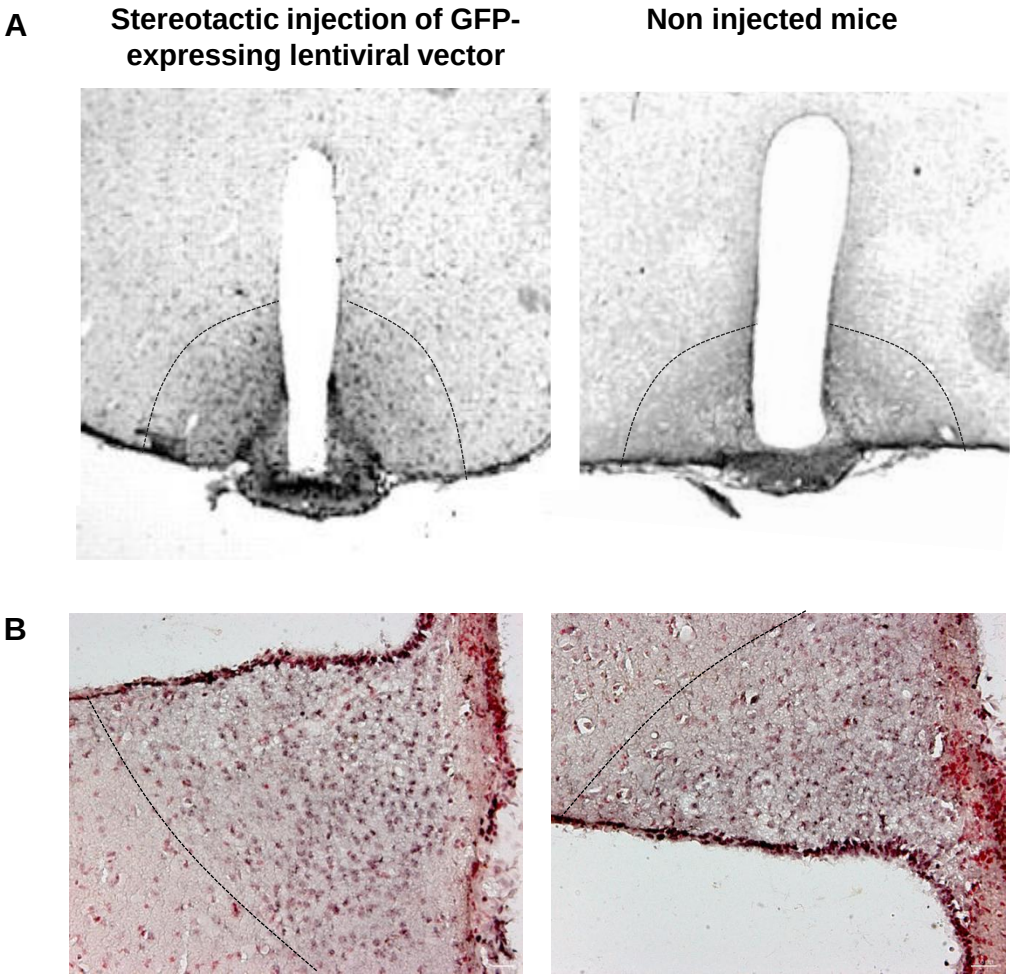

Figure S1 (Related to Figures 1-2)

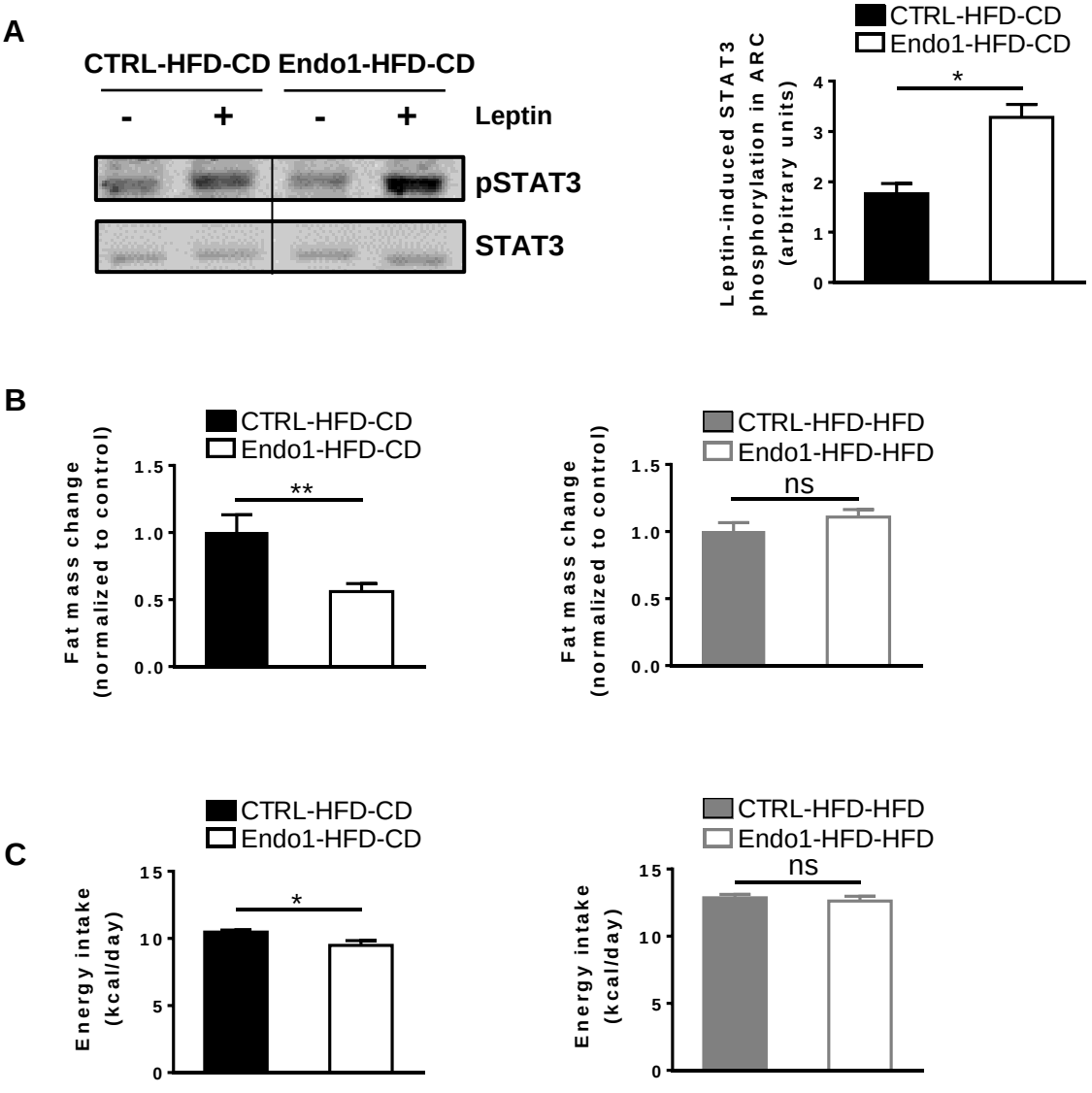

Figure S2 (Related to Figures 1-2)

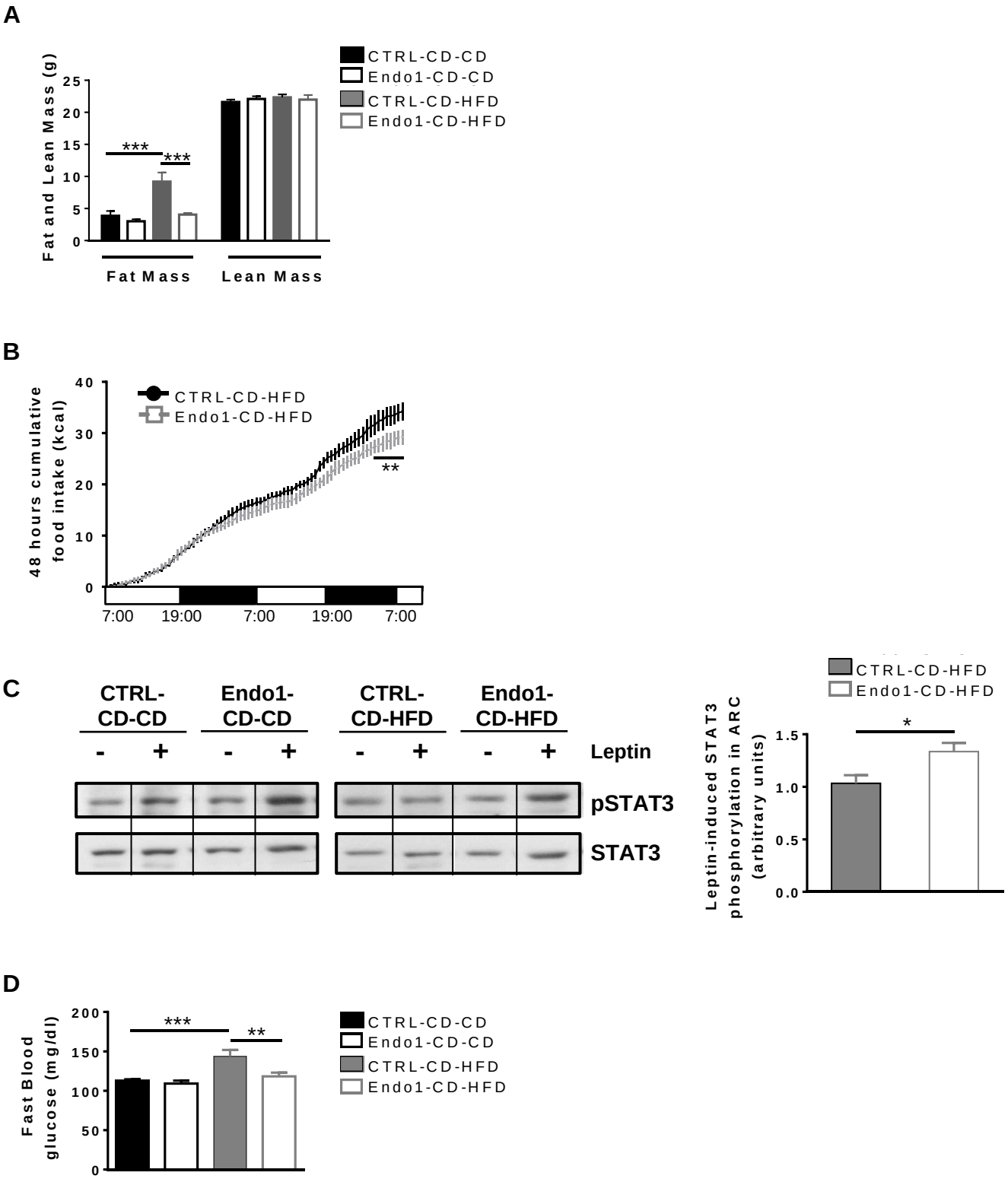

Figure S3 (Related to Figures 3-4)

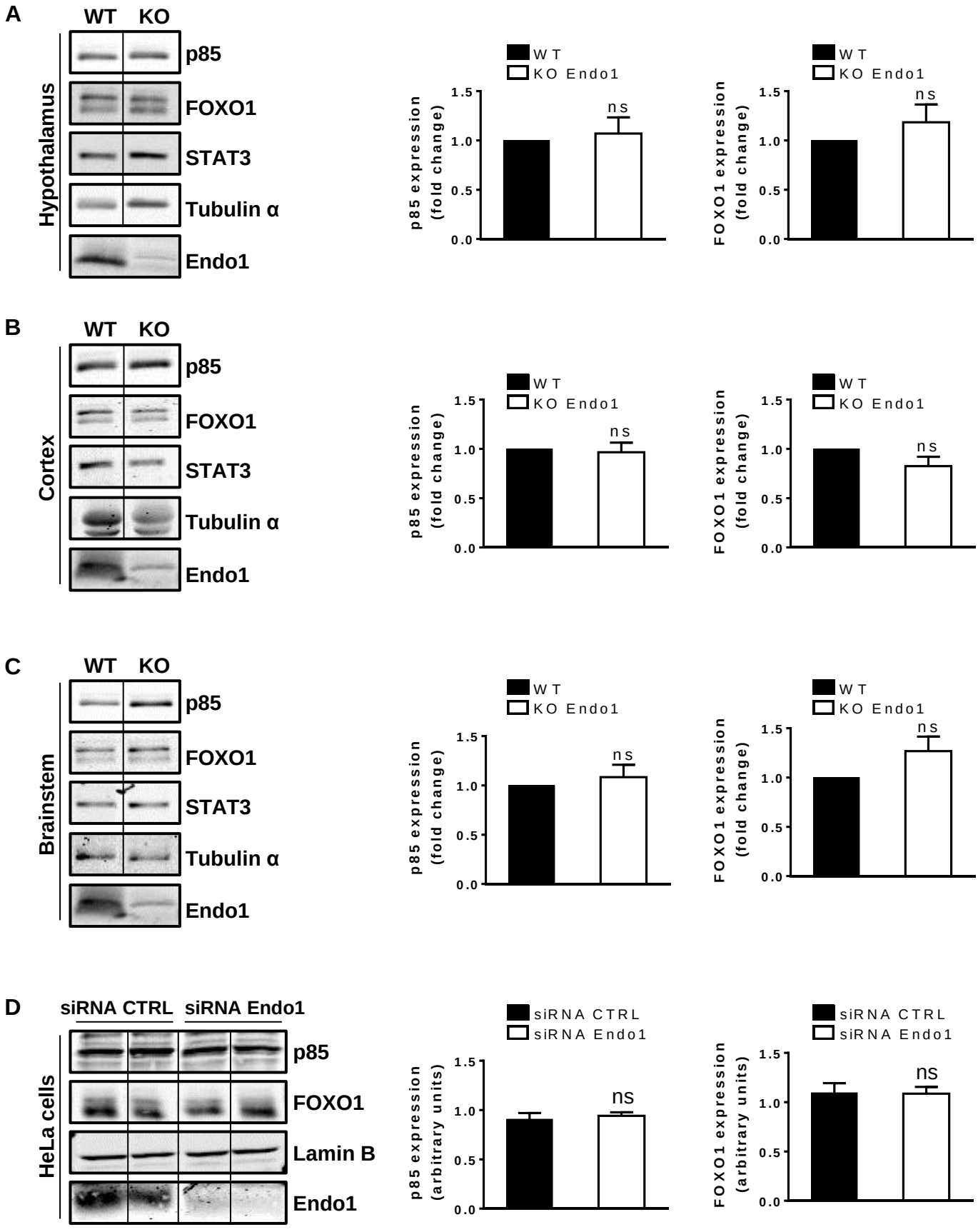

Figure S4 (Related to Figure 6)

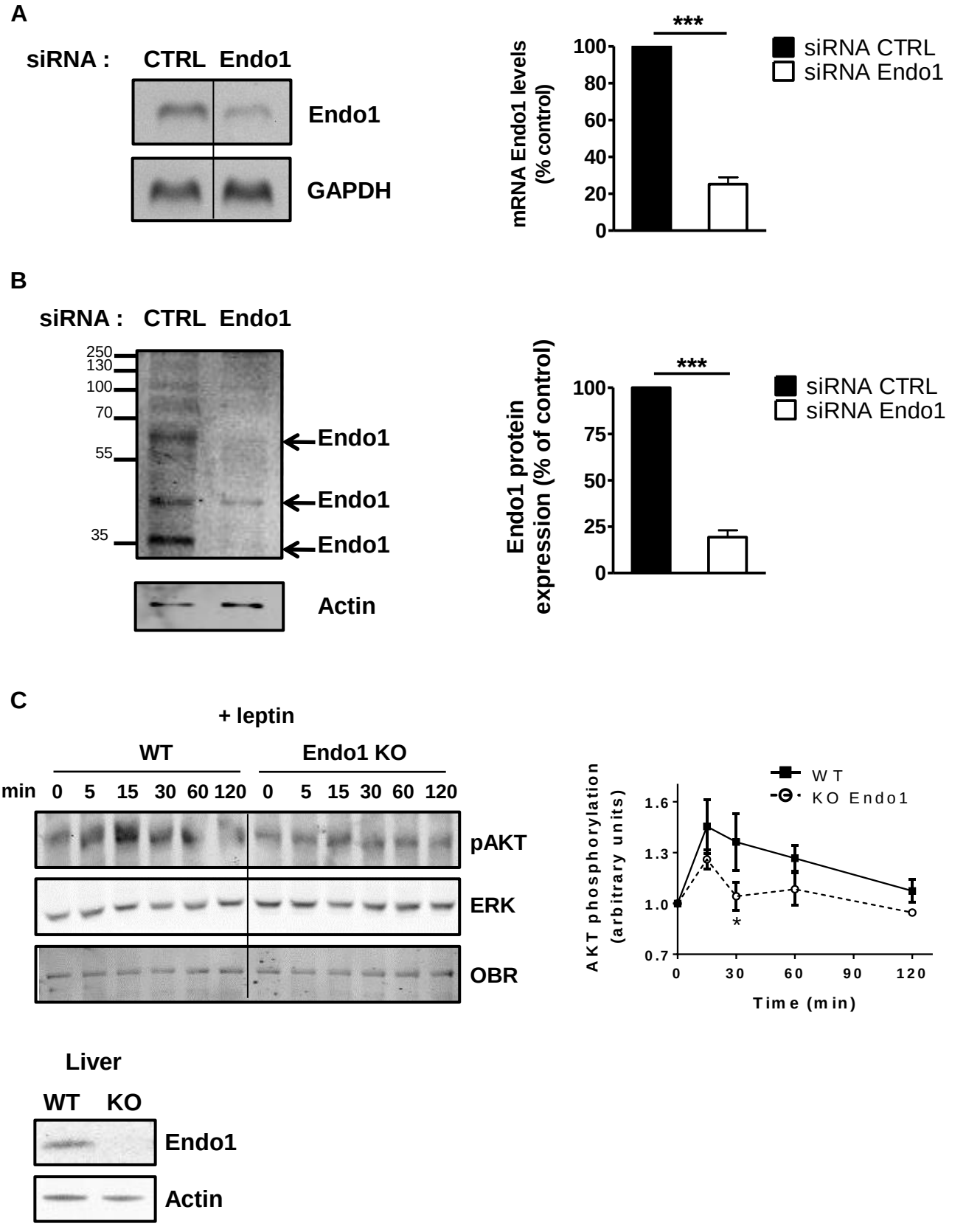

Figure S5 (Related to Figure 6)

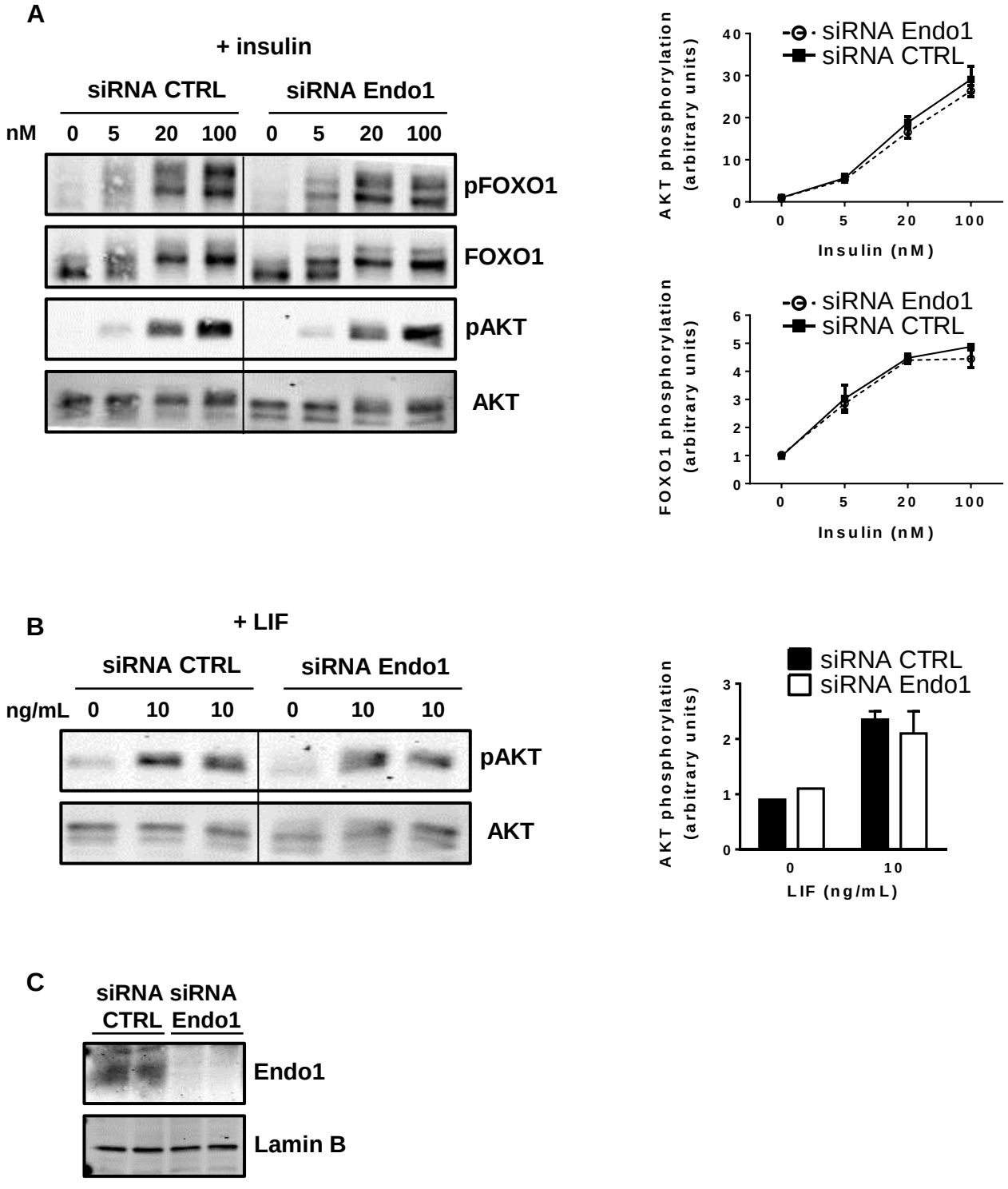

Figure S6 (Related to Figure 6)

SUPPLEMENTAL FIGURE LEGENDS

Figure S1 (Related to Figures 1-2)

(A) The specificity of the injection site in the mouse ARC, after stereotactic injection of a lentiviral vector-encoded GFP marker gene, was determined by immunohistochemical detection of the GFP protein expressed in transduced brain regions (using an antibody against GFP) (left panel), compared to non-injected mice (right panel). (B) Immunohistochemical detection of the expression of the GFP reporter gene in the ARC of injected mice by double labelling with anti-GFP (marker of lentivirus transduction, purple) and anti-Tyrosine Hydroxylase (marker of neurons, red). (C) Level of Endo1 mRNA expression in hypothalamic ARC punches after Endo1 silencing by stereotactic injection of shRNA CTRL or shRNA Endo1-expressing lentiviral vectors. As previously shown, the extend of Endo1 silencing in the punches is underestimated since ARC punches are enriched in ARC neurons but include also surrounding tissues. *, $p<0.05$; **, $p<0.01$. (D) Verification of the ARC isolation by micropunches: As expected for the DIO model, we observed an increase in the AgRP mRNA expression and a decrease in the POMC mRNA expression in the ARC micropunches of HFD mice versus CD controls.

Figure S2 (Related to Figures 1-2)

(A) Representative western blot of 1mg/kg leptin-stimulated STAT3 phosphorylation in the ARC of obese mice injected with control or Endo1 shRNA and switched to CD. Densitometry analysis is presented on the right panel. *, $p<0.05$ (n=3-4 per group). Data are presented as means +/- SEM. Dividing lines on western blots (A) indicate the grouping of images from different parts of the same gel. (B) Epigonadal fat-pad mass of obese mice treated with CTRL or Endo1 shRNA and kept on HFD or switched to CD. ns, $p>0.05$; **, $p<0.01$ (n=14-18 per group). (C) Cumulative caloric intake measured during 3 weeks (reported as mean per day). ns, $p>0.05$; *, $p<0.05$ (n=8-12 per group).

Figure S3 (Related to Figures 3-4)

(A) Lean and fat mass from EchoMRI analysis. ***, $p<0.005$ (n=6 per group). (B) Cumulative food intake (Kcal) for 48 hours using metabolic cages. **, $p<0.01$ (n=5-6 per group). (C) Representative western blot of STAT3 phosphorylation in the ARC of mice injected with control or Endo1 shRNA after 1mg/kg leptin ip administration. Densitometry analysis is presented on the right panel. *, $p<0.05$ (n=7 per group). Dividing lines on western

blots (C) indicate the grouping of images from different parts of the same gel. (D) Fasting blood glucose at week 14. **, p<0.01; ***, p<0.005 (n=8 per group). Data are presented as means +/- SEM.

Figure S4 (Related to Figure 6)

(A-C) Representative western blot of p85, FOXO1 and Endo1 expression at the protein level in mouse tissues (hypothalamus (A), cortex (B), brainstem (C)) of Endo1 KO versus WT mice (n=6). (D) Representative western blot of p85, FOXO1 and Endo1 expression at the protein level in HeLa cells silenced for Endo1 with two consecutive transfections of Endo1 siRNA during 4 days (n=2). Densitometry analysis of (A-D) is presented on the right panel. ns, p>0.05. Data are presented as means +/- SEM. Dividing lines on western blots (A-D) indicate the grouping of images from different parts of the same gel.

Figure S5 (Related to Figure 6)

(A-B) Expression of Endo1 both at the mRNA level (A) and protein level (B), after Endo1 silencing with two consecutive transfections of Endo1 siRNA in HEK293-OBRb during 4 days. Densitometry analysis is presented on the right panel. ***, p<0.005. (C) Representative western blot of 10nM leptin-stimulated AKT phosphorylation in primary hepatocytes from Endo1 KO or WT mice, transiently expressing OBRb. Densitometry analysis is presented on the right panel. *, p<0.05 (n=2). Western blot of Endo1 protein in liver from Endo1 KO or WT mice is also shown. Data are presented as means +/- SEM. Dividing lines on western blots (A; C) indicate the grouping of images from different parts of the same gel.

Figure S6 (Related to Figure 6)

(A) Representative western blot of insulin-stimulated FOXO1 and AKT phosphorylation in a dose response manner after Endo1 silencing with two consecutive transfections of Endo1 siRNA in HeLa cells during 4 days. Densitometry analysis is presented on the right panel. (B) Representative western blot of 10ng/mL LIF-stimulated AKT phosphorylation after Endo1 silencing with two consecutive transfections of Endo1 siRNA in HeLa cells during 4 days. Densitometry analysis is presented on the right panel. (C) Representative western blot of Endo1 expression at the protein level in HeLa cells silenced for Endo1 with two consecutive transfections of Endo1 siRNA during 4 days. Data are presented as means +/- SEM.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Lentivirus production and intracerebral stereotactic injection

Lentiviral production and stereotactic injection were performed as previously described (1). Stereotactic injection of Endo1 or control (1 mismatch on Endo1 sequence) shRNA was performed on 80 obese mice of 4 months old ("Obesity reversal experiment") or on 56 C57BL/6J male mice of 8 weeks old ("Obesity prevention experiment"). Two weeks after surgery, half of control or silenced mice were either fed a CD or a HFD during 5 months.

Generation of Endo1 KO mice

Conversely to humans, the mouse Endo1 gene localized on chromosome 4 contains four exons and is not genetically linked to OBR gene allowing the generation of transgenic mice with specific Endo1 gene disruption without affecting OBR expression. Endo1 floxed mice were generated using a targeting vector construct purchased from the NIH Knock-Out-Mouse Project (KOMP) where Endo1 exon 2 was flanked with loxP sites and introduced by homologous recombination in 129/SV CK35 embryonic stem cells (2). The neomycin resistance cassette flanked by FRT sites was excised by crossing Endo1$^{lox/wt}$ mice with FLP-expressing mice. Mice with Endo1 deletion were generated by intercrossing C57Bl/6 mice expressing Cre recombinase under the control of the mouse ubiquitous EIIa promoter (EIIa-Cre mice) (3) with Endo1 floxed mice. The disruption of Endo1 gene was assessed by PCR from DNA extract and the absence of Endo1 mRNA and protein expression was confirmed by RT-PCR and western blot.

RNA extraction and Quantitative Real-Time RT-PCR

RNAs were extracted from tissues with RNeasy® Mini Kit (Qiagen). Retrotranscription was performed using reverse transcriptase (Invitrogen) followed by qPCR with LightCycler SYBR Green (Roche). Primers for Endo1 (GGACTTCCTGTTGTTCTTGC and AAGACGAGGAAGAAGCCTTG) and RPLP0 (GGACCCGAGAAGACCTCCTT3' and GCACATCACTCAGAATTTCAATGG) were used as internal controls.

Food intake and fat mass

For the "Obesity reversal protocol", food intake (kcal) was measured at weeks 12–15 after shRNA injection in the ARC of obese mice after DIO. Perigonadal WAT was weighted to estimate the body fat mass. For the "Obesity prevention protocol", mice were housed individually and acclimated to metabolic chambers for 48 hours and spontaneous feeding was measured using automated feeding recording in Labmaster (TSE Systems). Whole lean and fat mass was extracted from the Echo Medical Systems (EchoMRI 100, Whole Body Composition Analyzers; EchoMRI, Houston, TX) analysis.

Cell culture and transfection

Human Embryonic Kidney (HEK) 293 cells stably expressing OBRb (4), human HeLa cells or murine hypothalamic N46 cells were grown in DMEM supplemented with 10% (vol/vol) FBS, 4.5 g/liter glucose, and 1mM glutamine (Life Technologies).

cDNAs for p85α and for p85α fused to the yellow variant of the green fluorescent protein (p85α-YFP), cDNAs for OBRb fused to Ypet (5) and for Endo1 fused to *Renilla* luciferase (Rluc), obtained by PCR from 6Myc-Endo1 (1), were transfected in cells with JetPEI® (Polyplus-transfection™).

Protein Endo1 downregulation was efficiently performed by transfecting cells twice for 96 hours using Lipofectamine 2000 (Invitrogen) with either CTRL (SR-CL000-005, Eurogentec) or Endo1 siRNA (ACAUGUGCACAUGCGGCAU).

Western Blot (WB)

Antibodies:

Cell or tissue lysates were separated by SDS/PAGE, transferred to nitrocellulose membranes and immunoblotted with anti-phosphotyrosine (Tyr-705) STAT3 (Cell Signaling), anti-phosphoserine (Ser-473) AKT (Cell Signaling), anti-STAT3 (Cell Signaling), anti-AKT (Cell Signaling), anti-ERK2 (SantaCruz), anti-Rluc (Millipore), anti-GFP (Roche), anti-phosphothreonine (Thr-24) FOXO1 (Cell Signaling), anti-FOXO1 (Cell Signaling), anti- α-tubulin (Sigma), anti-β-actin (Sigma), anti-lamin B1 (Sigma), anti-p85 (gift from Dr F. Verdier) and anti-Endo1 (6) antibodies. WB were scanned on the Odyssey infra-red Imaging System (Licor) and quantified with ImageJ.

Expression of p85 and FOXO1:

Hypothalamus, cortex and brainstem were dissected from 12-week old Endo1 KO and WT mice. Tissues were lysed in TEM buffer, supplemented with CHAPS 10mM (Sigma), on wheel at 4°C for 4 hours. Lysates were cleared by centrifugation at 14000 rpm for 1 hour at 4°C. Protein content was determined using the BCA method (Thermo Fisher), and equal amounts of proteins were mixed with Laemmli buffer supplemented with 30mM DTT. Protein expression was analyzed by SDS-PAGE and WB. In parallel, HeLa cells were silenced for Endo1 by siRNA transfection as described above. Cells were lysed in Laemmli buffer with protease inhibitors and analyzed in WB.

Co-immunoprecipitation

cDNAs for p85α and for p85α fused to the yellow variant of the green fluorescent protein (p85α-YFP), cDNAs for OBRb fused to Ypet (5) and for Endo1 fused to *Renilla* luciferase (Rluc), obtained by PCR from 6Myc-Endo1 (1), were transfected in HEK293 cells with JetPEI® (Polyplus-transfection™). Hypothalamic tissues were dissected from fasted 12-week old Endo1 KO and WT mice. Cells or hypothalamic tissues were lysed in TEM buffer (Tris HCl 25mM pH 7.5, EDTA 2mM, MgCl2 10mM, protease and phosphatase inhibitors), supplemented with CHAPS 10mM (Sigma), on wheel at 4°C for 4 hours. Lysates were cleared by centrifugation at 14000 rpm for 1 hour at 4°C and subjected to p85 or OBR immunoprecipitation, overnight on wheel at 4°C, with anti-GFP antibody (2µg, Roche) or anti-p85 (1:500, Millipore) as specified for each experiment. For endogenous experiments, control rabbit IgG (1:500, Santa Cruz Biotechnology) was used as negative control. Protein G Sepharose® (Sigma), previously saturated with 2% BSA, were then added for 2 additional hours on wheel at 4°C, followed by washes with lysis buffer. Protein complexes were denatured in Laemmli buffer supplemented with 50mM DTT, separated by SDS-PAGE and analyzed by Western Blot.

Leptin signaling in hypothalamic ARC

16 h-fasted mice were intraperitoneal injected either with 1mg/kg murine leptin (PLR Ltd, Rehovot) or saline and killed 60 min later. Hypothalamic ARC, recovered by micropunches of 200 µm frozen brain sections, were denatured in Laemmli buffer containing phosphatase inhibitors, separated by SDS-PAGE and analyzed by Western Blot.

Leptin signaling in primary culture of hepatocytes

Hepatocytes were isolated from dissected liver (25-30g) of fed 12-week old Endo1 KO and WT mice, by collagenase as previously described (7). Hepatocytes were seeded for 6h on type I collagen-coated dishes ($2*10^4$ cells /cm^2), in M199 medium (Invitrogen) supplemented with 10% (vol/vol) FBS, 4.5g/L glucose, 10 µg/ml streptomycin, 100 units/ml penicillin and 100 nM dexamethasone (Sigma). Before cDNA transfection, the medium was replaced by fresh supplemented M199 medium. Primary hepatocytes were transfected with OBRb-Rluc-expressing vector with Lipofectamine® 2000 (Invitrogen), following the instructions of the manufacturers. 16h-starved cells were stimulated with 10nM leptin in kinetics experiments at 37°C, before being lysed in Laemmli buffer supplemented with 10mM DTT and phosphatase inhibitors and analyzed in WB.

Immunohistochemistry

Animals were anesthetized with Ketamin/Rompon and perfused with 10mM phosphate buffer, pH 7.4, 140 mM NaCl (PBS) followed by 4% PFA in PBS. After dissection, the brains were 24h-fixed and dehydrated in PBS/20% sucrose. Brains were sectioned coronally at 14µm throughout the hypothalamus on a cryostat and collected on super frost plus glass slides and stored at -80°C. Brain sections were defrosted, rinsed in Tris Buffer Saline (TBS), incubated in -20°C methanol (15min) then in 0.1% H2O2 in methanol (15min) to quench endogenous peroxide, then washed in TBS followed by incubation in blocking solution (1h). Sections were incubated overnight at 4°C with the anti-phospho-AKT Ser473, IHC-specific (Cell Signaling Technology) diluted in blocking solution (1:50). On the next day, sections were washed in TBS (15min), incubated with a biotinylated secondary goat anti-rabbit antibody (1:1000, in blocking solution, 1h) and then treated with ABC (Vector Laboratories) solution according to manufactures instructions. Finally, the signal was developed by nickel-diaminobenzidine solution (Vector Laboratories), giving a gray/black precipitate. Positively

stained cells were counted on captured section images using ImageJ. The number of positive cells was compared to the total number of cresyl violet-stained neurons.

REFERENCES FOR THE SUPPLEMENTAL EXPERIMENTAL PROCEDURES

1. Couturier C, Sarkis C, Seron K, Belouzard S, Chen P, Lenain A, Corset L, Dam J, Vauthier V, Dubart A, et al. Silencing of OB-RGRP in mouse hypothalamic arcuate nucleus increases leptin receptor signaling and prevents diet-induced obesity. *Proceedings of the National Academy of Sciences of the United States of America.* 2007;104(49):19476-81.
2. Kress C, Vandormael-Pournin S, Baldacci P, Cohen-Tannoudji M, and Babinet C. Nonpermissiveness for mouse embryonic stem (ES) cell derivation circumvented by a single backcross to 129/Sv strain: establishment of ES cell lines bearing the Omd conditional lethal mutation. *Mammalian genome : official journal of the International Mammalian Genome Society.* 1998;9(12):998-1001.
3. Holzenberger M, Lenzner C, Leneuve P, Zaoui R, Hamard G, Vaulont S, and Bouc YL. Cre-mediated germline mosaicism: a method allowing rapid generation of several alleles of a target gene. *Nucleic acids research.* 2000;28(21):E92.
4. Hosoi T, Sasaki M, Miyahara T, Hashimoto C, Matsuo S, Yoshii M, and Ozawa K. Endoplasmic reticulum stress induces leptin resistance. *Molecular pharmacology.* 2008;74(6):1610-9.
5. Bacart J, Leloire A, Levoye A, Froguel P, Jockers R, and Couturier C. Evidence for leptin receptor isoforms heteromerization at the cell surface. *FEBS letters.* 2010;584(11):2213-7.
6. Seron K, Couturier C, Belouzard S, Bacart J, Monte D, Corset L, Bocquet O, Dam J, Vauthier V, Lecoeur C, et al. Endospanins regulate a postinternalization step of the leptin receptor endocytic pathway. *The Journal of biological chemistry.* 2011;286(20):17968-81.
7. Stephenne X, Foretz M, Taleux N, van der Zon GC, Sokal E, Hue L, Viollet B, and Guigas B. Metformin activates AMP-activated protein kinase in primary human hepatocytes by decreasing cellular energy status. *Diabetologia.* 2011;54(12):3101-10.