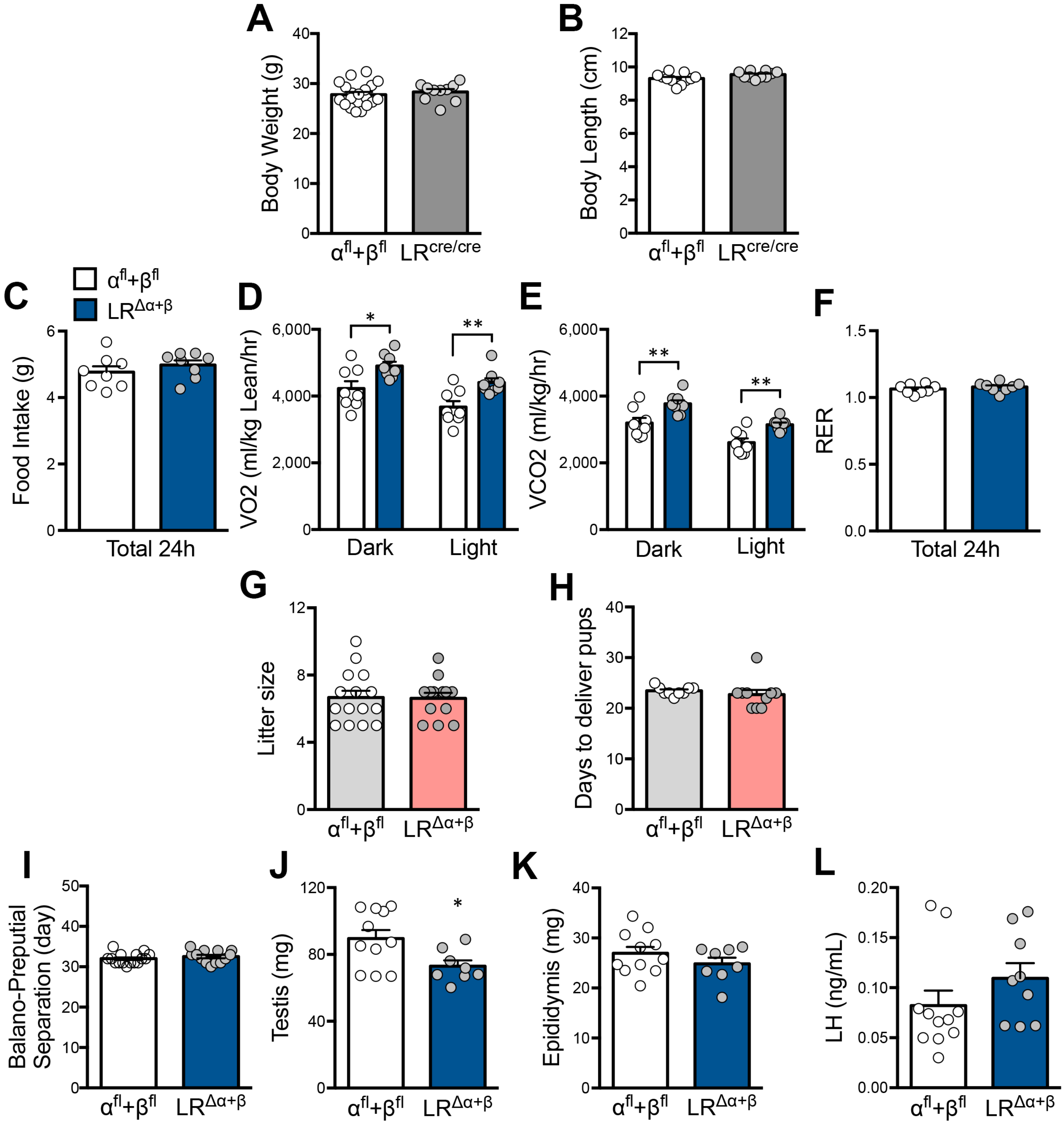
SUPPLEMENTAL MATERIAL

PI3Kα inactivation in leptin receptor cells increases leptin sensitivity but disrupts growth and reproduction

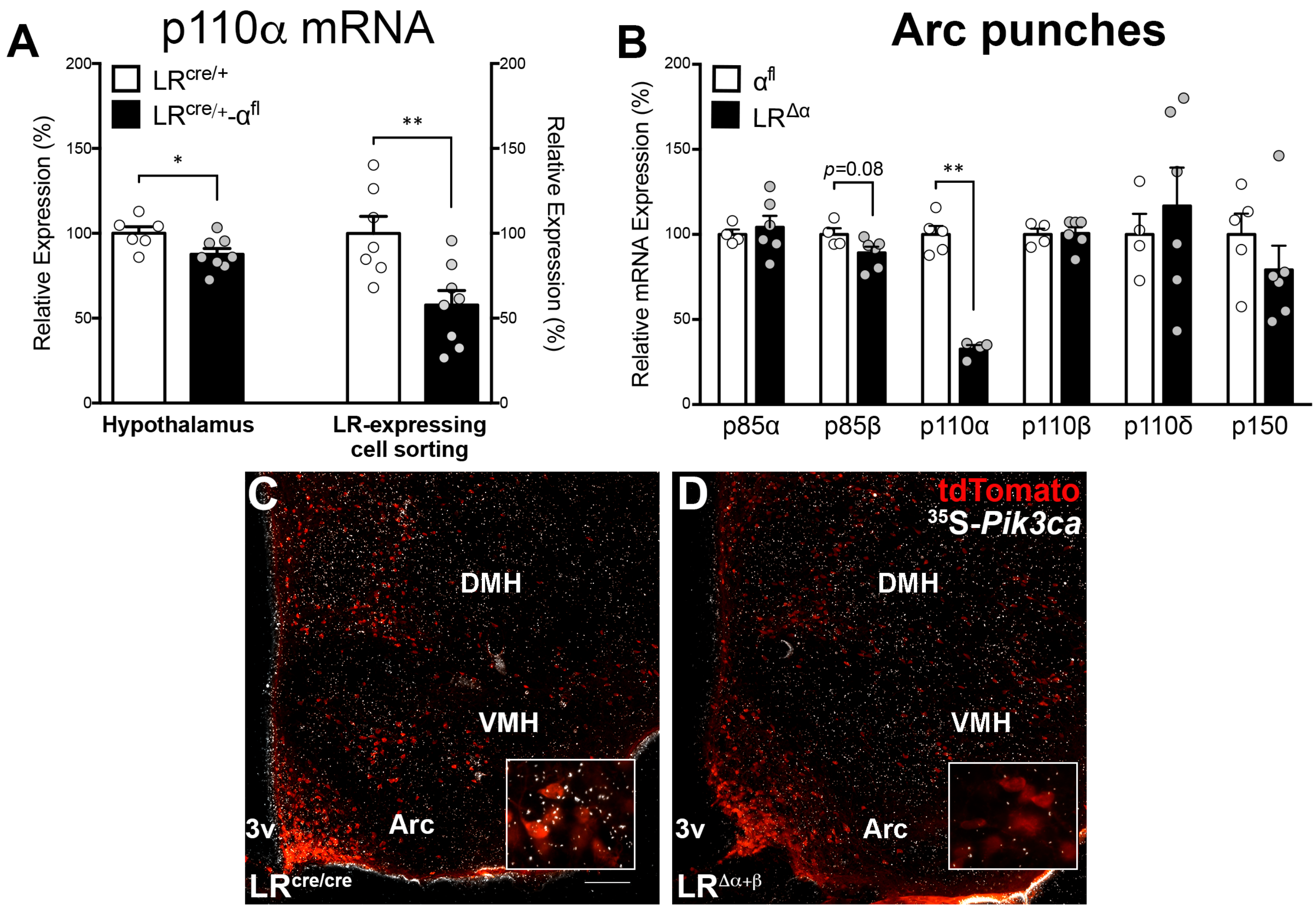
David Garcia-Galiano[1], Beatriz C. Borges[1,2], Jose Donato Jr[3], Susan J. Allen[1], Nicole Bellefontaine[1], Mengjie Wang[4], Jean J. Zhao[5], Kenneth M. Kozloff[6], Jennifer W. Hill[4], Carol F. Elias[1,7] *

[1]Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, USA;
[2]Department of Physiology, University of São Paulo, Ribeirão Preto, SP, Brazil;
[3]Department of Physiology and Biophysics, University of São Paulo, São Paulo, SP, Brazil;
[4]Department of Physiology and Pharmacology, University of Toledo, Toledo, OH, USA;
[5]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA;
[6]Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI, USA;
[7]Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, MI, USA.

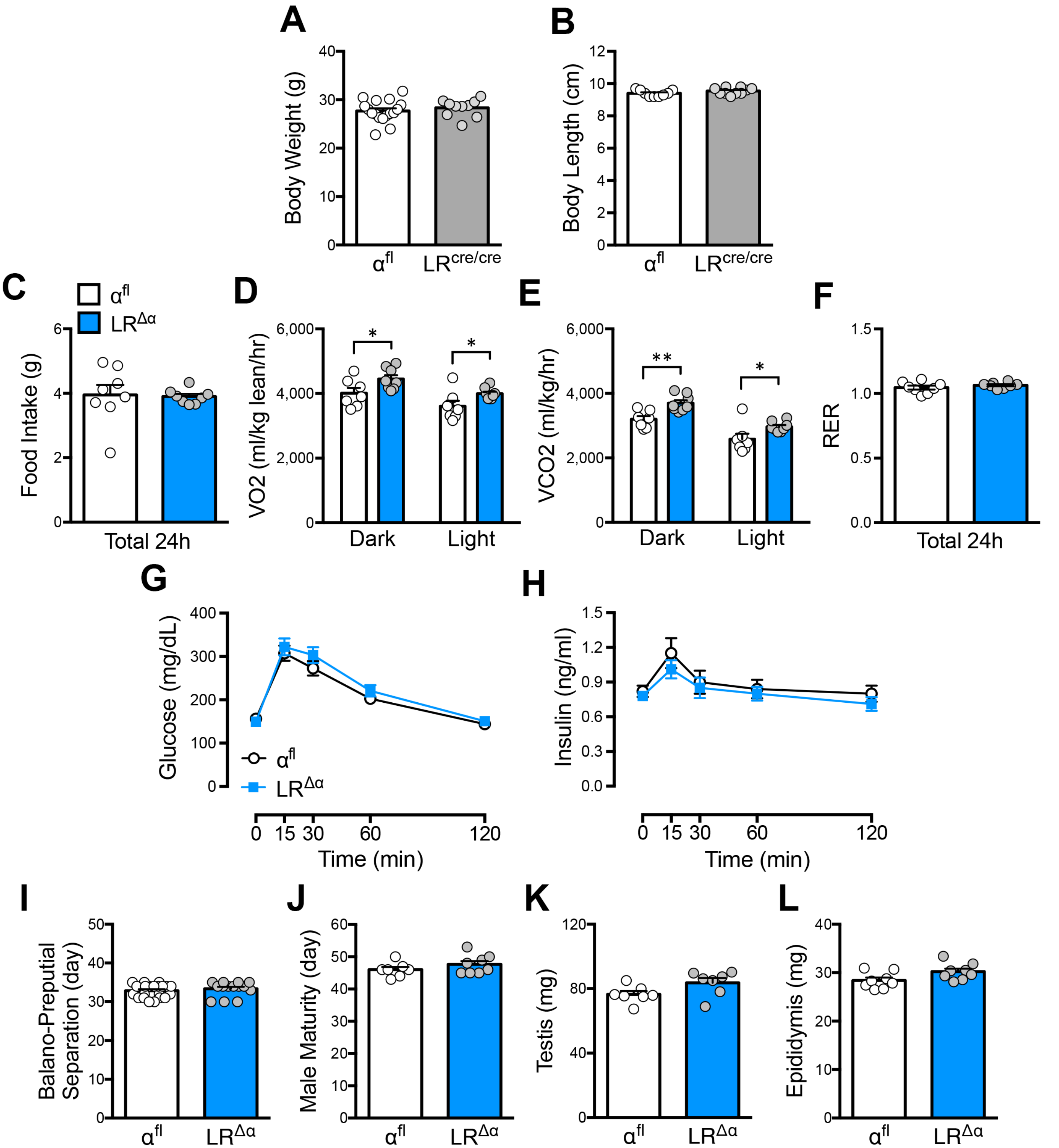
Supplementary Figures: 6



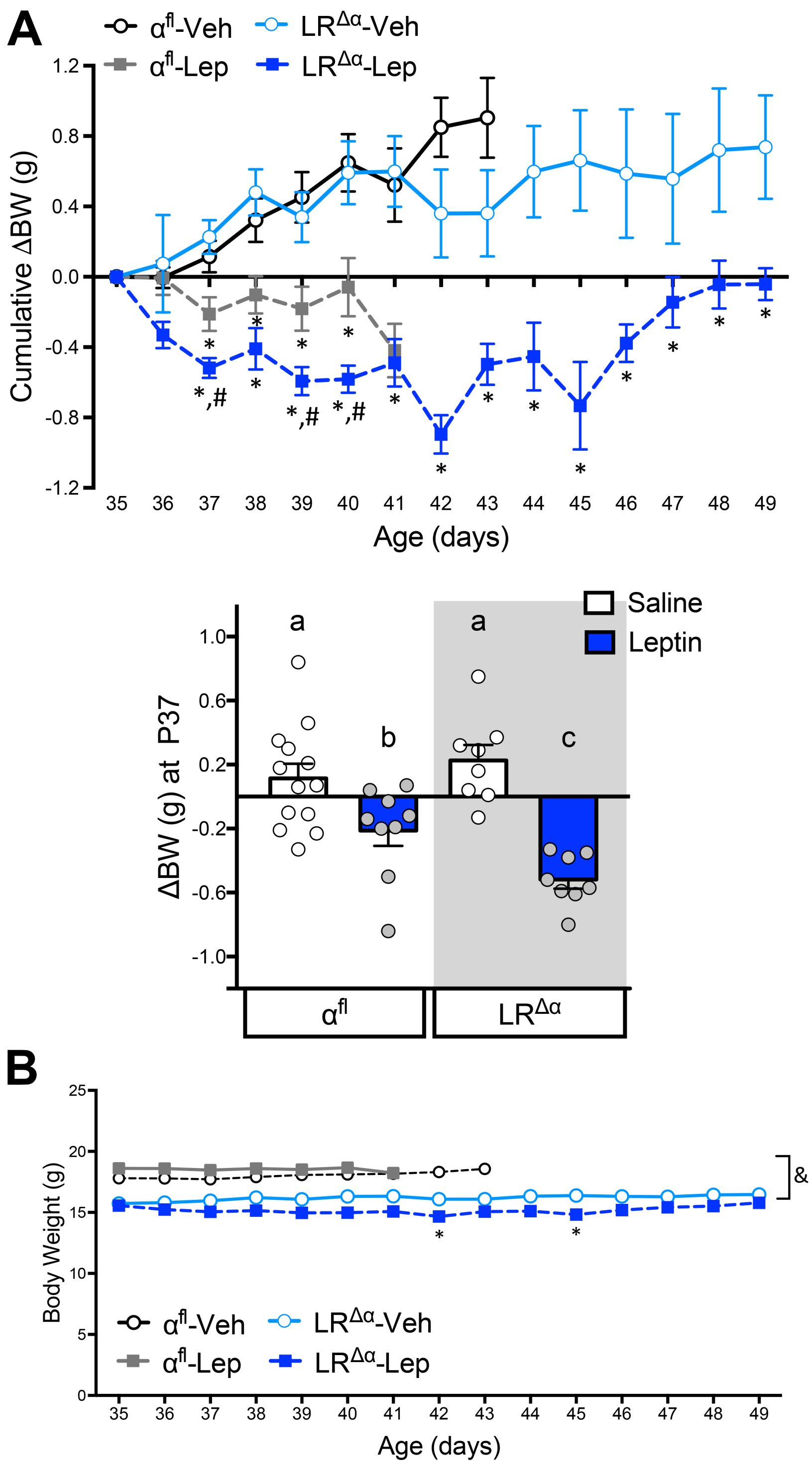
Supplementary Figure 1. Metabolic and reproductive phenotypes of $LR^{\Delta\alpha+\beta}$ and $LR^{cre/cre}$ mice. (**A**) Similar body weight of $\alpha^{fl}+\beta^{fl}$ (n=19) and $LR^{cre/cre}$ (n=10) males at 12 weeks of age, and (**B**) body length in adult $\alpha^{fl}+\beta^{fl}$ (n=11) and $LR^{cre/cre}$ (n=10) males. (**C**) Daily food intake in $\alpha^{fl}+\beta^{fl}$ and $LR^{\Delta\alpha+\beta}$ (n=8/group) males. (**D**) Increased O_2 consumption (t_{14}=2.68, p=0.018 in dark phase; t_{14}=3.33, p=0.005 in light phase); and (**E**) CO_2 production in dark and light phase in $LR^{\Delta\alpha+\beta}$ compared to $\alpha^{fl}+\beta^{fl}$ males (n=8/group; t_{14}=3.09, p=0.008 in dark phase; t_{14}=3.87, p=0.0017 in light phase), during CLAMS study. (**F**) No changes in respiratory exchange ratio (RER) average in 24 hours were detected between groups. (**G**) Litter size ($\alpha^{fl}+\beta^{fl}$, n=15; and $LR^{\Delta\alpha+\beta}$, n=13) and (**H**) days to deliver pups. (**I**) Similar day of pubertal completion in males by balano-preputial separation ($\alpha^{fl}+\beta^{fl}$, n=16; and $LR^{\Delta\alpha+\beta}$, n=13), but (**J**) reduced testis weight in $LR^{\Delta\alpha+\beta}$ mice (t_{17}=2.44, p=0.026). No changes in (**K**) epididymis weight, (**L**) or serum LH levels in $\alpha^{fl}+\beta^{fl}$ (n=11) and $LR^{\Delta\alpha+\beta}$ (n=8) mice at 9 weeks of age. Each point represents one individual mouse, and all values are presented as mean ± s.e.m. *$p < 0.05$, **$p < 0.01$ *vs* control mice; by two-tailed Student's *t*-test.



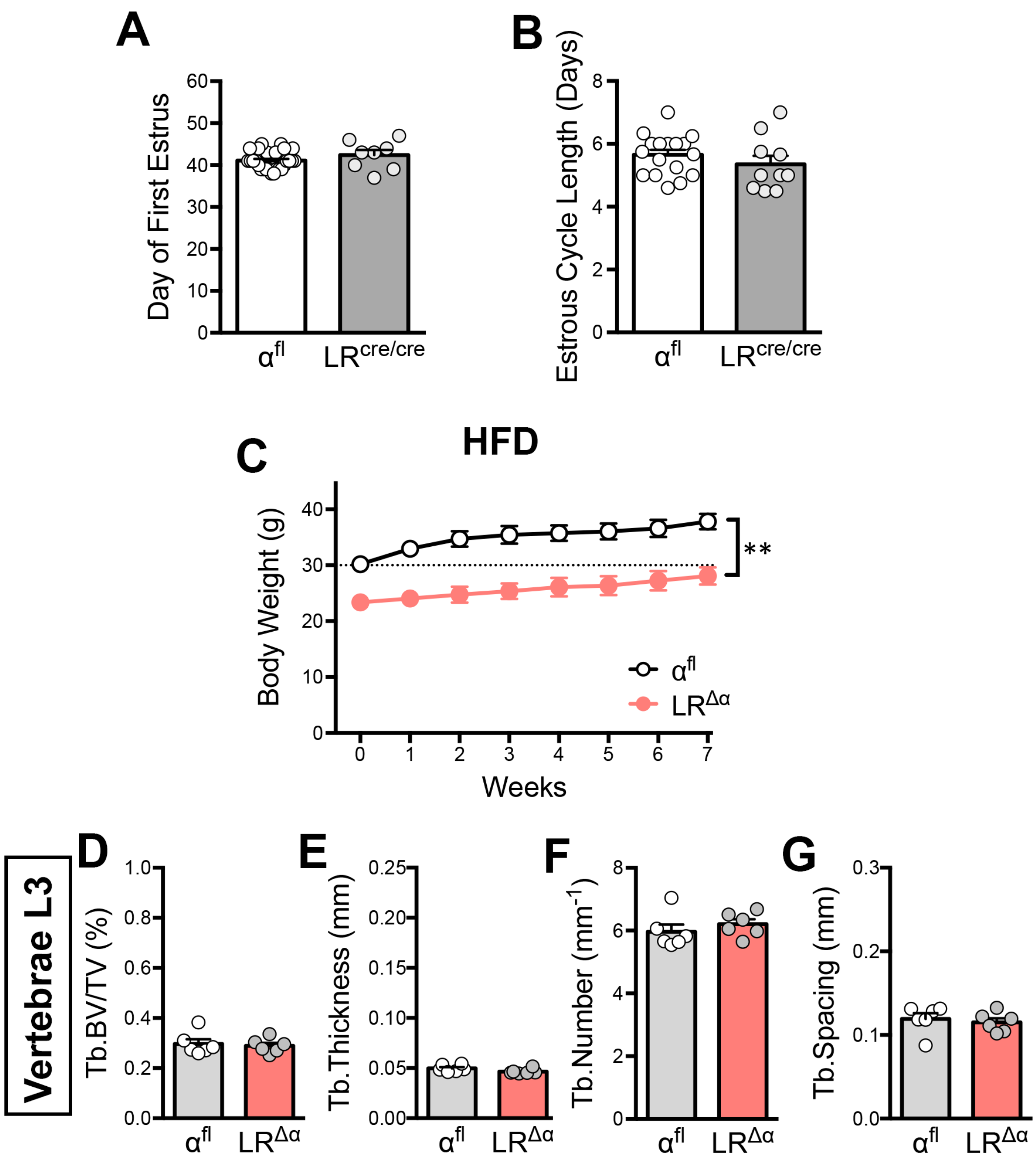
Supplementary Figure 2. Validation of PI3Kα mRNA deletion in LR cells. (**A**) Significant reduction of Pik3ca mRNA (coding for p110α/PI3Kα subunit) expression in whole hypothalamus of $LR^{cre/+}$-α^{fl} (n=8) respect to $LR^{cre/+}$ mice (n=6; t_{12}=2.41, *p*=0.033), and in neurons that putatively express the LR isolated from by fluorescence-activated cell sorting (FACS), using the tdTomato fluorescent protein ($LR^{cre/+}$, n=7; $LR^{cre/+}$-α^{fl}, n=8; t_{13}=3.25, *p*=0.006). Reduction of 12% and 42.2% in Pik3ca expression, respectively, were observed compared to controls. (**B**) Detection of mRNA expression levels of different PI3K catalytic and regulatory subunits in arcuate nucleus (Arc) punches of adult $LR^{\Delta\alpha}$ (n=6) and α^{fl} females (n=4-5; t_8=2.04, *p*=0.08 for p85β) showed a 70% reduction of Pik3ca expression in Arc of $LR^{\Delta\alpha}$ compared to α^{fl} mice (t_7=11.22, *p*<0.0001). Representative images of Pik3ca mRNA (silver grains) distribution in the Arc, dorsomedial and ventromedial nucleus of the hypothalamus (DMH and VMH, respectively) over (**C**) $LR^{cre/cre}$ and (**D**) $LR^{\Delta\alpha+\beta}$ tdTomato (n=3/group) positive cells. Dual-label ISH and IHC shows minimal hybridization signal over tdTomato cells in $LR^{\Delta\alpha+\beta}$ mice. Each circle represents one individual mouse, and all values are showed as mean ± s.e.m. Scale bar: 100 µm. **p*< 0.05, ***p*< 0.01 *vs* control; by two-tailed Student's *t*-test.



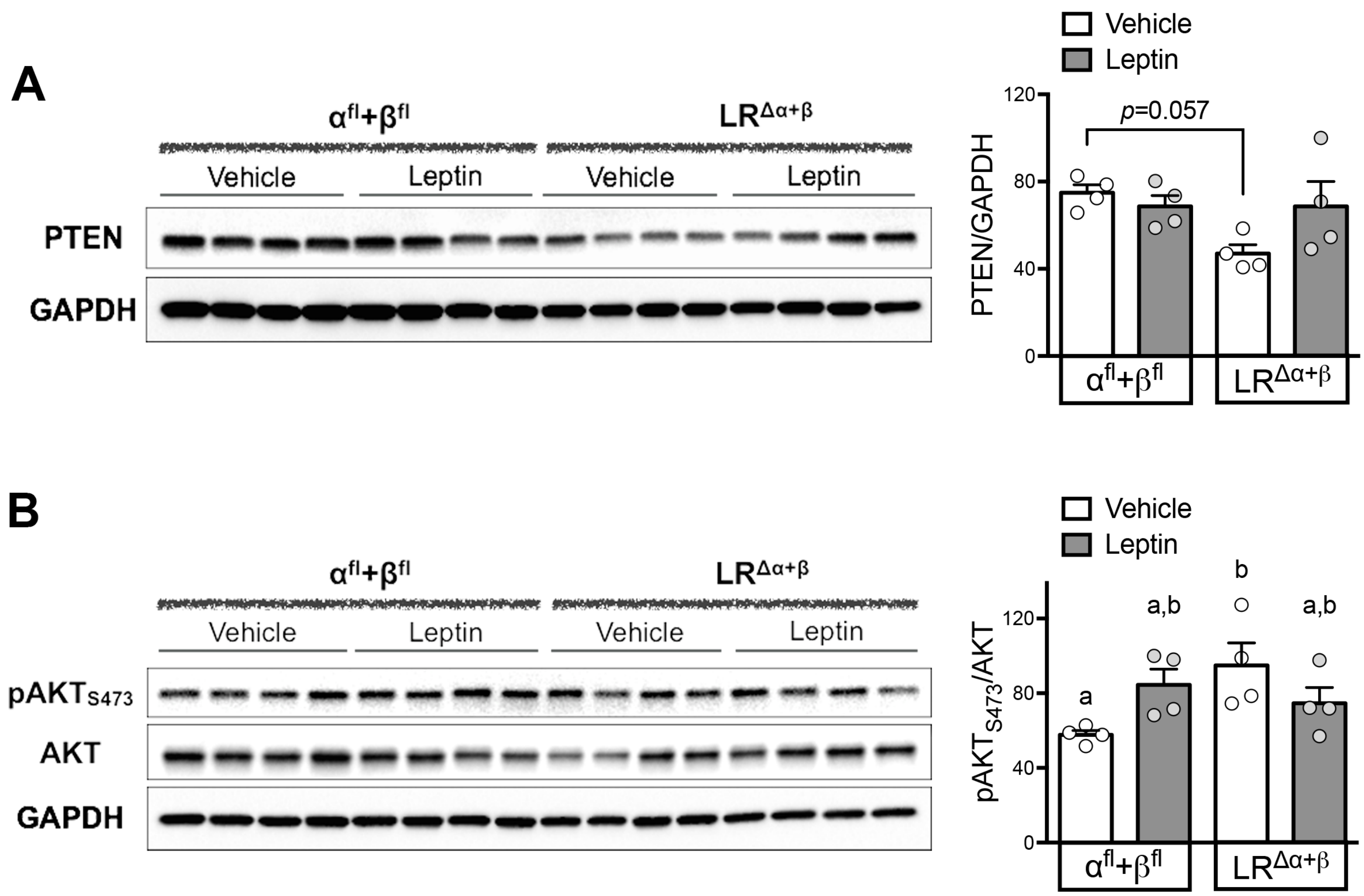
Supplementary Figure 3. Metabolic and reproductive phenotypes of LR$^{\Delta\alpha}$ mice. (**A**) Similar body weight in young adult α^{fl} (n=17) and LR$^{cre/cre}$ (n=10) males, and (**B**) body length in α^{fl} (n=9) and LR$^{cre/cre}$ (n=10) males. (**C**) Total daily food intake in α^{fl} and LR$^{\Delta\alpha}$ males (n=8/group) during CLAMS study. Increased (**D**) O_2 consumption (t_{13}=2.24, *p*=0.04 in dark and light phases), and (**E**) CO_2 production in dark and light phases in LR$^{\Delta\alpha}$ respect to control males (n=8/group; t_{13}=3.72, *p*=0.0026 in dark phase; t_{13}=2.3, *p*=0.039 in light phase). (**F**) No changes in respiratory exchange ratio (RER) between groups (n=8/group) were detected, or in (**G**) glucose and (**H**) insulin levels in glucose tolerance test at basal (0'), 15, 30, 60 and 120 after oral glucose administration in α^{fl} (n=8) and LR$^{\Delta\alpha}$ (n=6) males. (**I**) Day of balano-preputial separation in α^{fl} (n=22) and LR$^{\Delta\alpha}$ (n=14). (**J**) Day of male sexual maturation was calculated after mating pubertal (P32) males with experienced wild-type females and subtracting 21 days after first litter was born (α^{fl}, n=7; and LR$^{\Delta\alpha}$, n=8). Similar (**K**) testis and (**L**) epididymis weight at 10 weeks of age (n=8/group). Each point represents one individual mouse, and all values are showed as mean ± s.e.m. **p*< 0.05, ***p*< 0.01 *vs* control; by two-tailed Student's *t*-test.



Supplementary Figure 4. Effect on body weight progression after daily leptin supplementation to pubertal females. (**A**) Cumulative body weight gain (ΔBW) in pubertal α^{fl} females treated with ip vehicle (n=13) or leptin (n=9; 2 µg/g of body weight), and $LR^{\Delta\alpha}$ females treated with vehicle or leptin (n=8/treatment; $F(3,34)=11.68$, $p<0.0001$ for genotype analysis; $F(18,204)=6.57$, $p<0.0001$ for interaction analysis). In $LR^{\Delta\alpha}$ females, dose of leptin was reduced to 1.8 µg/g of body weight when cummulative weight loss was higher than 0.8 g test. Note that α^{fl} and $LR^{\Delta\alpha}$ females at P37, after two days of vehicle or leptin treatment showed higher decrease in ΔBW in $LR^{\Delta\alpha}$ respect to α^{fl} females ($F(1,34)=33.95$, $p<0.0001$ for treatment analysis; $F(1,34)=5.37$, $p=0.027$ for interaction analysis). (**B**). Body weight progression in pubertal α^{fl} and $LR^{\Delta\alpha}$ females treated with ip vehicle or leptin ($F(1.5,9)=396$ $p<0.0001$ for interaction analysis). Each point represents one individual mouse, and all values are presented as mean ± s.e.m. * $p<0.05$ *vs* vehicle (Veh) group; # $p<0.05$ *vs* α^{fl} + leptin (Lep) group; & $p<0.001$ *vs* control group; groups with different superscript letters are statistically different; by two-way ANOVA with Holm-Sidak's *post hoc* analysis.



Supplementary Figure 5. Metabolic, reproductive and bone phenotypes in mice with deletion of PI3Kα in LR cells. (**A**) Similar day of first estrus in α^{fl} (n=27) and $LR^{cre/cre}$ (n=8) mice, and (**B**) estrous cycle length in α^{fl} (n=17) and $LR^{cre/cre}$ (n=10) animals. (**C**) Reduced body weight progression in $LR^{\Delta\alpha}$ (n=12) compared to α^{fl} (n=9) mice for 7 weeks on HFD (F(1,19)=26.9, p<0.0001). (**D-G**) MicroCT analysis of vertebrae in α^{fl} and $LR^{\Delta\alpha}$ (n=6/group) females did not detect changes between groups for (**D**) trabecular (Tb) bone volume respect to total volume (Tb.BV/TV), (**E**) trabecular thickness, (**F**) trabecular number, or (**G**) trabecular spacing.
HFD: high fat diet; Tb: trabecular; Ct: cortical; BV: bone volume; TMD: tissue mineral density. Each point represents one individual mouse, and all values are presented as mean ± s.e.m. **p< 0.01 *vs* control mice; by repeated measures two-way ANOVA with Holm-Sidak's *post hoc* analysis in c; and two-tailed Student's *t*-test in **A**, **B**, and **D-G**.



Supplementary Figure 6. Altered hypothalamic PTEN and $pAKT_{S473}$ levels in $LR^{\Delta\alpha+\beta}$ mice. Representative western-blotting and relative protein quantification of (**A**) PTEN in $LR^{\Delta\alpha+\beta}$ respect to control $\alpha^{fl}+\beta^{fl}$ mice ($F(1,12)=4.18$, $p=0.057$); (**B**) pAKT at Ser473 ($pAKT_{S473}$) residue from hypothalamic extracts of diestrous fasted mice injected with vehicle or leptin showed increased $pAKT_{S473}$ levels in $LR^{\Delta\alpha+\beta}$ compared to $\alpha^{fl}+\beta^{fl}$ females (n=4/treatment; $F(1,12)=7.468$, $p=0.018$). Each point represents one individual mouse, and all values are presented as mean ± s.e.m. Groups with different superscript letters are statistically different; by two-way ANOVA with Tukey's *post hoc* analysis.

Table 1 – List of primers used for genotyping

Mice	**Forward primers**
InsR-floxed	F 5' CTGAATAGCTGAGACCACAG 3' R 5' GATGTGCACCCCATGTCT 3'
LR-Cre	F 5' TGCTTCTGTCCGTTTGCCGGT 3' R 5' GTGAAACAGCATTGCTGTCAC 3'
p110α-floxed	F 5' CTGTGTAGCCTAGTTTAGAGCAACCATCTA 3' R 5' CCTCTCTGAACAGTTCATGTTTGATGGTGA 3'
p110β-floxed	F 5' CTCAAACTAGTGACTAGAAGCTGTGA 3' R 5' CTGATCGAGGCCATTAGAGAAGACCG 3'
R26-tdTomato	F 5' CTGTTCCTGTACGGCATGG 3' R 5' GGCATTAAAGCAGCGTATCC 3'

Table 2 – List of primers used for qPCR

Target Genes	Reference Sequence	Primers
Agrp	NM_001271806	F 5' AAGCTCAGGGCACAAGAGAC 3' R 5' CAGTGCCAACAGCAGAACAC 3'
Fgf21	NM_	F 5' CTCTATGGATCGCCTCAC 3' R 5' GTACACATTGTAACCGTCC 3'
Gapdh	NM_001289726	F 5' GCTCATGACCACAGTCCATGC 3' R 5' GTTGGGGGGGGATAGGGCCTCTCTTG 3'
Gh	NM_008117	F 5' CCTCAGCAGGATTTTCACCA 3' R 5' CTTGAGGATCTGCCCAACAC 3'
Ghr	NM_001048178	F 5' GATTTTACCCCAGTCCCAGTTC 3' R 5' GACCCTTCAGTCTTCTCATCCACA 3'
Ghrh	NM_001329683	F 5' CAACTGTACCCTGTTACTTC 3' R 5' TTTTTGCAGATGAGAATGGG 3'
Igf1	NM_001111274	F 5' GACAAACAAGAAAACGAAGC 3' R 5' ATTTGGTAGGTGTTTTCGATG 3'
Insr	NM_010568	F 5' CAAACAGATGCCACTAATCC 3' R 5' CTTTGAGACAATAATCCAGCTC 3'
Lepr	NM_001122899	F 5' CCTCTTGTGTCCTACTGCTCG 3' R 5' GAAATTCAGTCCTTGTGCCCAG 3'
Npy	NM_023456	F 5' CAGAAAACGCCCCCAGAACAAGC 3' R 5' GGCAGACTGGTTTCAGGGGATGGAT 3'
Pik3ca (p110α)	NM_008839	F 5' GAACCAGTAGGCAACCGTGA 3' R 5' GCTCTGCTATGAGGCGAGTT 3'
Pik3cb (p110β)	NM_029094	F 5' TTCTGCCCACCGGGATTTAT 3' R 5' GCTCTGCTATGAGGCGAGTT 3'
Pik3cd (p110δ)	NM_001029837	F 5' GAACAAGGCAGACATCTAAG 3' R 5' AGTCTTCGTGTTTCGTCTTCCA 3'
Pik3r1 (p85α)	NM_001024955	F 5' ACATCTCAAGGGAAGAAGTG 3' R 5' GGATCAGAGAAGCCATATTTTC 3'
Pik3r2 (p85β)	NM_008841	F 5' TTGGAGGATCTTCTGAGTC 3' R 5' CTTACTGTAGCATTCACTGTGTC 3'
Pik3r4 (p150)	NM_001081309	F 5' AGCCAGTGATACAGATTGAG 3' R 5' GCATAGTCAAATATGGAGCG 3'
Pomc	NM_001278581	F 5' TGAAAACCCCCGGAAGTACG 3' R 5' ACGTTGGGGTACACCTTCAC 3'
Socs3	NM_007707	F 5' CCAAAGAAATAACCATCCC 3' R 5' GATCTGCGAGGTTTCATTAG 3'
Sst	NM_009215	F 5' TCTGCATCGTCCTGGCTTT 3' R 5' CTTGGCCAGTTCCTGTTTCC 3'
Ucp1	NM_009463	F 5' CTTTTTCAAAGGGTTTGTGG 3' R 5' CTTATGTGGTACAATCCACTG 3'