Supplemental Information

Role of the mixed-lineage protein kinase pathway in the metabolic stress response to obesity

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Extended Experimental Procedures

RNA analysis

The expression of mRNA was examined by quantitative PCR analysis using a 7500 Fast Real Time PCR machine (Applied Biosystems). Taqman© assays were used to quantitate *Agrp* (Mm00475829_g1), *Bc021891* (also known as *Mlk4)* (Mm00524049_m1), *Dio2* (Mm00515664_m1), *Gapdh* (4352339E), *Gh1* (Mm00433590_g1), *Ldhb* (Mm00493146_m1), *Lepr* (Mm00434759_m1), *Lpl* (Mm00434770_m1), *Map3k9* (also known as *Mlk1)* (Mm00616834_m1), *Map3k10* (also known as *Mlk2)* (Mm01175535_m1), *Map3k11* (also known as *Mlk3)* (Mm01233534_m1), *Pck1* (Mm00440636_m1), *Pmch* (Mm242886_g1), *Pomc* (Mm00599949_m1), *Ppargc1a* (also known as *Pgc1*α)(Mm00447183_m1), *Slc2A4* (also known as *Glut4)* (Mm00436615-m1), *Sst* (Mm 00436671_m1), *Thrsp* (also known as *Spot14*) (Mm01273967_m1), *Trh* (Mm01963590_s1), *Ucp1* (Mm01244861_m1), and *Ucp3* (Mm00494077_m1) mRNA (Applied Biosystems). Standard curves were constructed using the threshold cycle (Ct) values for each template dilution plotted as a function of the logarithm of the amount of input template. The number of mRNA copies for each gene-sample combination was calculated using the slope of the standard curve. To obtain a normalized abundance, copy numbers were corrected for the amount of *Gapdh* mRNA in each sample.

Preparation of tissue extracts and immunoblot analysis

Mice were fasted overnight and treated with insulin (1.5 mU/g) or saline solution by intraperitoneal injection (15 mins). The mice were euthanized and tissues were freeze clamped in liquid nitrogen. Tissue extracts were prepared using Triton lysis buffer [20 mM Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/mL of aprotinin plus leupeptin]. Extracts (50 µg of protein) and immunoprecipitates were examined by protein immunoblot analysis. Immunecomplexes were detected by enhanced chemiluminescence (NEN). Primary antibodies were obtained from Cell Signaling (AKT, pThr^{308}-AKT, pSer^{473}-AKT, phosphor-ERK, phospho-JNK, p38 MAPK, and phospho-p38 MAPK), Millipore (phosphor-tyrosine), Pharmingen (JNK), Santa Cruz (ERK2), Sigma (α-Tubulin), and Upstate (IRS-1).

Metabolic cages

The metabolic studies were performed at the UMASS Mouse Metabolic Phenotyping Center. Food/water intake, V_{O2}, V_{CO2}, respiratory exchange ratio, energy expenditure, and physical activity were measured using metabolic cages (TSE Systems).

Hyperinsulinemic-euglycemic clamp studies

The clamp studies were performed at the UMASS Mouse Metabolic Phenotyping Center. Following an overnight fast, a 2-hr hyperinsulinemic-euglycemic clamp was conducted in conscious mice with a primed and continuous infusion of human insulin (150 mU/kg body weight priming followed by 2.5 mU/kg/min; Humulin; Eli Lilly), and 20% glucose was infused at variable rates to maintain euglycemia.

Analysis of tissue sections

Histology was performed using tissue fixed in 10% formalin for 24 h, dehydrated, and embedded in paraffin. Sections (7 µm) were cut and stained using hematoxylin and eosin (American Master Tech Scientific). The total number of adipocytes per epididymal fat pad was measured using methods previously described (Jo et al. 2009).

Supplemental References

Jo, J., Gavrilova, O., Pack, S., Jou, W., Mullen, S., Sumner, A.E., Cushman, S.W., and Periwal, V. 2009. Hypertrophy and/or Hyperplasia: Dynamics of Adipose Tissue Growth. *PLoS Comp. Biol.* **5**: e1000324.

Supplemental Figure Legends

Figure S1. Expression of MLK isoforms, Related to text.

(**A**) Total RNA isolated from liver and epididymal white adipose tissue of WT mice fed a chow diet (ND) or a high fat diet (HFD) for 16 wks. was employed to measure the expression of *Map3k9, Map3k10, Map3k11,* and *Bc021891* mRNA by quantitative Taqman© RT-PCR analysis. The data are normalized for the amount of *Gapdh* mRNA detected in each sample (mean ± SE; n = 4).

(**B**) The expression of *Map3k9, Map3k10, Map3k11,* and *Bc021891* mRNA by gastrocnemius muscle, brown adipose tissue, brain, and hypothalamus of chow-fed WT mice is presented.

Figure S2. Comparison of WT and MLK-deficient mouse tissue mass, Related to Figure 1.

Map3k10$^{-/-}$ *Map3k11*$^{-/-}$ and WT mice were fed a chow diet (ND) or a HFD (16 weeks). The weight of epididymal white fat, intrascauplar brown fat, quadriceps muscle, liver, and heart were measured (mean ± SE; n = 10). Statistically significant differences between *Map3k10*$^{-/-}$ *Map3k11*$^{-/-}$ and WT are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

White epididymal fat pad mass in MLK-deficient mice was decreased compared with WT mice. However, the total number of adipocytes per epididymal fat pad was greater in MLK-deficient mice (1,390,000 ± 140,000 adipocytes) than WT mice (720,000 ± 120,000 adipocytes) (mean ± SEM; n = 5; $P < 0.05$).

Figure S3. Pituitary hormones and hypothalamic gene expression, Related to text.

(**A**) Blood concentration of pituitary peptide hormones. WT and *Map3k10*$^{-/-}$ *Map3k11*$^{-/-}$ mice were fed a chow diet (ND) or a HFD (16 wks.) and then fasted overnight. The concentration of adrenocorticotropic hormone (ACTH), thyroid stimulating hormone (TSH), and growth hormone (GH) in the blood was measured by multiplexed ELISA (mean ± SE, n = 10). Statistically significant differences between WT and *Map3k10*$^{-/-}$ *Map3k11*$^{-/-}$ mice are indicated (*, $P < 0.05$).

(**B**) Hypothalamic gene expression. WT and *Map3k10*$^{-/-}$ *Map3k11*$^{-/-}$ mice were fed a chow diet (ND) or a HFD (16 wk) and then fasted overnight. The expression of *thyrotropin releasing hormone* (*Trh)*, *leptin receptor* (*Lepr*), *pro-opiomelanocortin* (*Pomc)*, Agouti-related peptide (*Agrp)*, *somatostatin* (*Sst*) and *pro-melanin-concentrating hormone* (*Pmch)* mRNA in the hypothalamus was measured by quantitative RT–PCR. The data were normalized to the expression of *Gapdh* mRNA in each sample and are presented as the mean ± SE (n = 6).

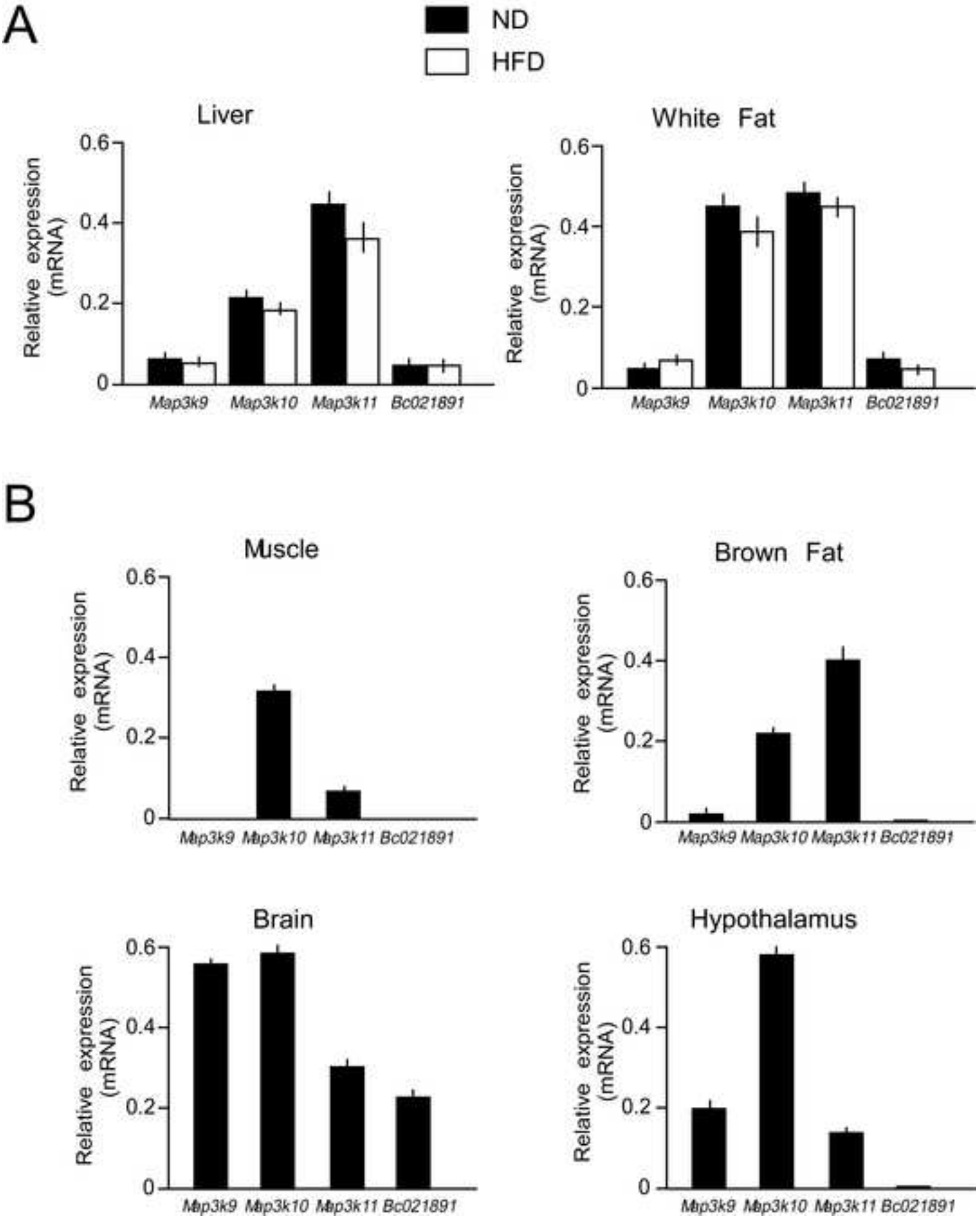
Statistically significant differences between WT and *Map3k10*$^{-/-}$ *Map3k11*$^{-/-}$ mice are indicated (*, P < 0.05; **, P < 0.01).

Figure S4. Thyroid hormone-stimulated gene expression in WT and *Map3k10*$^{-/-}$ *Map3k11*$^{-/-}$ mice, Related to text.

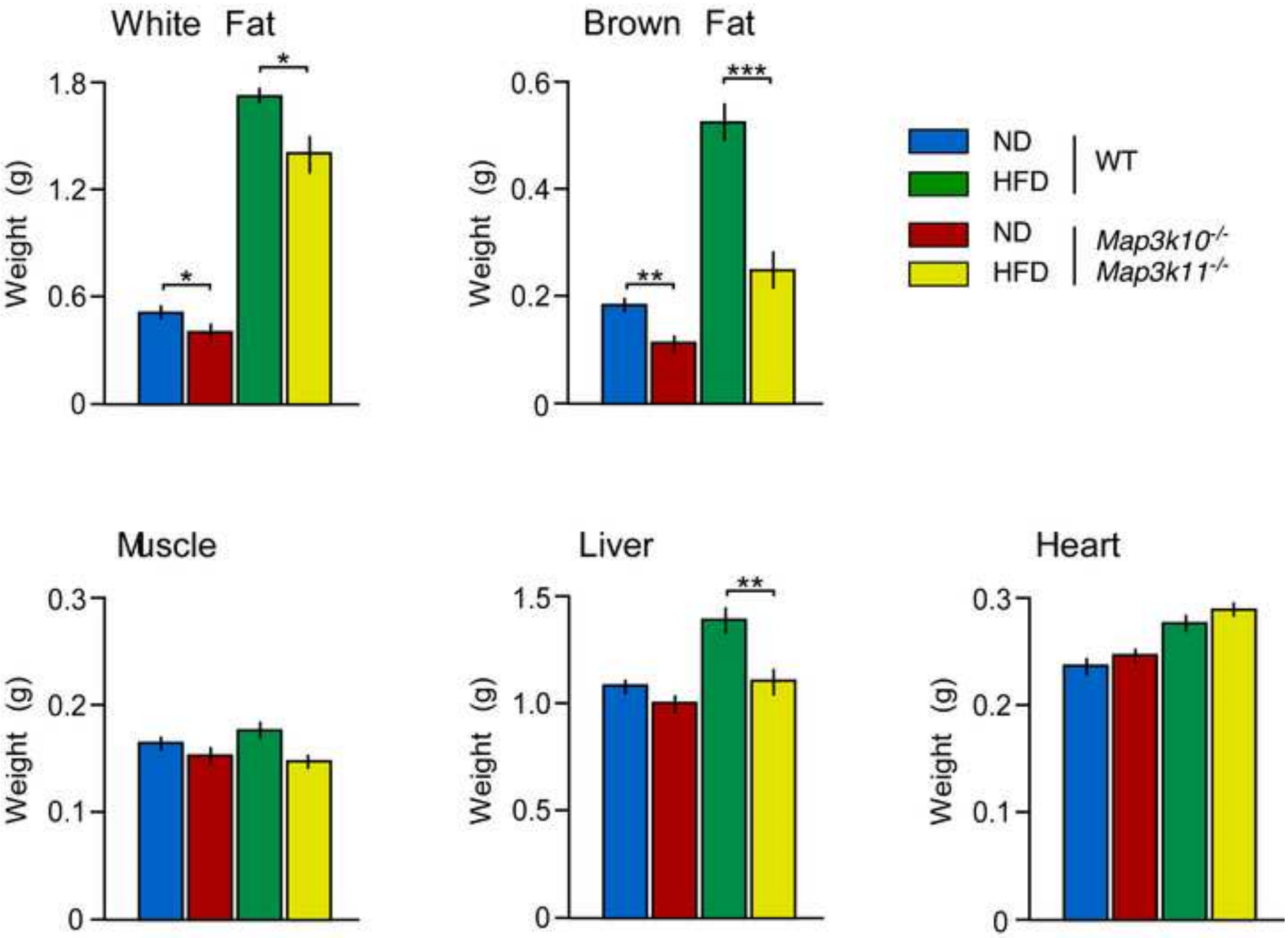
(**A**) The expression of *uncoupling protein 1 (Ucp1), phosphoenolpyruvate carboxykinase (Pck1), Spot14 (Thrsp), Lactate dehydrogenase* β *(Ldhb), and glucose transporter 4 (Slc2A4*) in brown fat of WT and *Map3k10*$^{-/-}$ *Map3k11*$^{-/-}$ mice was measured by quantitative RT–PCR. The data were normalized to the expression of *Gapdh* mRNA in each sample and are presented as the mean ± SE (n = 6). Statistically significant differences between WT and *Map3k10*$^{-/-}$ *Map3k11*$^{-/-}$ mice are indicated (*,P < 0.05; **,P < 0.01).

(**B-G**) WT and *Map3k10*$^{-/-}$ *Map3k11*$^{-/-}$ mice were fed a HFD (16 weeks) together with PTU in the drinking water. No significant difference (P > 0.05) between the WT and MLK-deficient mice were detected in body temperature (B), body weight (C), insulin tolerance (D), blood glucose concentration of fed (E) and over-night starved (F) mice or fasted insulin concentration (G). The data are presented as the mean ± SE (n = 10). No statistically significant differences between WT and *Map3k10*$^{-/-}$ *Map3k11*$^{-/-}$ mice were detected.

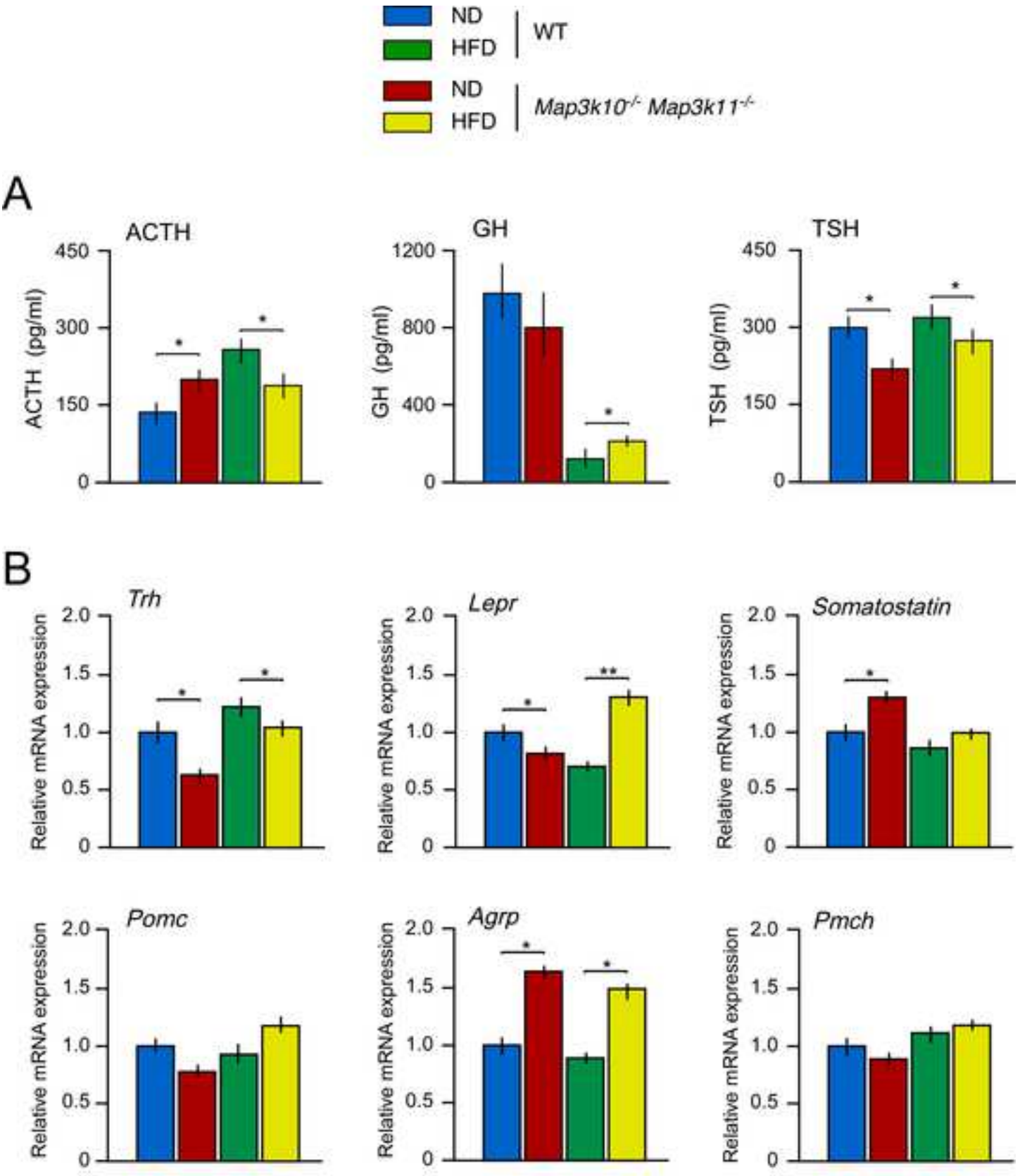
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4