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Supplemental Information

IRS2 Signaling in LepR-b Neurons Suppresses FoxO1 to Control Energy Balance Independently of Leptin Action

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Supplemental Experimental Procedures

In situ hybridization: Mice were perfused with room temperature 4% formaldehyde in PBS. Brain samples were postfixed in 4% formaldehyde in PBS for 18–24 h at 4°C, dehydrated, and embedded in paraffin. Brain sections (5 μm in thickness) were subjected to *in situ* hybridization (ISH) by using RNAscope (Advanced Cell Diagnostics) following the manufacturer's protocol.

Western Blotting: Mouse whole brain, hypothalamus, liver, muscle, islets, WAT and BAT were removed, homogenized and applied for direct immunoblotting (50 μg) as described previously (Dong et al., 2008). To assess the expression levels of Irs2, rabbit polyclonal IRS2 antibody (Upstate Biotechnology Inc. NY, USA; now known as EMD Millipore) was used. Irs2 expression was quantified by densitometry and levels were normalized to β-actin levels for presentation.

Islet morphology and immunohistochemistry: For immunohistochemistry, pancreatic sections were fixed for 16 hours in 4% paraformaldehyde and then transferred to PBS until embedding in paraffin as previously described (Lin et al., 2004). Following rehydration and permeabilization with 1% Triton X-100, sections were incubated overnight at 4°C with guinea pig anti-insulin (Zymed Laboratories Inc.). Alexa-Flour (Invitrogen) secondary antibodies were used. Mounting media included 4′,6′-diamino-2-phenylindole (DAPI) to stain nuclei (ProLong Gold; Invitrogen). For quantification of β cell area, we analyzed 4-5 pancreata per using 4 sections at least 150 μm apart from each other. For each section, the total area occupied by insulin-positive cells was analyzed using Spot Imaging Solutions, Ver 5.0, advanced. Results are expressed as the percentage of the total pancreatic area. Hematoxylin and eosin (H&E) staining was performed using paraffin embedded pancreatic tissue sections as previously described (Norquay et al., 2009).

Microdissection and PCR analysis: Mice were weighed just prior to euthanasia, after which brains were microdissected to obtain the ARC, VMH, PVN, NTS and LHA which were snap frozen and stored at −80°C as previously described (Leinninger et al., 2009). To detect Cre-mediated recombination at the genomic DNA levels of various tissues by PCR, we utilized primers for the floxed *Irs2* and for the *Irs2* coding region as

previously described (Hennige et al., 2003). For the analysis of *Irs2* and *Socs3* mRNA expression, RNA was extracted as described in the main text, and analyzed using quantitative real-time PCR with customized primers. Actin gene expression was used to normalize RNA content and the relative gene product amounts were reported as mean ±SEM of several animals. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Endocrine profile: Mouse trunk blood was collected from 3-month-old mice and assayed for hormone levels. Serum corticosterone levels were measured by EIA (Arbor Assays) from fasted mice. Serum thyroxine (T4) was measured according to the manufacturer's instructions (Siemens).

Fertility assessment: Female mice for study were housed individually from the time of weaning at 21 days old. Beginning at 28 days of age female mice were monitored daily for vaginal opening and then for vaginal estrus by cellular histology through three cycles, or until 10 weeks old as previously described (Holzenberger et al., 2003). All data were collected between 14:00 and 16:00. Mating success rates were determined by pairing experimental mice with unrelated control mice (known to be fertile) for 2 months or until a litter was produced. Pairs were monitored regularly for signs of visible pregnancy.

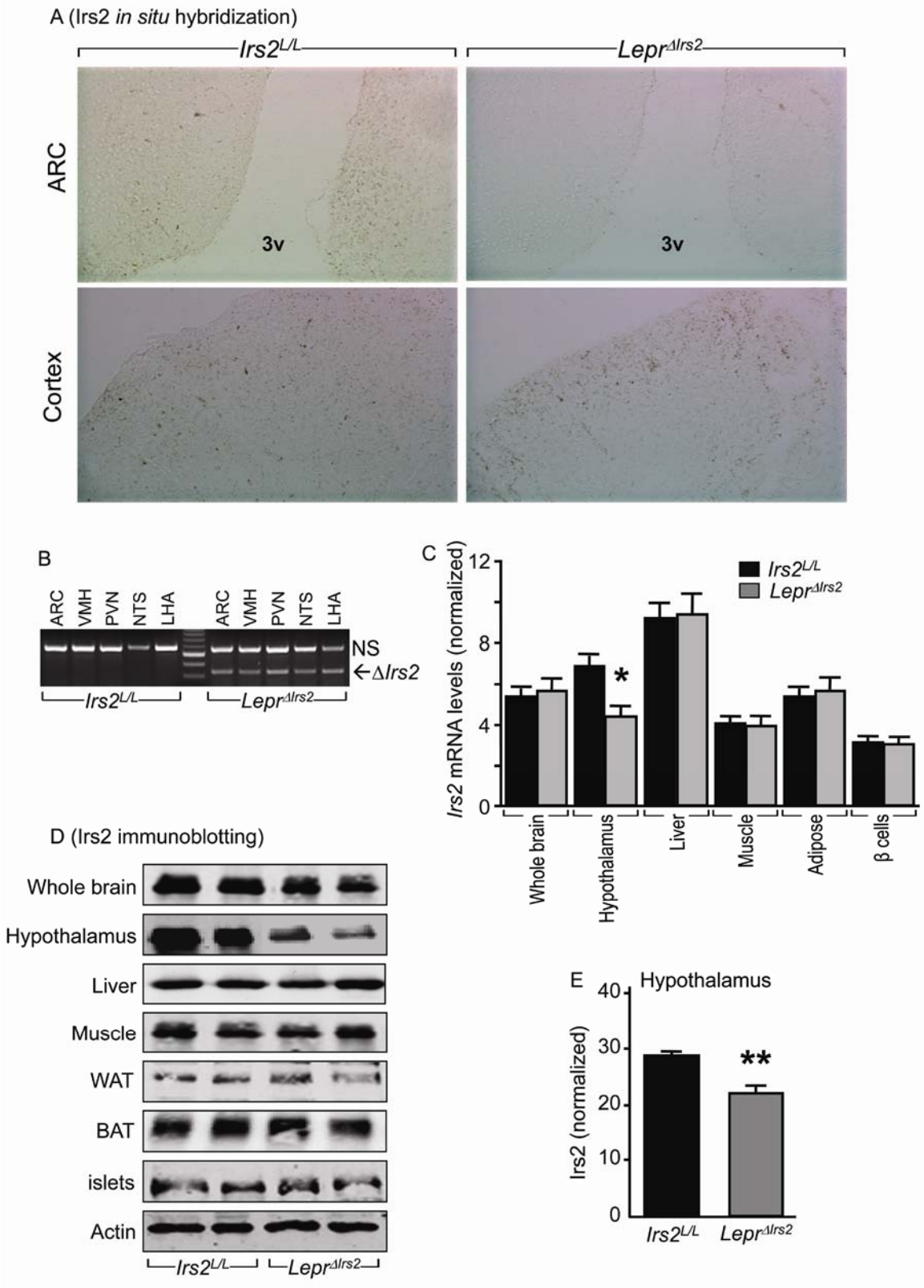

Figure S1. Related to Figure 1. (A) Representative images from *in situ* hybridization for *Irs2* (brown) in ARC and cortex from control and *Lepr*$^{\Delta Irs2}$ mice. 3v= 3rd cerebral

ventricle. (B) PCR analysis of genomic DNA from microdissected brain areas of control and *Lepr*$^{\Delta Irs2}$ mice to detect intact *Irs2*^{L} and *Irs2*$^{\Delta}$ (following cre-mediated recombination). (C) *Irs2* mRNA expression in the indicated tissues of control and *Lepr*$^{\Delta Irs2}$ mice. The mRNA levels were normalized to β-actin and are expressed as a unitless ratio of Irs2/b-actin. (D) Western blot analysis of Irs2 in the indicated tissues of control and *Lepr*$^{\Delta Irs2}$ mice. (E) Quantification of Irs2 protein expression in hypothalamus (as in (D)), normalized to β-actin (n=4/genotype). Data are presented as a unitless ratio of Irs2 density/b-actin density (mean ± SEM), **, $p < 0.01$.

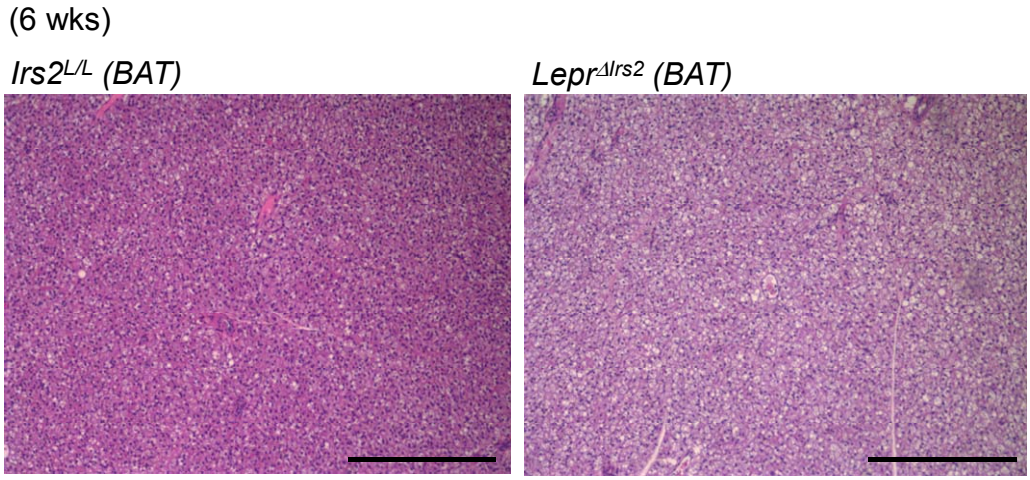

Figure S2. Related to Figure 2. Representative H&E staining of brown adipose tissue (BAT) of *Irs2*$^{L/L}$ (left panel) and *Lepr*$^{\Delta Irs2}$ mice (right panel) aged 6 weeks. Scale bar: 500 μm.

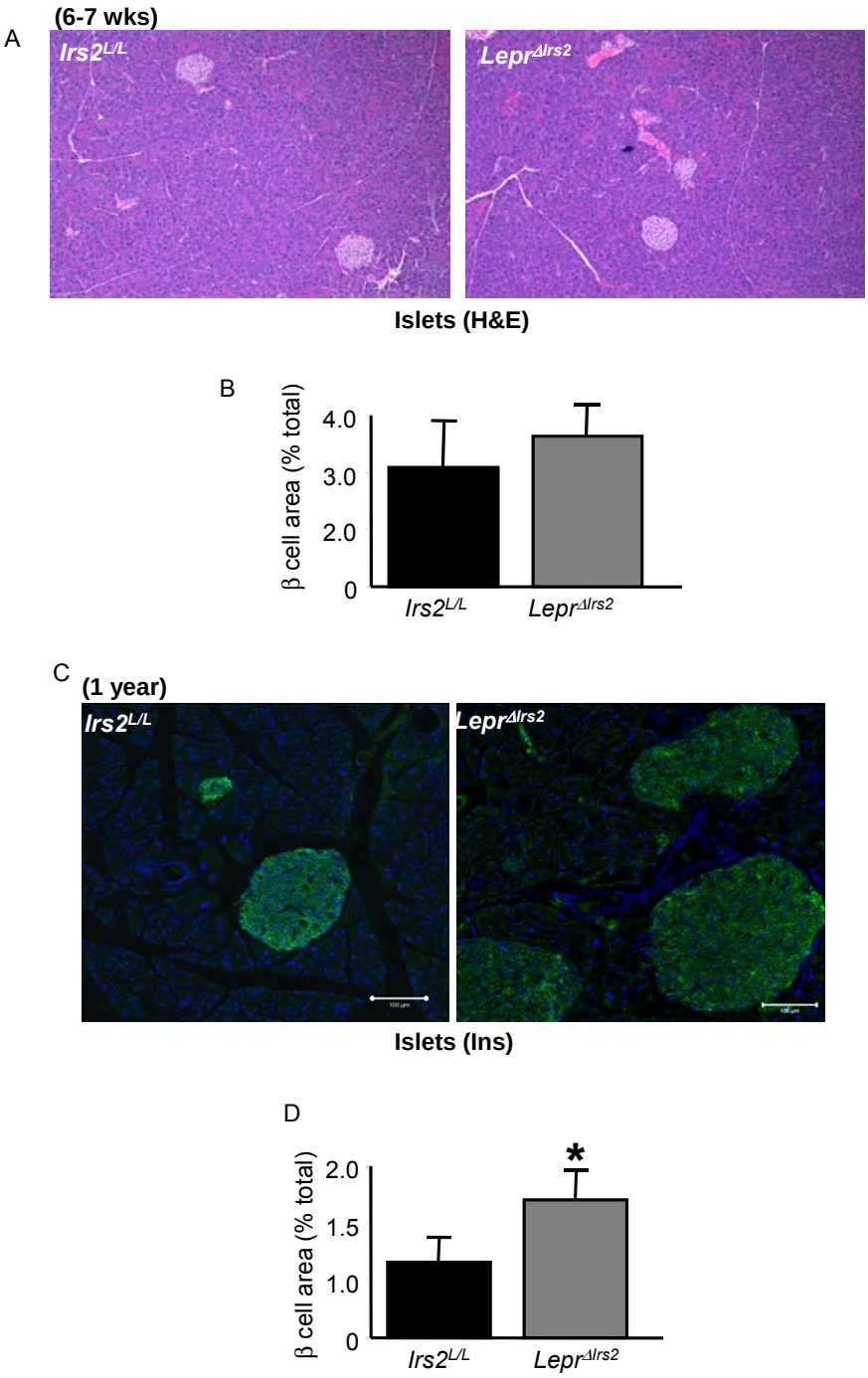

Figure S3. Related to Figure 3. (A) Representative H&E staining of pancreatic islets of *Irs2*$^{L/L}$ and *Lepr*$^{\Delta Irs2}$ mice aged 6-7 weeks. (B) The percentage of the total pancreatic area occupied by β cells in 6-7 weeks old male mice of the indicated genotypes. (C) Immunofluorescence staining for insulin (green) in islets from 1-year-old mice (DNA staining- DAPI, blue). Scale bar: 100 μm. (D) The percentage of the total pancreatic area occupied by β cells in 1-year-old male mice of the indicated genotypes (n=4-5/genotype). Data in (B, D) are presented as mean ± SEM; *, p< 0.05.

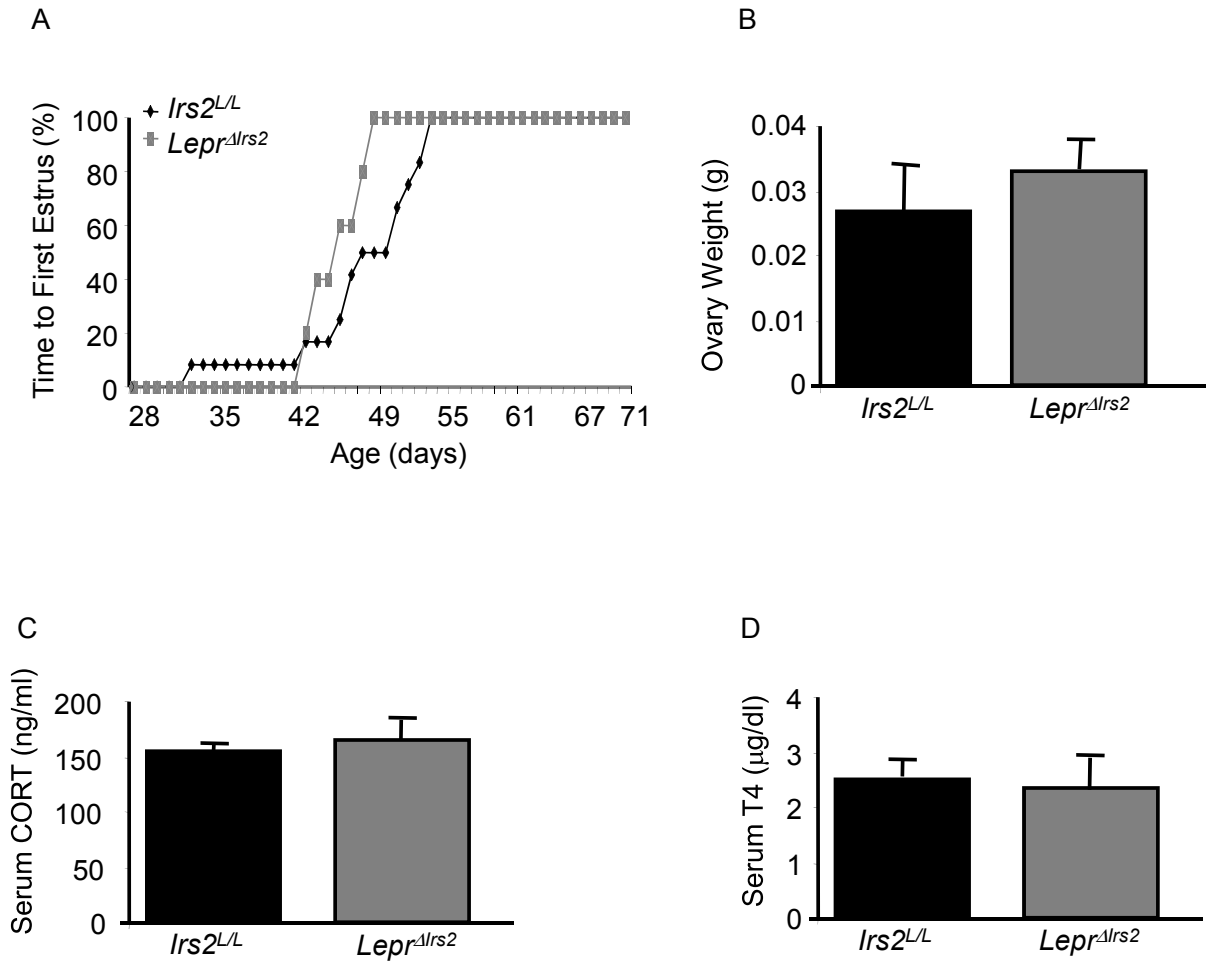

Figure S4. Related to Figure 3. (A) Onset of estrus in female mice of the indicated genotypes. (B) Ovarian weight of female mice of the indicated genotypes (10 weeks of age, n=8-12) (C) Fasting CORT levels in male mice of the indicated genotypes at 8 weeks (n=4/ genotype). (D) Serum T4 levels in male mice of the indicated genotypes at 12 weeks of age (n=6/genotype). Data are presented as mean ± SEM; *, $p<0.05$.

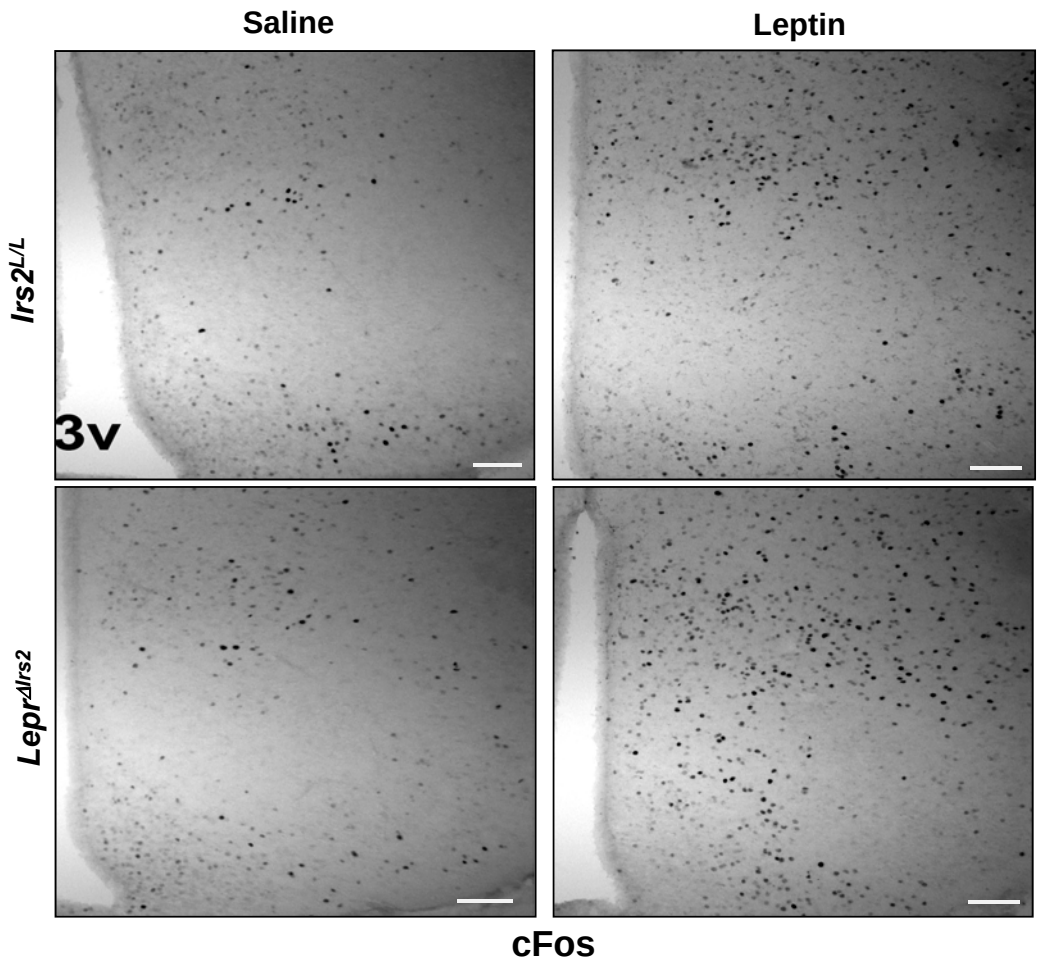

Figure S5. Related to Figure 4. Representative images of cFos-immunoreactivity in the hypothalamus of 6-week-old mice of the indicated genotypes following treatment with vehicle or leptin (5 mg/kg, i.p., 2 hours). 3v- 3rd cerebral ventricle. Scale bar: 100μm

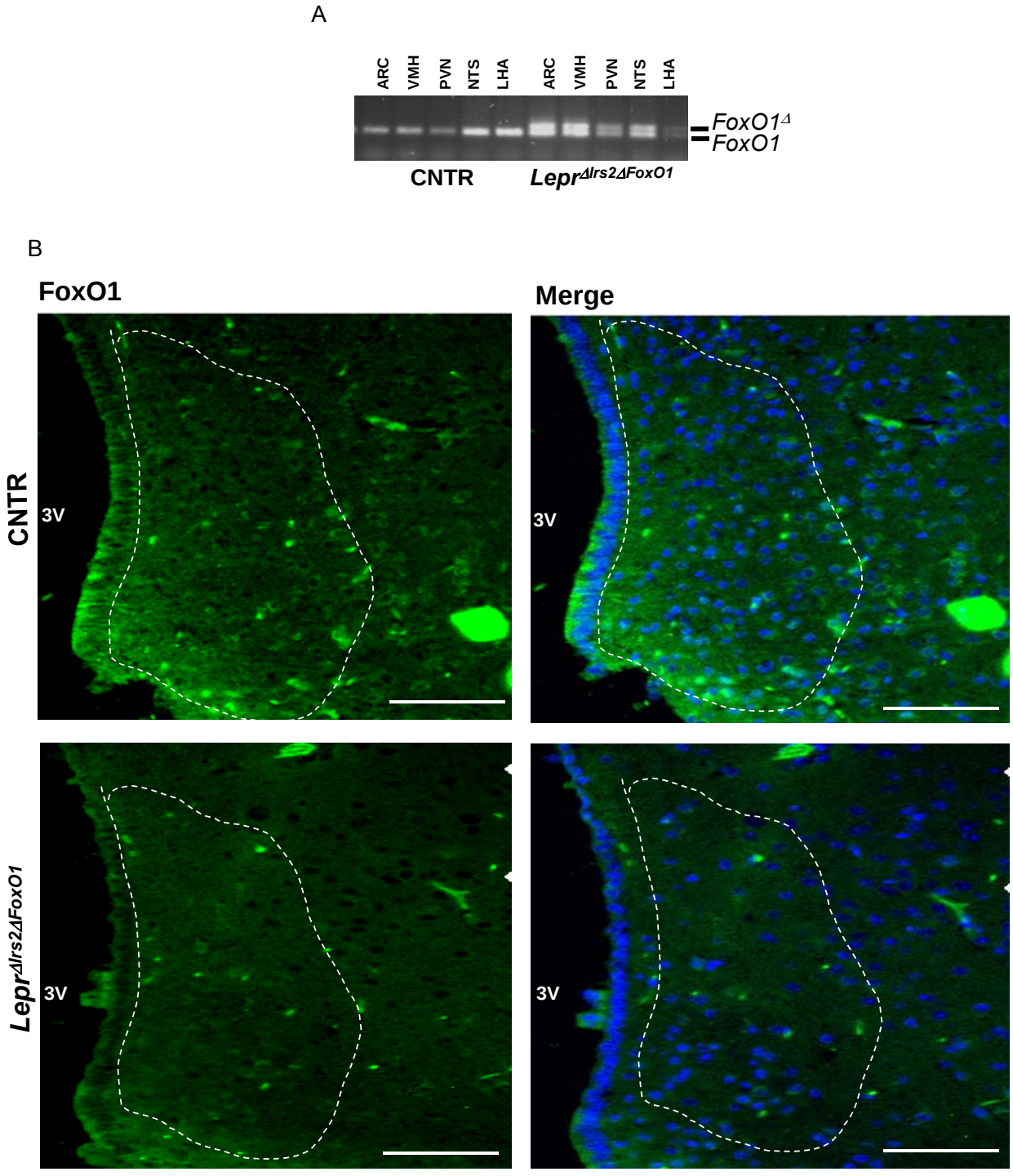

Figure S6. Related to Figure 6. (A) PCR analysis of genomic DNA from microdissected brain tissue of control (CNTR) and *Lepr*$^{\Delta Irs2\Delta FoxO1}$ mice to detect intact *Foxo1*^{L} and *Foxo1*$^{\Delta}$ (following cre-mediated recombination) in the indicated brain regions. (B) Representative images of immunofluorescent detection of FoxO1 in the ARC of 12-week-old CNTR and *Lepr*$^{\Delta Irs2\Delta FoxO1}$ mice. Green: FoxO1, blue: DAPI (DNA labeling). Scale bar: 100μm. 3v= 3rd cerebral ventricle

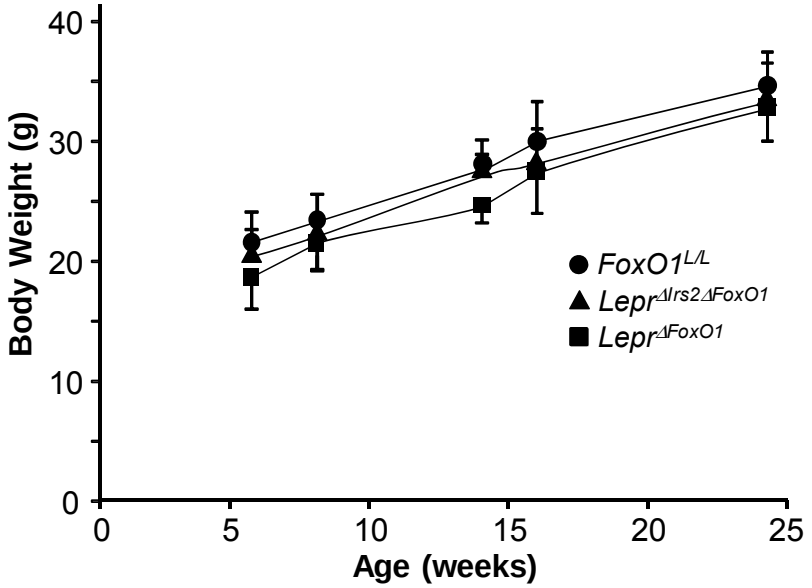

Figure S7. Related to Figure 6. Body weight of chow-fed male mice of the indicated genotypes. Data are presented as mean ± SEM at the indicated time intervals; p=NS at all ages.

Table S1. Fertility Assessment in Control and *Lepr*$^{\Delta lrs2}$ Mice, Related to Figure 3

Cross	No. sets	No. litters	No. offspring per litter
Male control X female control	3	3	8.8±0.6
Male control X female *Lepr*$^{\Delta lrs2}$	3	3	9.1±0.9
Male *Lepr*$^{\Delta lrs2}$ X female control	3	3	10.5±0.5
Male *Lepr*$^{\Delta lrs2}$ X female *Lepr*$^{\Delta lrs2}$	3	3	9.2±1.3

Supplemental References

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