Supplementary Data: Table of Contents

Supplementary Experimental Procedures

Plasmids, nucleofection, and luciferase assay.

The leptin UTRs constructs were generously provided by Susan Fried, Boston University. The leptin UTRs were cloned into the pGL3 control vector containing SV40 promoter and enhancer sequences (Promega) to drive a high and stable expression of luciferase. UTR plasmids and Renilla as an internal control were nucleofected into well differentiated 3T3-L1 preadipocytes using an Amaxa Nucleofector with Kit L (Lonza) and an electroperator (Nucleofector II AAD-1001N, device no. 400414; Amaxa Biosystems, Cologne, Germany). The proximal 764bp of the murine leptin promoter were amplified with BamHI-overhung primers from genomic epididymal fat pad DNA and inserted into the pGL4.14 vector (Promega). Plasmids were nucleofected into undifferentiated 3T3-L1 preadipocytes using an Amaxa Nucleofector with Kit V (Lonza). After overnight incubation, positively transfected cells were selected by hygromycin. After two weeks' selection, pools of cells were differentiated and also used to select clonal cell lines. Luciferase activity was quantified using the Luciferase Reporter Assay System (Promega) and a 96-well plate luminometer. Luciferase levels in different constructs were normalized to Renilla or protein levels.

Labeling, Analysis, and Sorting of Adipose Progenitor Cells.

Subcutaneous and epididymal adipose tissue was excised from C57BL/6 mice and the stromal vascular fraction (SVF) isolated as described (1). The SVF was resuspended in ice-cold HBSS with 10% FBS for labeling. Antibody incubations were performed on ice for 20 min. Cells were washed and resuspended in HBSS for sorting. Samples were sorted on a BD FACSAria cell sorter and analyzed on a BD LSRII (BD Biosciences) flow cytometer, each equipped with BD FACSDiva Software. For sorting, the cells were separated on the basis of the cell-surface markers indicated in the literature (2).

Holo-transferrin exposure.

Cell culture grade lyophilized bovine diferric transferrin (holo-transferrin; sigma #T1283) was dissolved in serum-free MEM containing 5.5 mM glucose (MEM-α). On the day before the experiment, the serum concentration of the cultures was reduced to 3.3% by replacing two-thirds of the medium with MEM-α. Remaining serum was washed out the following day, and holo-transferrin or apo-transferrin was immediately added in MEM-α (5.5mM glucose, no FBS) at the concentration of 10 mg/ml. Cultures were then incubated at 37 °C in a 5% CO2 atmosphere for 24 hours before harvesting for RNA extraction.

Cyclic AMP EIA and Calcium assay

3T3-L1 adipocytes differentiated for 10-14 days were starved by DMEM containing 0.5% BSA overnight, following by iron treatment. Cyclic AMP or calcium levels were measured by cyclic AMP EIA kit or calcium assay kit (Cayman Chemicals Ann Arbor, MI).

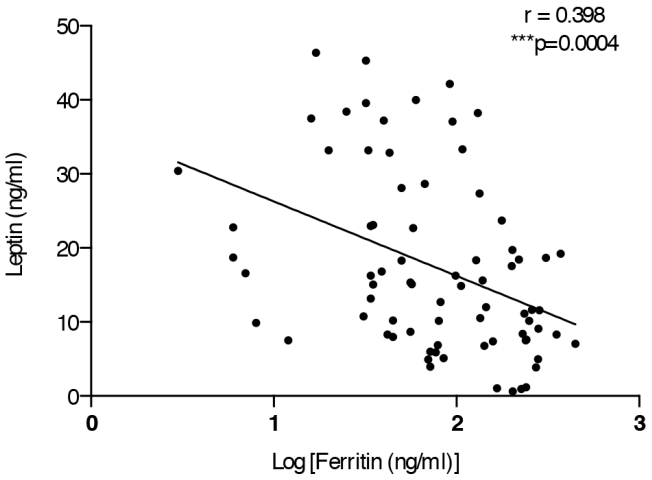


Figure S1. Serum ferritin levels are inversely associated with serum leptin levels. Serum leptin and ferritin levels were measured and correlated in a cohort of patients with Type 2 Diabetes as well as in obese subjects with metabolic syndrome (n = 76, Pearson's *r* = 0.396, *P* = 0.0004).

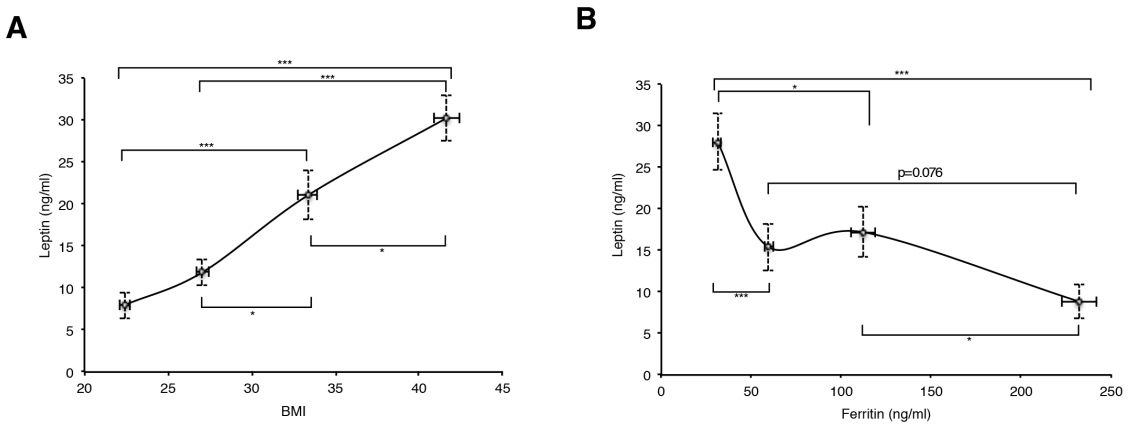


Figure S2. The association between leptin and BMI or ferritin was analyzed by quartiles in humans. (A) Leptin in the high BMI group (upper 25th percentile BMI) is significantly higher than that in the other three low BMI groups. **(B)** The leptin in the high ferritin group (upper 25th percentile ferritin) is significantly lower than that in the other three low ferritin groups (n = 64). Significance denoted *P < 0.05, and ***P < 0.001 using Two-tailed Student's t tests. Data represent mean ± SEM.

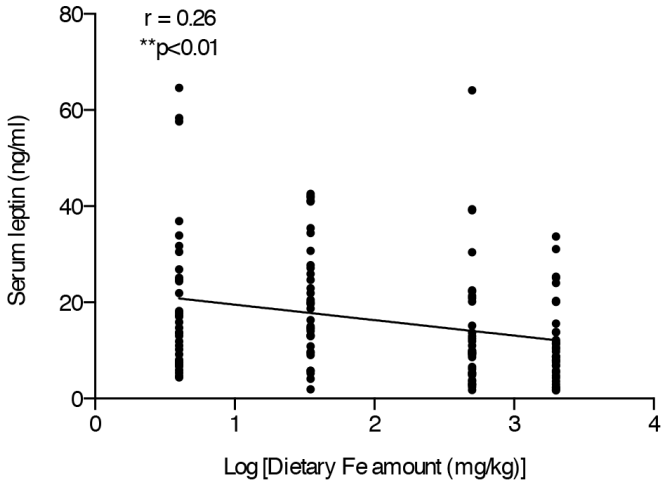


Figure S3. Serum leptin decreased with dietary iron amounts (n = 36, Pearson's *r* = 0.26, *P* < 0.01). Serum leptin levels were determined in 5-month-old C57BL/6J mice following 9 weeks of being fed low-iron (4 mg Fe /kg diet), normal chow (35 mg Fe/kg and 500 mg Fe/kg), or high-iron (2000 mg Fe/kg) diets.

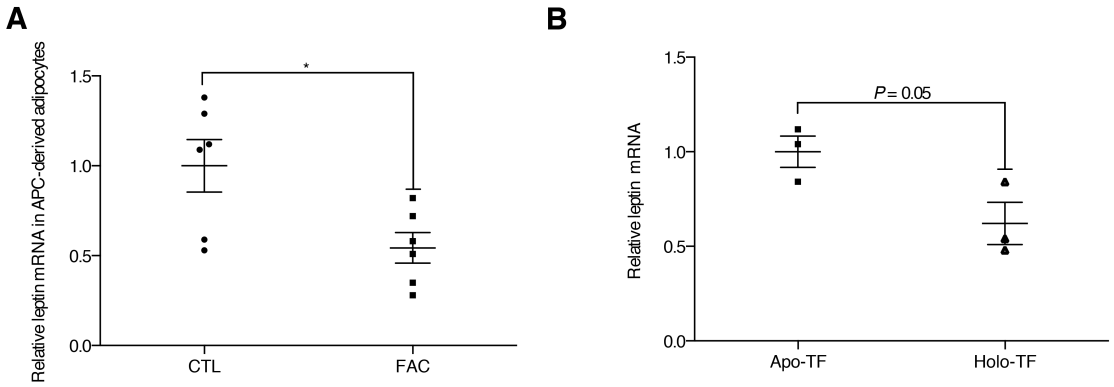


Figure S4. (A) Leptin mRNA levels decrease in adipocyte progenitor cells (APC)-derived primary adipocytes by fluorescence-activated cell sorting (FACS) (n = 6). **(B)** Leptin expression was decreased with holo-transferrin (holo-TF, 10 mg/ml) treatment (n = 3). Significance denoted *$P <$ 0.05 using Two-tailed Student's t test. Data represent mean ± SEM.

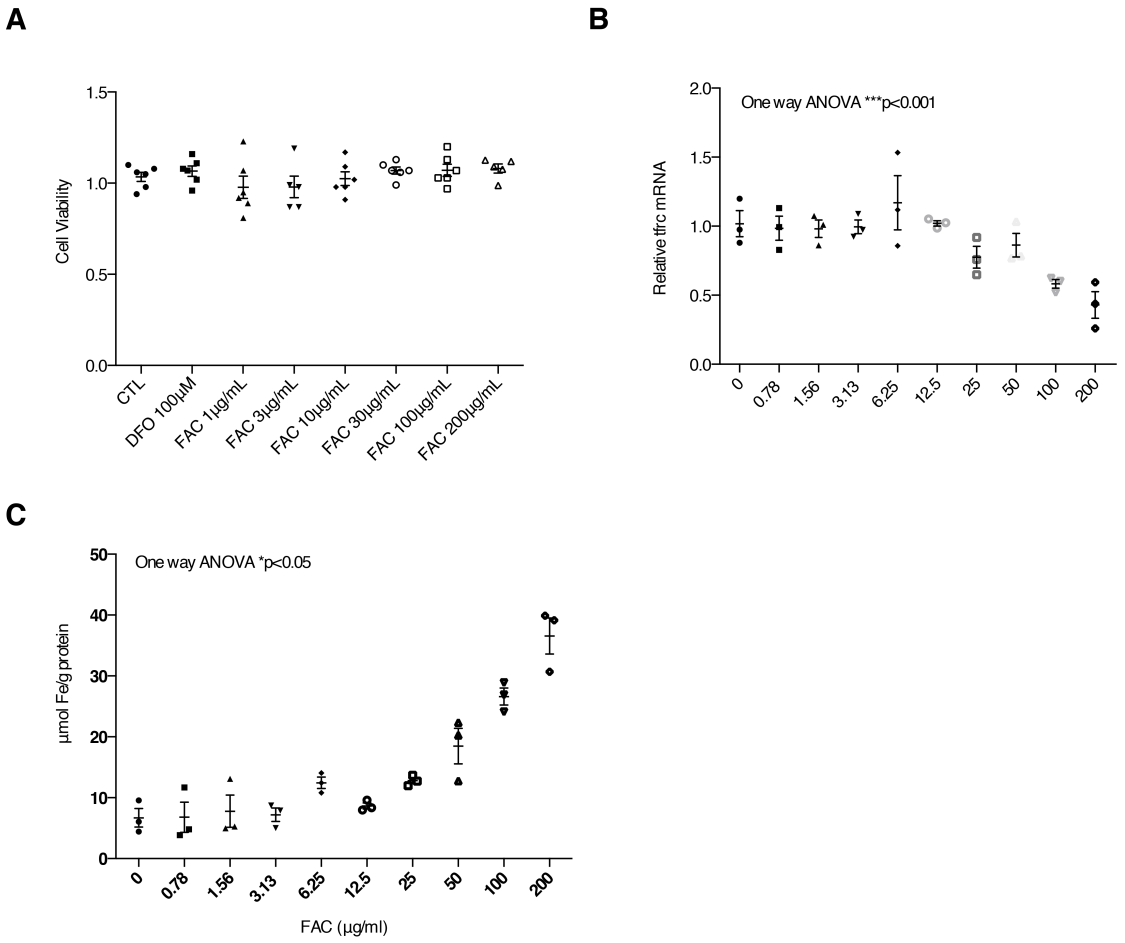


Figure S5. (A) MTT assay in 3T3-L1 adipocytes after exposure to FAC for 24 h (n = 6). **(B)** FAC resulted in dose-dependent regulation of the mRNA levels of the transferrin receptor, a physiologic reporter of intracellular iron levels that operates through Iron-Responsive Elements (IRE) (n = 3, One-way ANOVA, ***$P < 0.001$). **(C)** FAC resulted in dose-dependent increase of iron level (n = 3, One-way ANOVA, *$P < 0.05$). Data represent mean ± SEM.

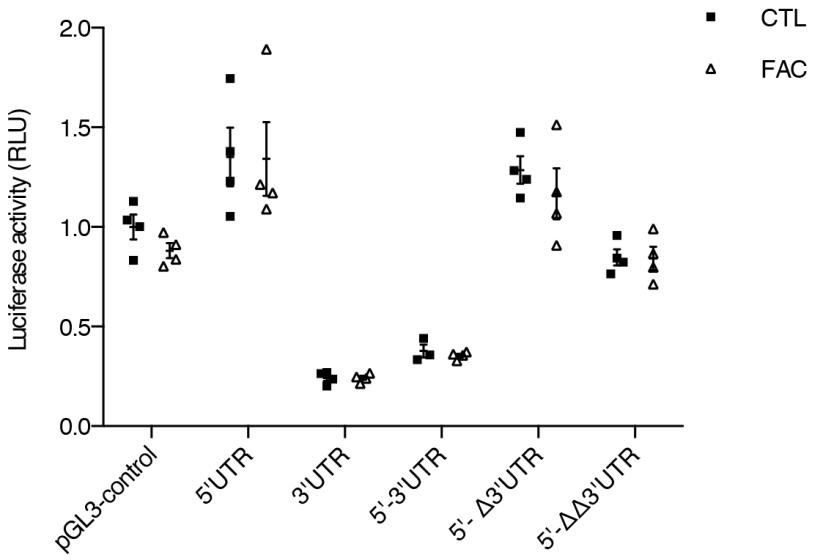


Figure S6. Iron had no effect on translational regulation of leptin by its 5'- or 3'- UTRs. Differentiated 3T3-L1 cells were transiently co-transfected with the chimeric leptin UTR-LUC constructs and the pRL-TK (*Renilla* luciferase vector) as described under "Supplementary Experimental Procedures" (n = 4). Chimeric UTR-LUC constructs carrying the 5'-UTR and/or various lengths of 3'-UTR of leptin mRNA (56). The pGL3 control contained no leptin UTR sequences and was used as control. Data represent mean ± SEM.

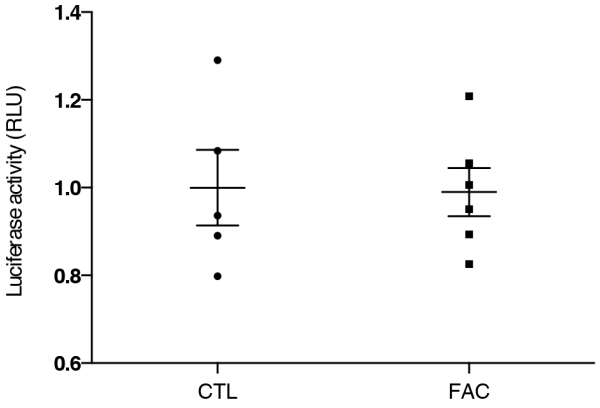


Figure S7. Proximal leptin promoter (-764 bp)-driven luciferase activity is unchanged in the presence or absence of 100 µg/ml FAC (n = 5 or 6). Data represent mean ± SEM.

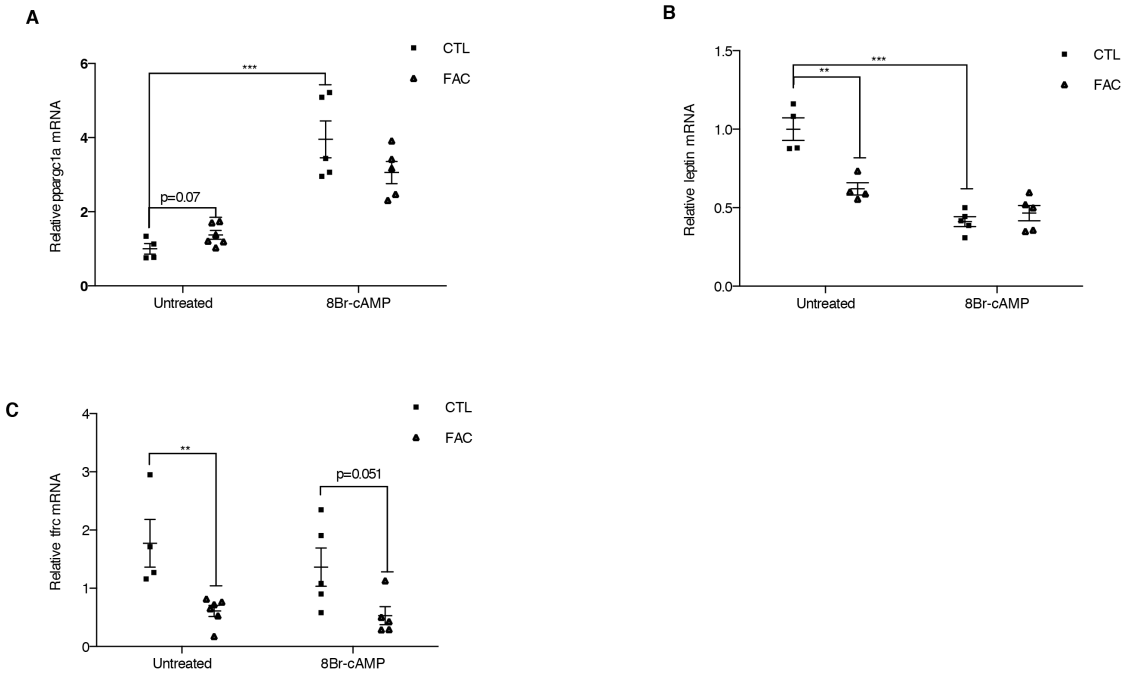


Figure S8. Gene expression with iron and/or 8Br-cAMP treatment. (A) 8Br-cAMP abolished increment of ppargac1a expression seen after 6h iron treatment (n = 4 to 6). **(B)** Leptin expression in 3T3-L1 adipocytes treated with cAMP agonist, 8Br-cAMP (1 mM) and/or 100 µg/ml FAC for 6 h (n = 4 to 5). **(C)** *Tfrc* mRNA in 3T3-L1 adipocytes treated with or without cAMP agonist, 8Br-cAMP (1 mM) for 6 h and no iron or 100 µg/ml FAC for 6 h (n = 4 to 6). Significance denoted $^{**}P < 0.01$ and $^{***}P < 0.001$ using Two-tailed Student's t test. Data represent mean ± SEM.

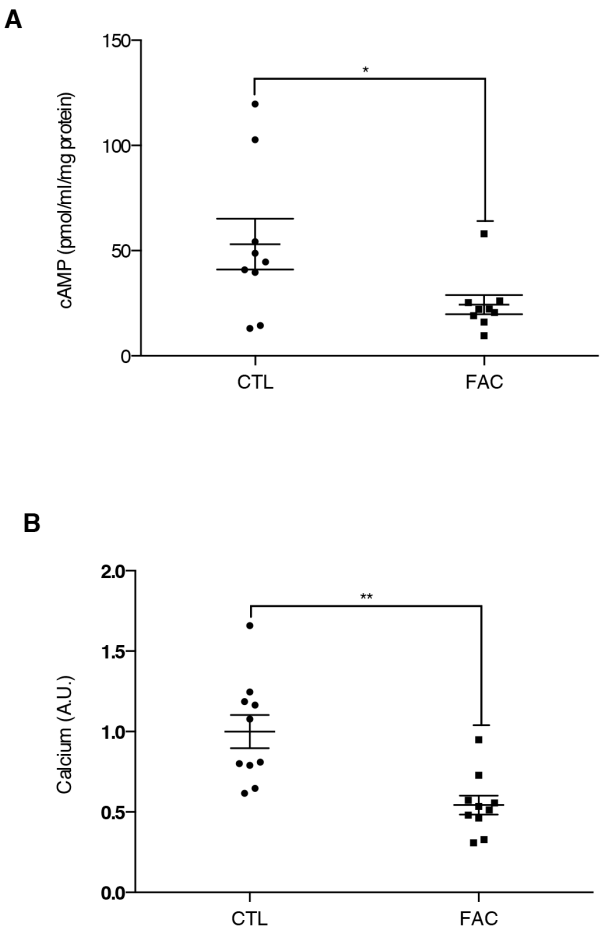


Figure S9. cAMP (n = 9) and calcium (n = 10) levels were decreased in FAC treated 3T3-L1 adipocytes. Significance denoted *$P < 0.05$ and **$P < 0.01$ using Two-tailed Student's t test. Data represent mean ± SEM.

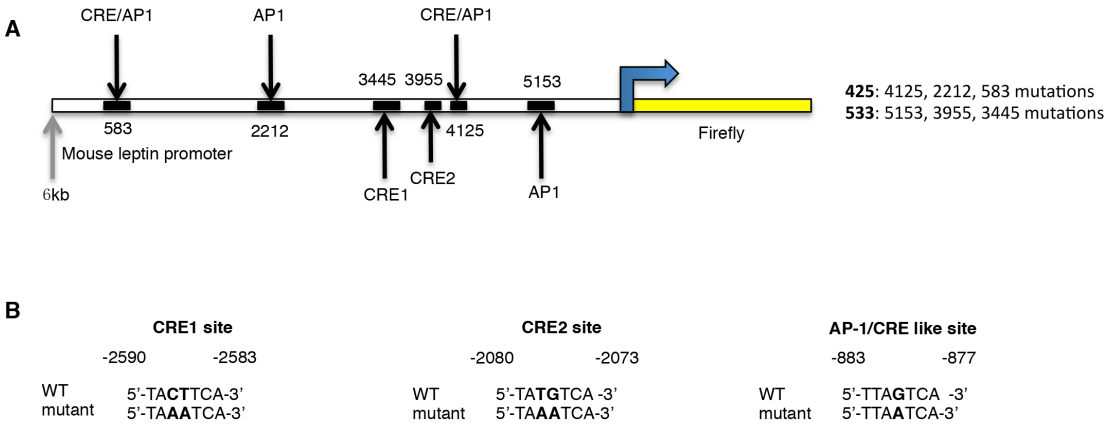


Figure S10. (A) Mouse leptin promoter and mutagenized constructs showing with potential CRE.

(B) Three potential CRE sites and mutagenesis used in plasmids or EMSA assay.

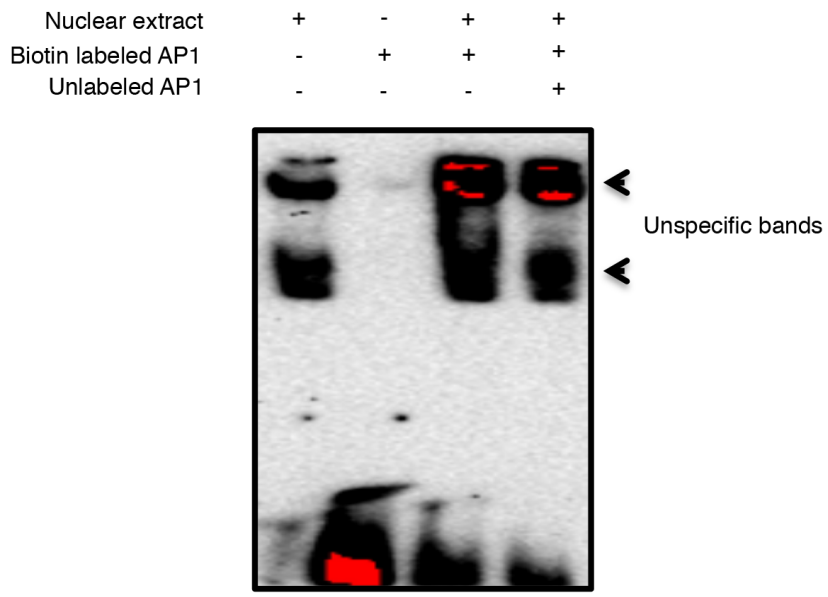


Figure S11. EMSA with the AP1 oligos as indicated.

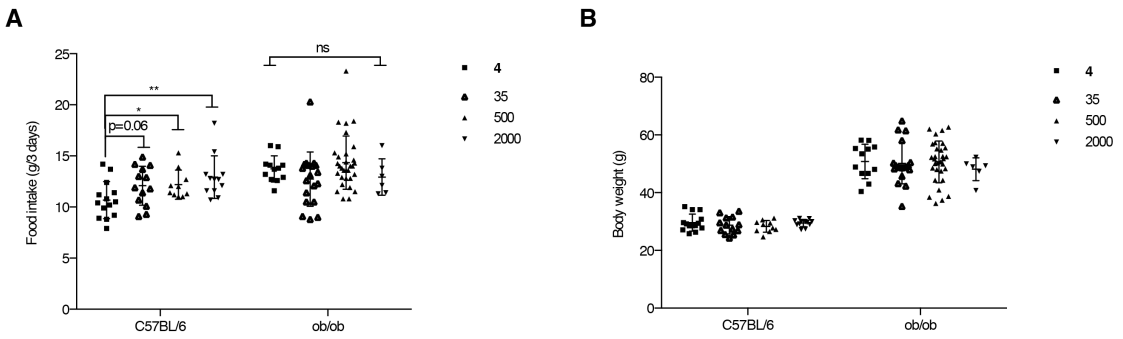


Figure S12. Accumulated food intake and body weight in C57/BL6 and ob/ob mice on different iron diets. (A) Mice fed with high iron diet (2000 mg/kg) had increased food intake compared to low iron diet. $*P < 0.05$ (One-way ANOVA for food intake in C57BL/6 mice under different dietary iron); $P = 0.255$ (One-way ANOVA for food intake in ob/ob mice under different dietary iron). Significance denoted $*P < 0.05$, and $**P < 0.01$ using Two-tailed Student's t test. (B) Body weights did not change with different dietary iron after 9 weeks on diet. $P = 0.524$ (One-way ANOVA for body weight in C57BL/6 mice under different dietary iron); $P = 0.856$ (One-way ANOVA for body weight in ob/ob mice under different dietary iron). Data represent mean ± SEM.

Supplementary Table 1. Patient clinical characteristics.

N = 76	
Age (years), mean (SD)	48.2 (13.7)
Females (%)	44
BMI, mean (SD)	31.3 (7.1)
Diabetes (%)	47
Ferritin, ng/ml, mean (SD)	119.1 (102.5)
Iron, g/dl, mean (SD)	87.7 (33.8)
TIBC, µg/dl, mean (SD)	331.3 (51.8)
hs CRP, ng/ml, mean (SD)	3069.8 (4207)
Adiponectin, ng/ml, mean (SD)	3.0 (1.86)
Leptin, ng/ml, mean (SD)	17.5 (12.0)

Supplementary Table 2

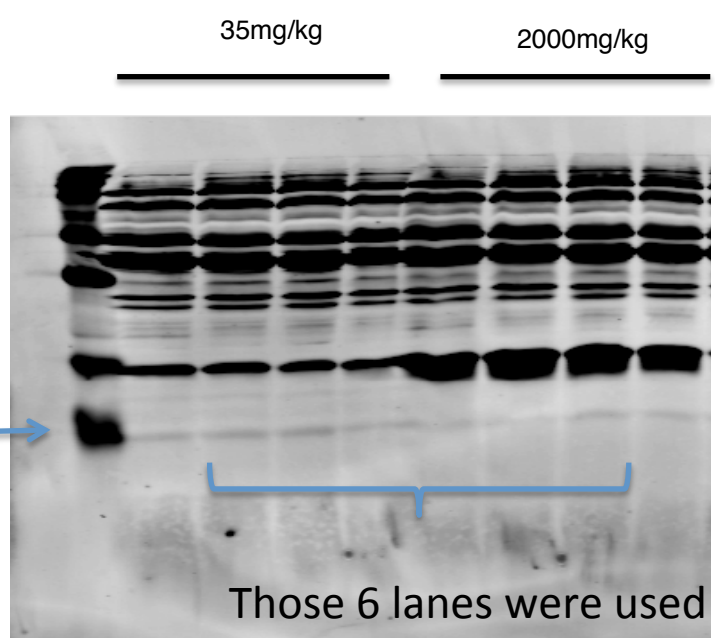
Multivariable linear regression analysis with leptin as the dependent variable and ferritin, BMI, diabetes, age, CRP, and gender as predictive variables in a cohort of patients with all the ferritin levels except those missing CRP values (n = 70). Multivariate logistic regression was analyzed by STATA.

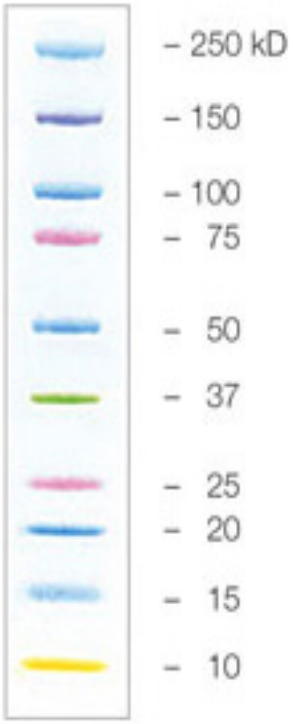
Variables	Coefficient	SEM	*P* values
Log (Ferritin)	- 5.56	1.95	0.006
BMI	0.94	0.14	< 0.001
Diabetes	2.72	2.19	0.218
Age	- 0.06	0.08	0.468
log(CRP)	1.56	1.91	0.418
Gender	- 10.14	1.92	< 0.001

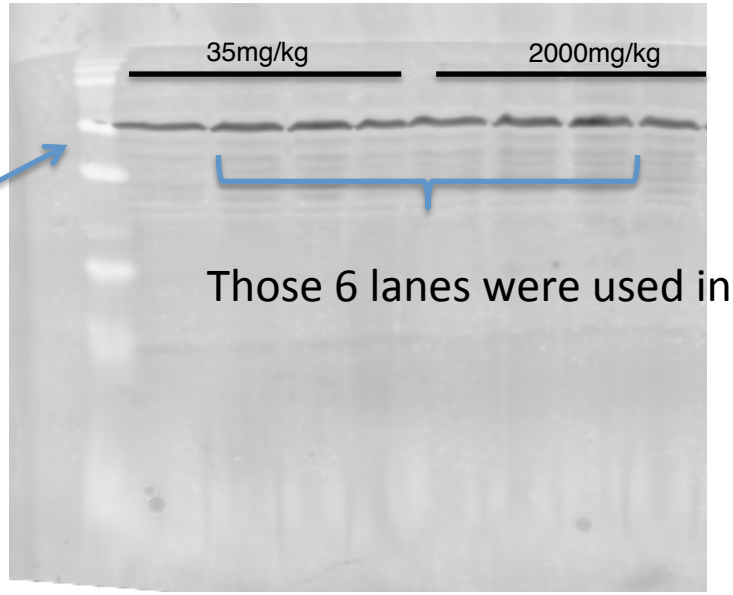
Reference

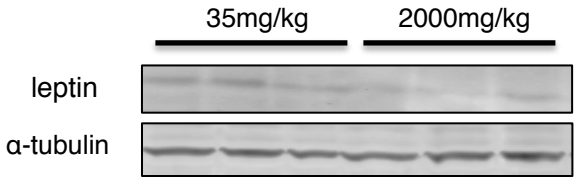
1. Galton DJ, and Fain JN. Effects of prolonged incubation of isolated fat cells on their response to hormones stimulating lipolysis and glucose metabolism. *Biochem J.* 1966;98(2):557-61.
2. Rodeheffer MS, Birsoy K, and Friedman JM. Identification of white adipocyte progenitor cells in vivo. *Cell.* 2008;135(2):240-9.

Full unedited gel for Figure 2D









Full unedited gel for Figure 4B and 4C

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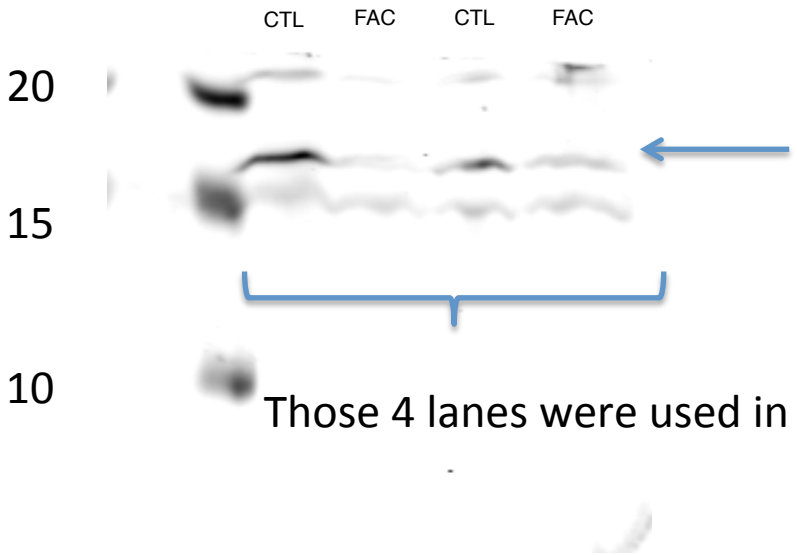

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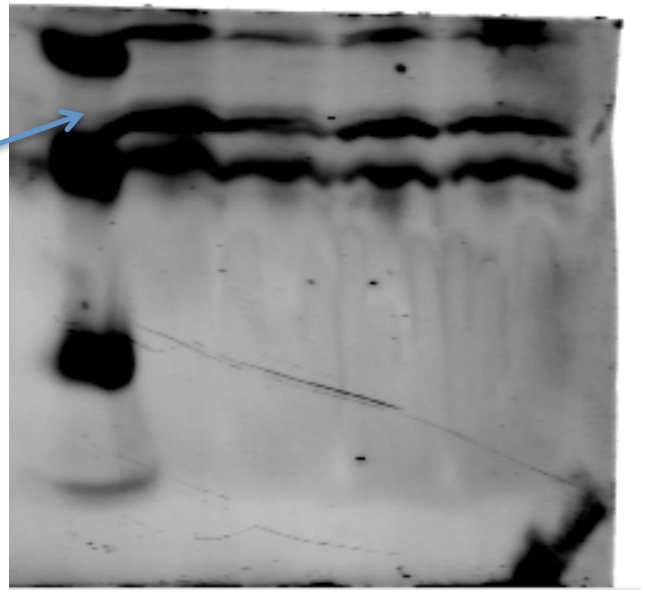

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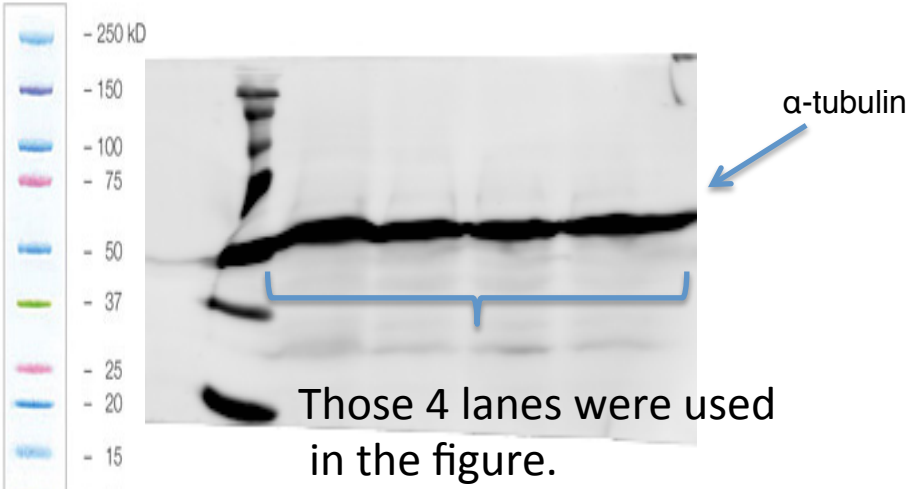

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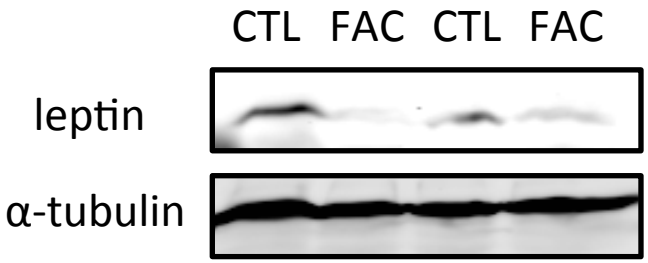
Figure 4B



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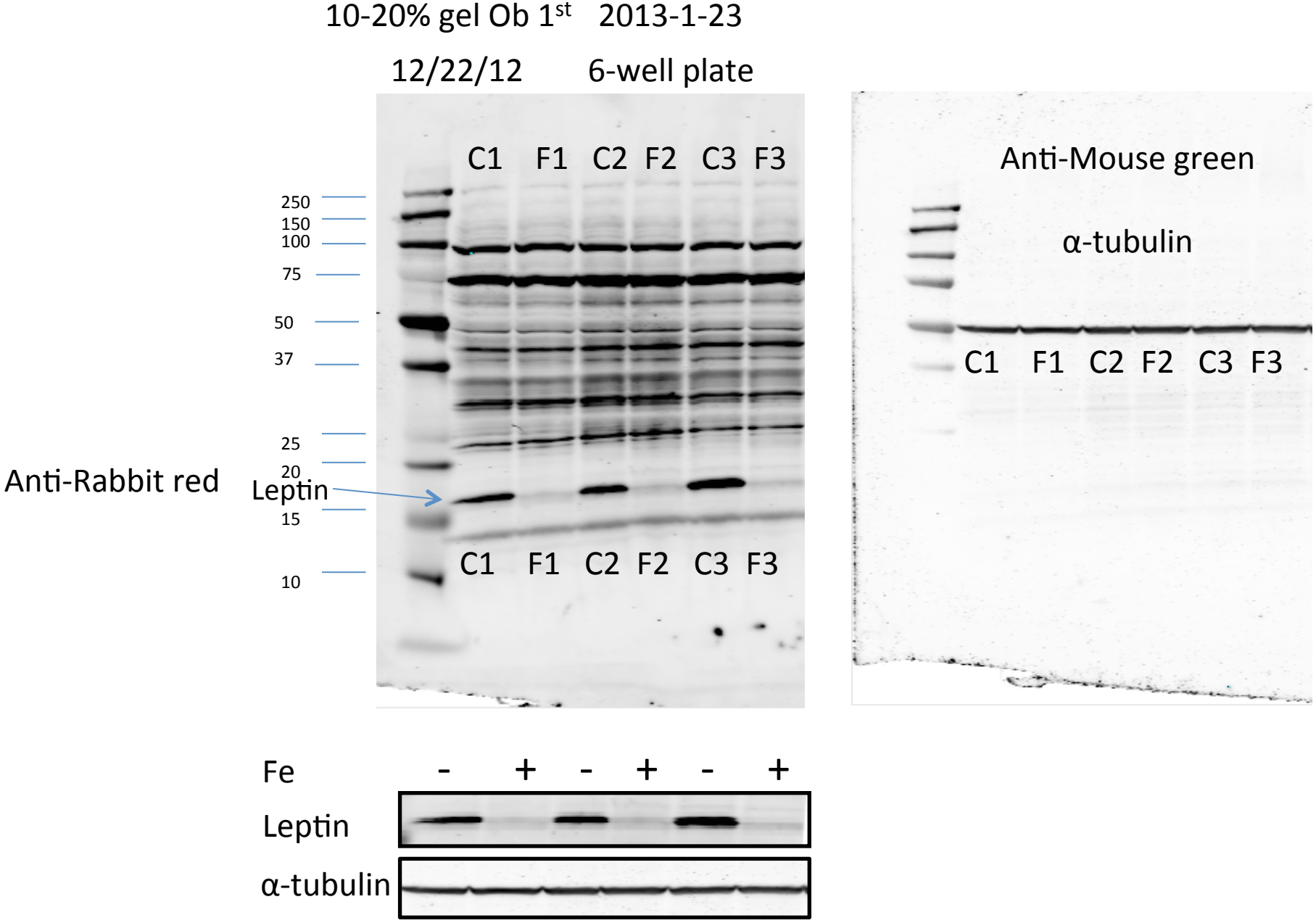

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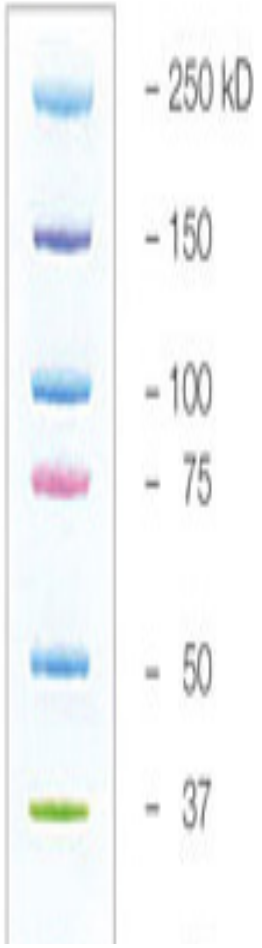

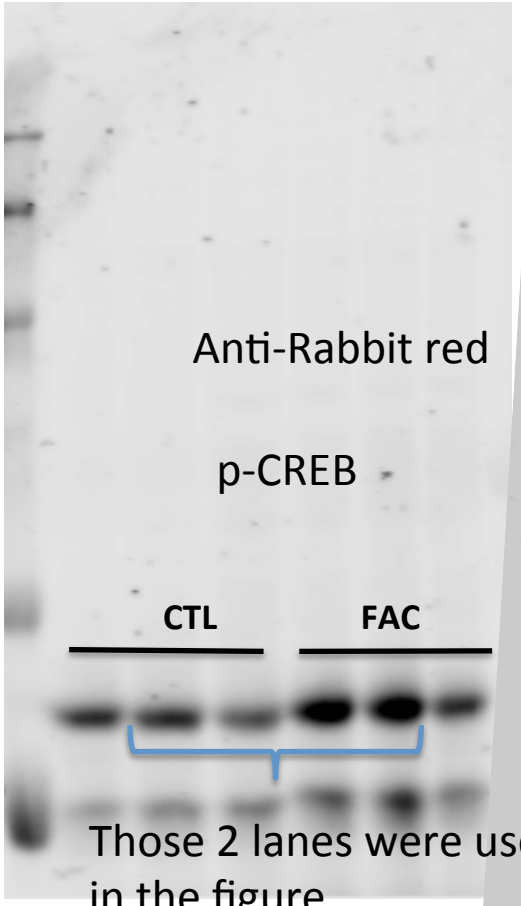

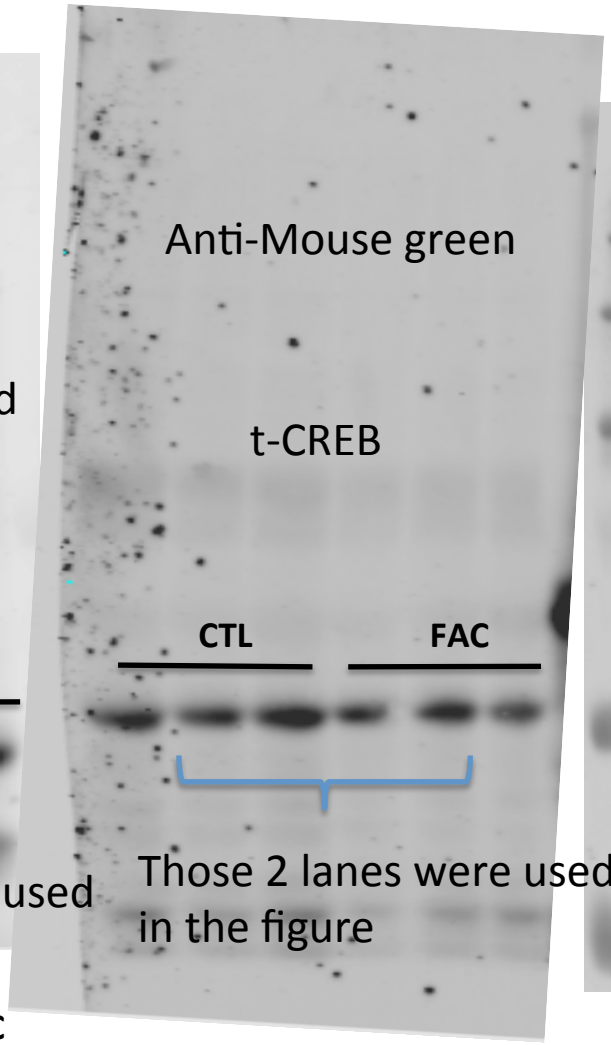

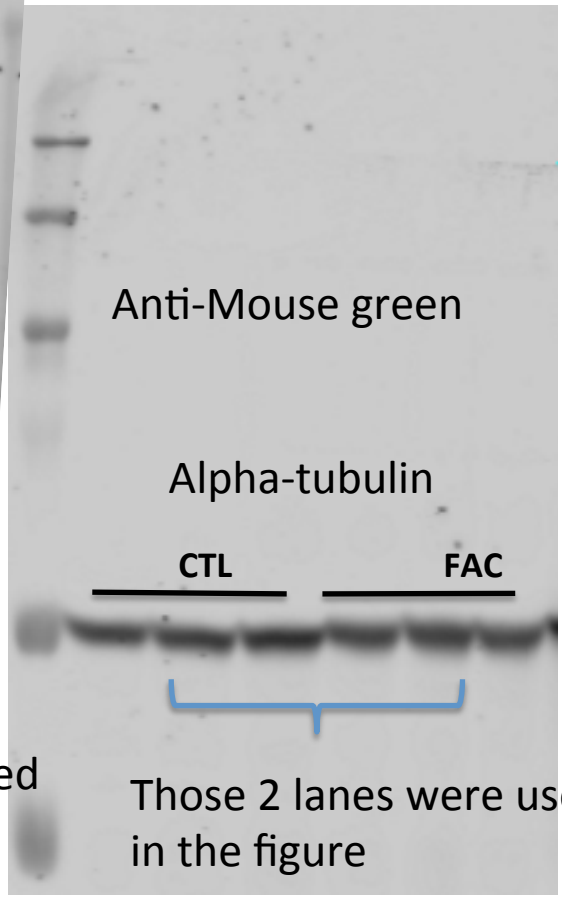

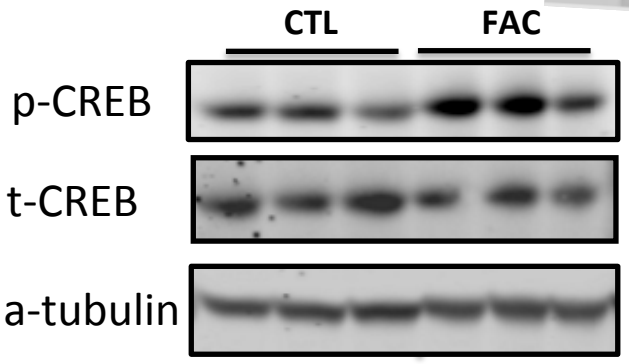

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