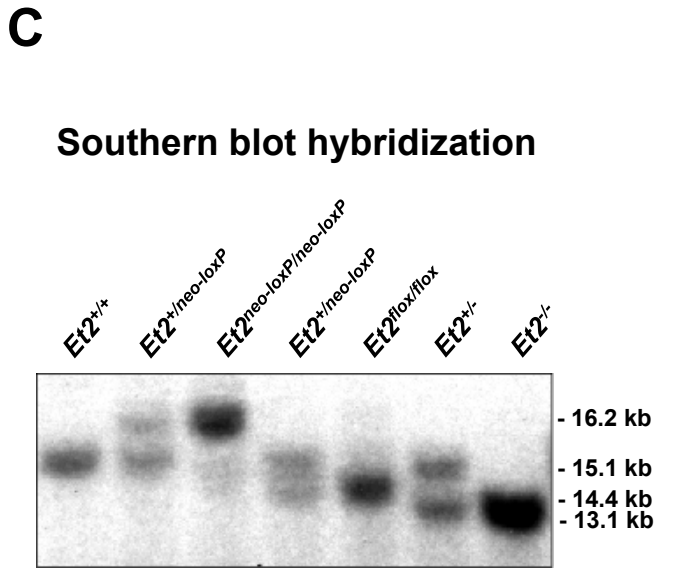
Supplemental Figure S1



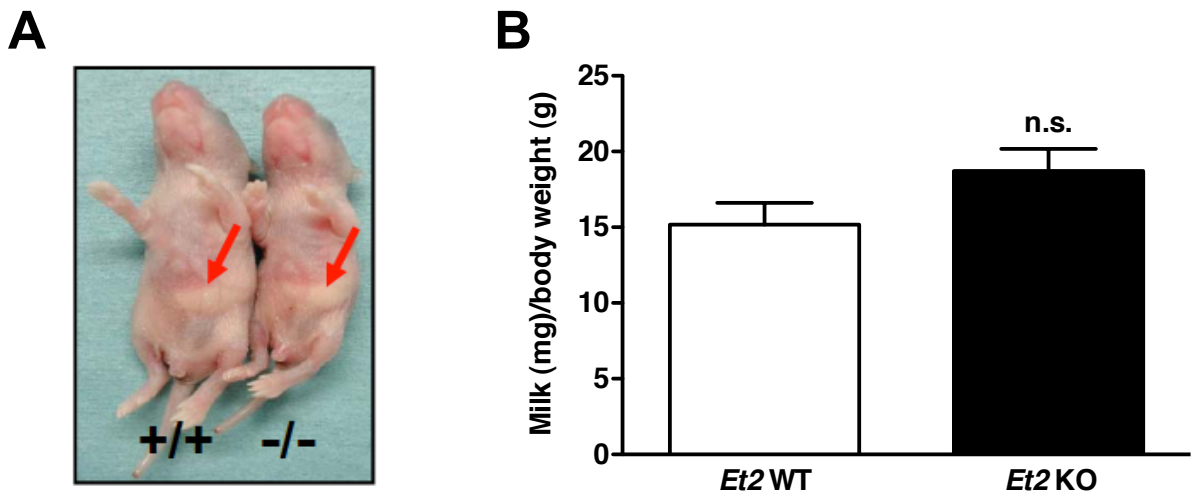
Supplemental Figure S2







Supplemental Figure S3



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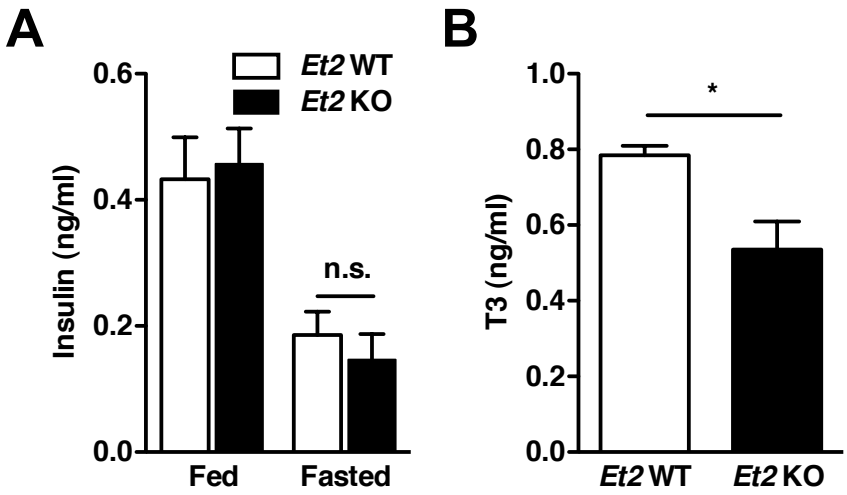

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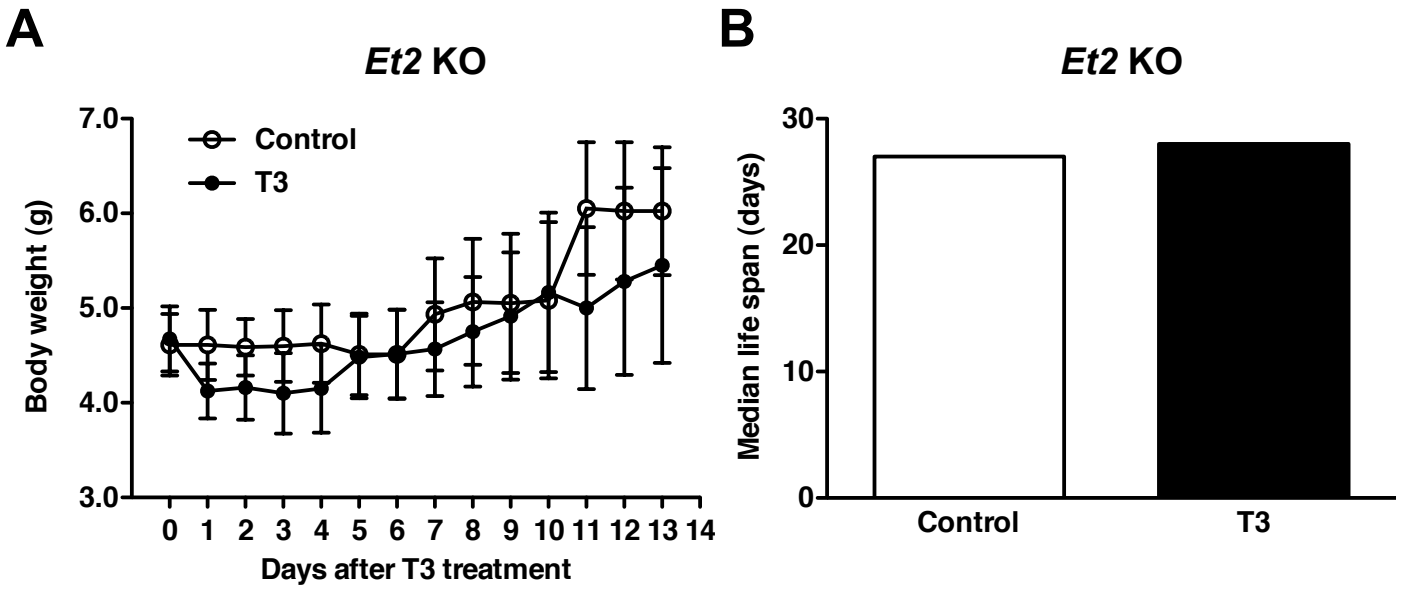

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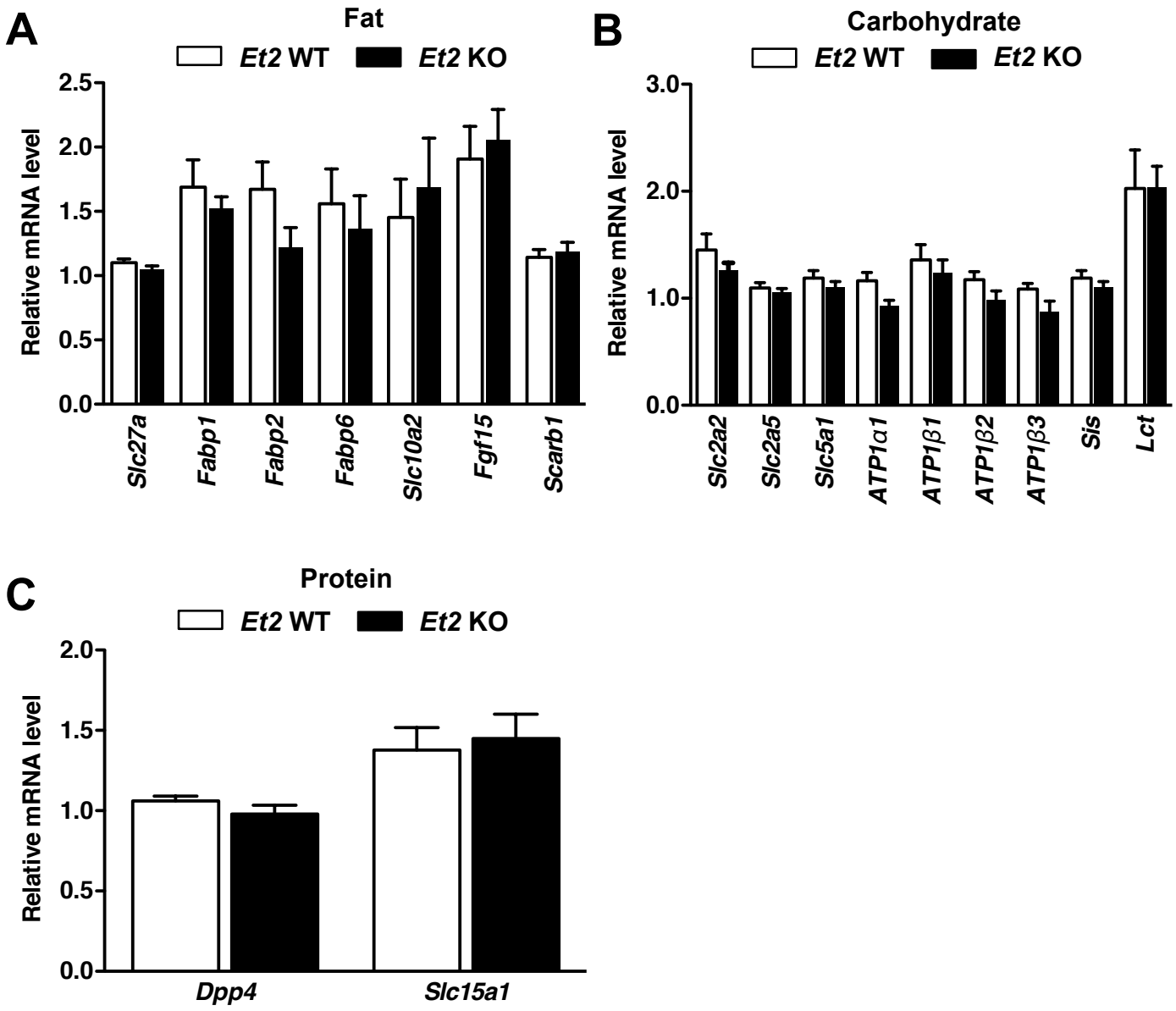

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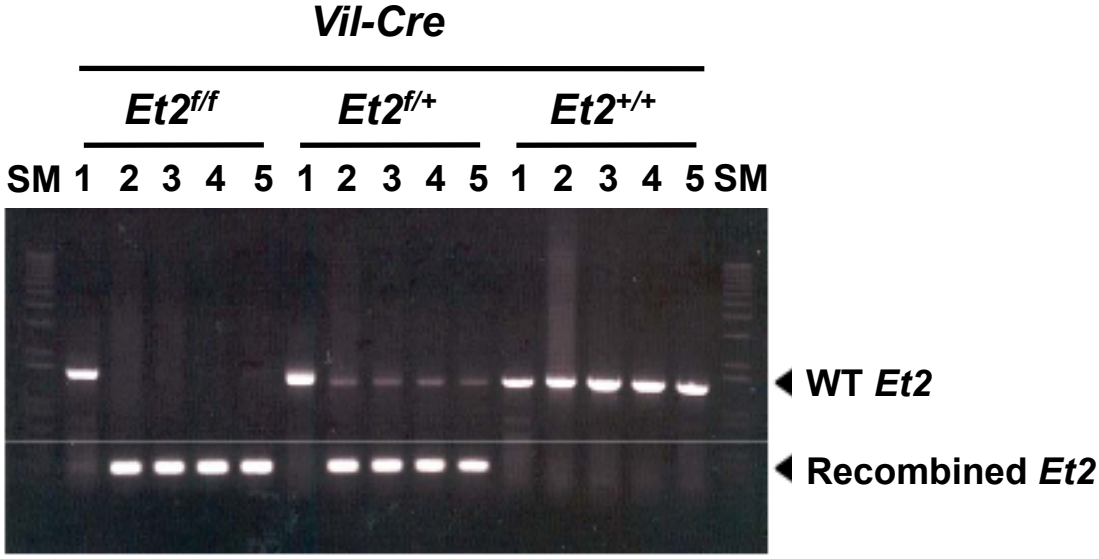
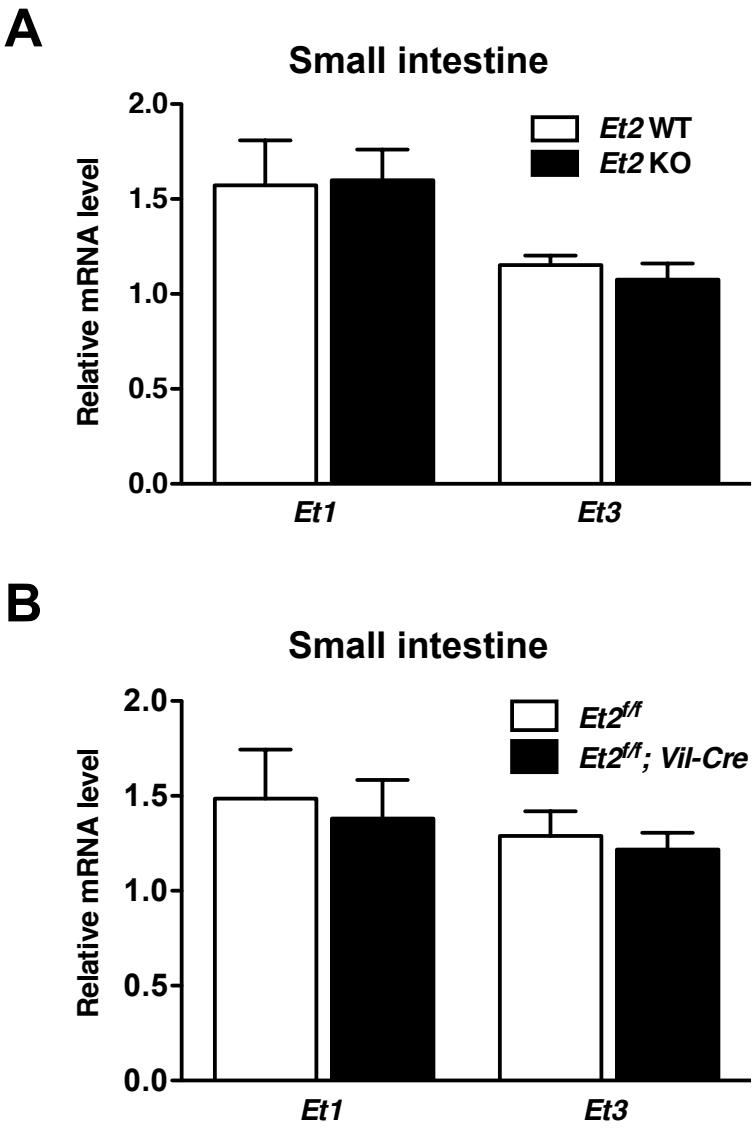


SM size marker

1. **Stomach**
2. **Duodenum**
3. **Jejunum**
4. **Ileum**
5. **Colon**

Supplemental Figure S8



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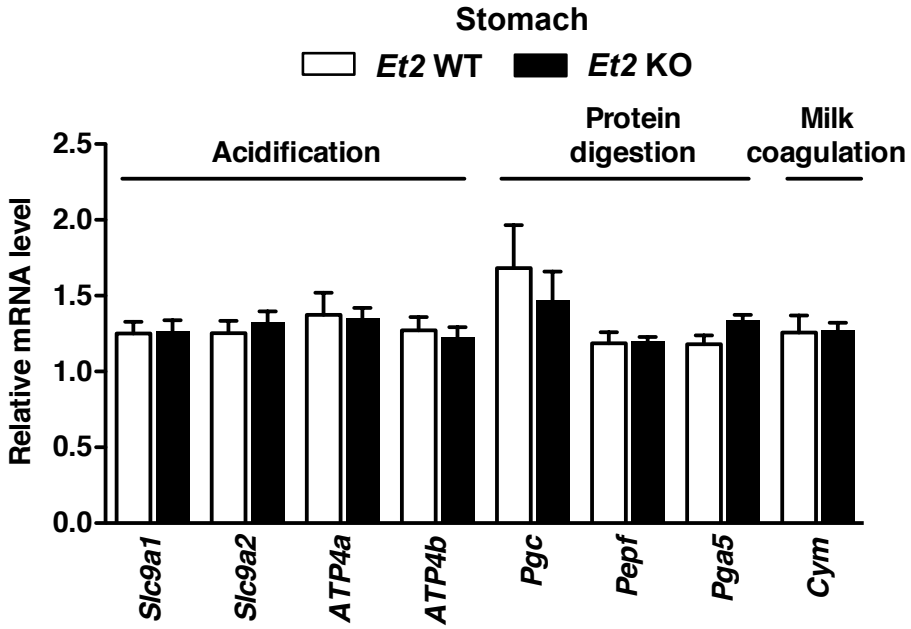

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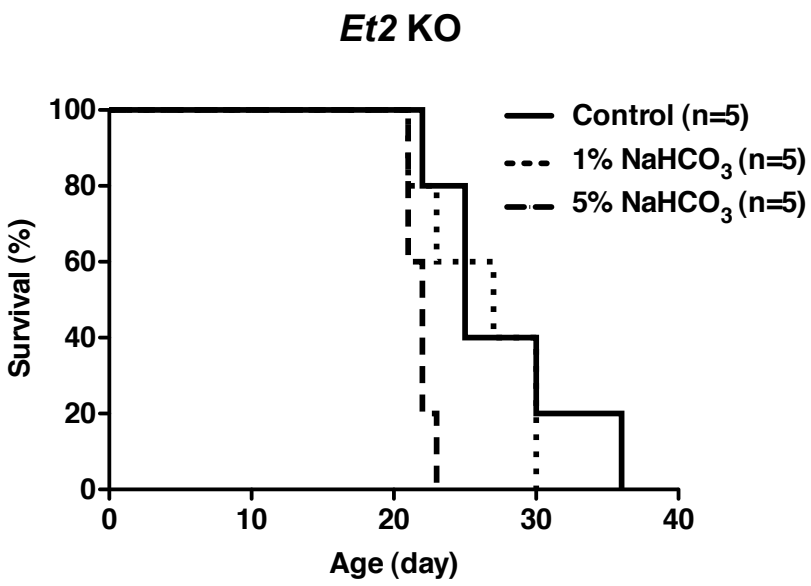

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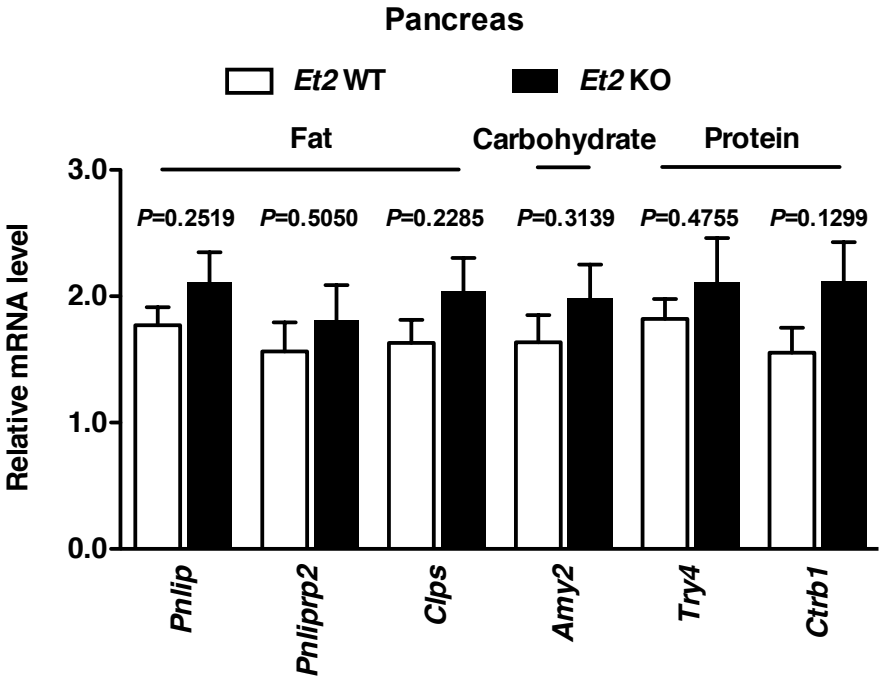

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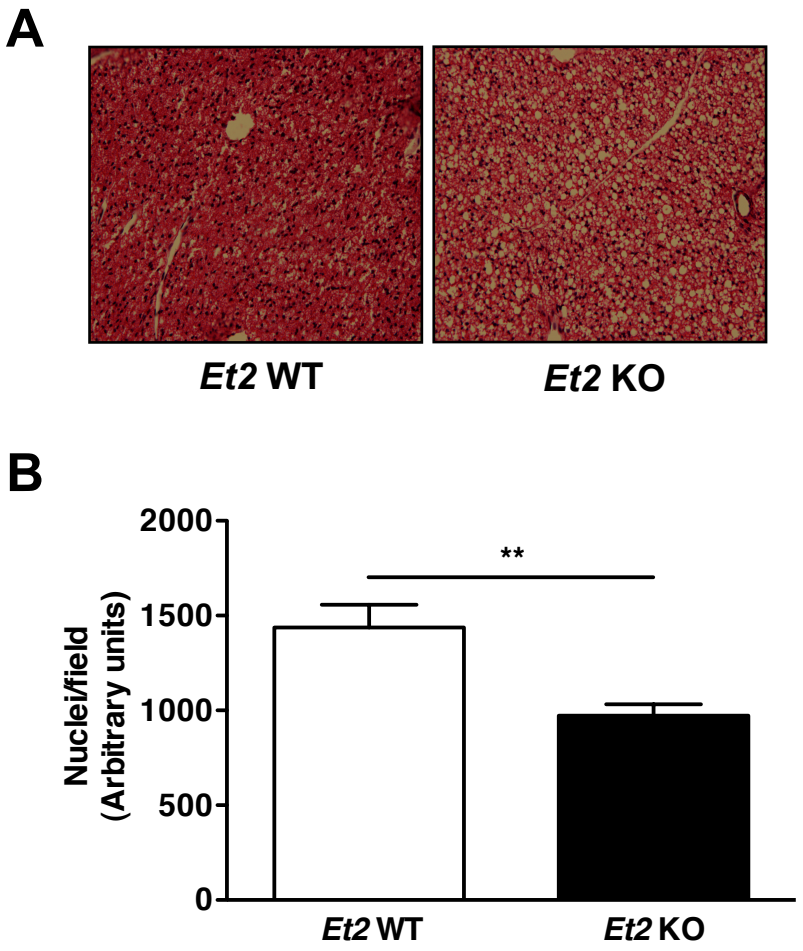

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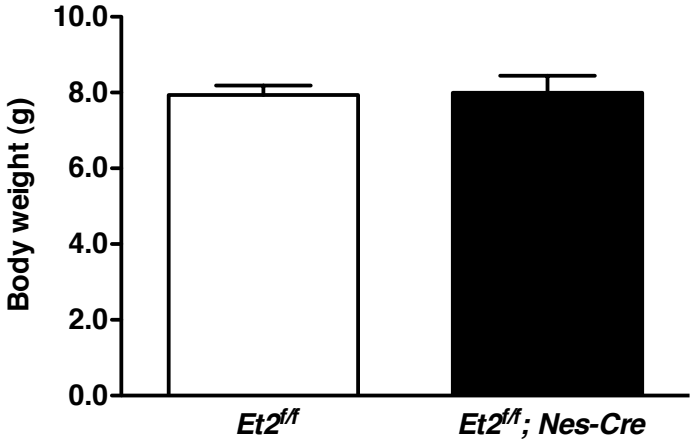

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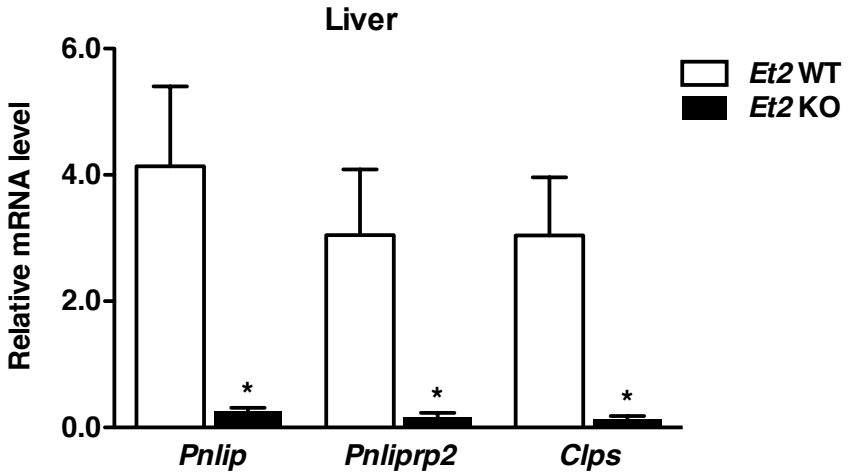

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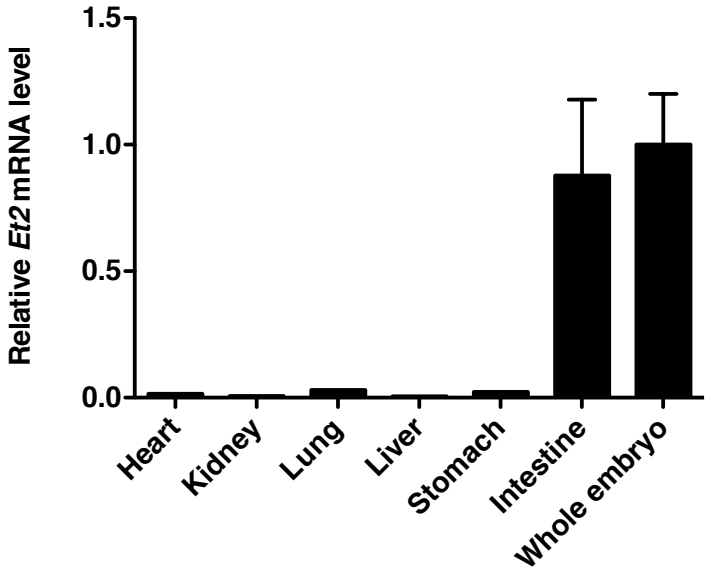

Supplemental Table S1. Blood chemistry and hormone levels of constitutive *ET-2* null and littermate WT mice

	ET-2 WT	*ET-2* KO
Sodium (mEg/dl)	136.3±3.2	126.8±5.7
Calcium (mEg/dl)	9.5±0.2	9.6±0.2
Phosphorous (mEg/dl)	7.7±0.8	7.4±0.2
Potassium (mEg/dl)	9.16±0.62	8.98±0.33
Magnesium(mEg/dl)	1.92±0.03	1.84±0.04
Chloride (mEg/dl)	107.8±0.23	103.3±0.43
Creatine (mg/dl)	0.24±0.04	0.26±0.02
Glucose (mg/dl)	147.4±8.6	42.6±4.4 ***
Uric acid (mg/dl)	0.84±0.05	1.37±0.11 *
BUN (mg/dl)	16.5±1.1	32.5±2.4 **
Total protein (mg/dl)	4.09±0.14	3.86±0.09
Albumin (g/dl)	1.57±0.07	1.41±0.09
Cholesterol (mg/dl)	129.5±4.5	179.0±11.0 *
Glucocorticoid (ug/dl)	18.43±1.0462	17.43±0.9861
Growth hormone (ng/ml)	7.438±0.6808	8.455±1.098
T4 (ug/dl)	3.127±0.2273	3.329±0.3001

Supplemental Table S2. List of QPCR primers used in this study

Gene	5'-Forward sequence-3'	5'-Reverse sequence-3'	Species
ET-1	TGTATCTATCAGCAGCTGGTGGAA	AAAAGATGCCTTGATGCTATTGC	mouse
ET-2	TCTGCCACCTGGACATCATC	GAGCACTCACAACGCTTTGG	mouse
ET-3	GGCCCTGGTGAGAGGATTGT	TCCTGCCCAGCGCTTTC	mouse
Rn18s	ACCGCAGCTAGGAATAATGGA	GCCTCAGTTCCGAAAACCA	mouse
Pck1	CACCATCACCTCCTGGAAGA	GGGTGCAGAATCTCGAGTTG	mouse
Pdk4	CAAAGACGGGAAACCCAAGCC	CGCAGAGCATCTTTGCACAC	mouse
Hmgcs2	CCGTATGGGCTTCTGTTCAG	AGCTTTGTGCGTTCCATCAG	mouse
Fgf21	CCTCTAGGTTTCGCCAACAG	AAGCTGCAGGCCTCAGGAT	mouse
Dio2	GTTGCTTCTGAGCCGCTC	GCTCTGCACTGGCAAAGTC	mouse
Ucp1	AAGCTGTGCGATGTCCATGT	AAGCCACAAACCCTTTGAAAA	mouse
Epo	AGGAATTGATGTCGCCTCCA	AGCTTGCAGAAAGTATCCACTGTG	mouse
Slc27a	TTGCAAGTCCCATCAGCAACT	GCATACAGAGGCAGCTCCTTTT	mouse
Fabp1	CCAGGAGAACTTTGAGCCATTC	TGTCCTTCCCTTTCTGGATGA	mouse
Fabp2	ACTGACAATCACACAGGATGGAA	CCGAGCTCAAACACAACATCA	mouse
Fabp6	CAAGGCTACCGTGAAGATGGA	CCCACGACCTCCGAAGTCT	mouse
Slc10a2	TGTCTGTCCCCCAAATGCA	TGCATTGAAGTTGCTCTCAGGTA	mouse
Fgf15	GAGGACCAAAACGAACGAAATT	ACGTCCTTGATGGCAATCG	mouse
Scarb1	TCCCCATGAACTGTTCTGTGAA	TGCCCGATGCCCTTGA	mouse
Pnlip	ACAAACAGAAAAACCCGTATCATTAT	TGCACATGTCAGATAGCCAGTT	mouse
Pnliprp2	CCCCTGTTCCTCCTATGAGAAG	CCATTTTGGGACACCCTTGT	mouse
Clps	ACCAACACCAACTATGGCATCT	CCAGCTAACTGCGTGATCTCA	mouse
Slc2a2	TTCATGTCGGTGGGACTTGT	TCATGCTCACGTAACTCATCCA	mouse
Slc2a5	GGGCCGTCAATGTGTTCAT	CCGACGAGGAGTAGGAATCG	mouse
Slc5a1	GGCAGCTGTAATTTACACAGATAC	GAAAGCAAACCCAGTCAGGATA	mouse
Atp1a1	GGGTTGGACGAGACAAGTATGAG	TTGGCCTTTTTGCCCTTTT	mouse
Atp1b1	TTTCGAGGACTGTGGCAATG	CTCCTCGTTCGTGATTGATGTC	mouse
Atp1b2	GAATGTTGAATGCCGCATCA	CACGGCCAGCGAACTTG	mouse
Atp1b3	AGCCTGGCCGAGTGGAA	GGTGCGCCCCAGAAACT	mouse
Sis	AACCTCGGCAAAACCTTTATAGTT	TGCCTACATCTGGATAACAAGTGA	mouse
Lct	TGTATGTCCTCTTCGCTCTTGTG	GGAGCGCTTGCAGTAGTATTTGTA	mouse
Amy2	CCTTCTGACAGAGCCCTTGTG	GATGATCCTCCAGCACCATGT	mouse
Dpp4	GAAGACACCGTGGAAGGTTCTT	TTGGCACGGTGATGATGGT	mouse
Slc15a1	CCACGGCCATTTACCATACG	TGCGATCAGAGCTCCAAGAA	mouse
Try4	TCTGTGGAGGTTCCCTCATCA	GGATGCGGGACTTGTAGCA	mouse
Ctrb1	GGTTCCAAGATCACCGATGTG	AGAGTCACCCATGCAGGAAGA	mouse
Slc9a1	TGTGACTTCAGACCGCATATTG	GCCGTTGCCGGGTTTT	mouse
Slc9a2	CCATCCAGACCGTAGACGTGTT	AATCAGCACCCCGCAATT	mouse
Atp4a	CGGCGGACACCACAGAAG	CAGCGCTCGCCATGTCT	mouse
Atp4b	TGCAGGAGAAGAAGTCATGCA	GTCCGGGTTCCAACAGTAGTG	mouse
Pgc	TAGGAGCCCAGGAAGGAGAGT	GTGAGGGTAGGCAGGCTACTG	mouse
Pepf	CGAAAGGATCGGTCACACAA	GGGTCTGATGGGTCATCCAT	mouse
Pga5	CGAAAGGATCGGTCACACAA	GGGTCTGATGGGTCATCCAT	mouse
Cym	CCCATCCAAGTCCATCACCTT	GCCCTCCATTCTACCAGTACCA	mouse
ET-2	TGCAGCTCCTGGCTCGA	AGTTCCCTCACTGCCACCTGTTGT	rat
ETA	TTCCCTCTTCACTTAAGCCGAA	GCAACAGAGGCATGACTGAAAA	rat
ETB	GCCACCCACTAAGACCTCCT	ATGCCTAGCACGAACACGAG	rat
Krt18	CAAGATCATCGAAGACCTGAGGG	GAGAGGAGTTAGACG	rat
Vim	AGATCGATGTGGACGTTTCC	CACCTGTCTCCGGTATTCGT	rat
Gapdh	GAGGTGACCGCATCTTCTTG	CCGACCTTCACCATCTTGTC	rat

Supplemental Table S3. List of genotyping PCR primers used in this study

Gene		Direction	5'-sequence-3'
ET-2	WT	Forward	AGGTGACACAAAATATTCTGTTCAGTCCAC
		Reverse	GCAGTGTTCACCCAGATGATGTCCAGGTG
ET-2	Recombined	Forward	AGGTGACACAAAATATTCTGTTCAGTCCAC
		Reverse	GAGGATTGGGAAGACAATAGCAGGCATGC
Floxed ET-2	WT	Forward	CATAGAGCGGTGAGGCCACAG
		Reverse	AAGTTGGCACCCTTGGTGTTC
Floxed ET-2	Recombined	Forward	CATAGAGCGGTGAGGCCACAG
		Reverse	CTGTTCAGCTGGCAGAGTGAAGC
CAGGCre-ER^{TM}		Forward	ATTTGCCTGCATTACCGGTC
		Reverse	ATCAACGTTTTCTTTTCGG
Vil-Cre		Forward	GTGTGGGACAGAGAACAAACCG
		Reverse	TGCGAACCTCATCACTCGTTGC
Nes-Cre		Forward	CCGGGGTGTCTGGCTGTATCTCAA
		Reverse	CGGTGCTAACCAGCGTTTTC