Supplementary Materials and Methods

Materials. Akt and pAkt-ser473 antibodies were obtained from Cell Signaling. IR and myc antibodies were purchased from Santa Cruz. pIRβ antibody was from Calbiochem. F4/80 antibody was from Serotec. Flag antibody was purchased from Sigma. TIARP polyclonal antibody was generously provided by A.M. Chambaut-Guerin (Moldes et al. 2001). Anti-rabbit Alexa 488 was from Molecular Probes/ Invitrogen. Myc-Glut4-GFP construct was a kind gift from M. Czech. Insulin ELISA kits were purchased from Crystal Chem (Downer's Grove, IL), and IL-6 and adiponectin ELISA kits were from R&D Systems (Minneapolis, MN). Plasma leptin, resistin, IL-6, TNFα, and MCP-1 levels were measured using LINCOplex mouse adipokine kits, per manufacturer's instructions (Linco Research Inc., St Charles, MO). We thank Dr. I Kramnik for the use of the Luminex system in adipokine measurements.

Metabolic Cages. Metabolic cage experiments were performed as previously described (Maeda 2005), using 6-week-old male wild type (n = 4) and STAMP2-/- (n = 4) mice.

Quantitative RT-PCR primers. *Adiponectin* (F- GAT GGC AGA GAT GGC ACT CC; R- CTT GCC AGT GCT GCC GTC AT); *Catalase* (F- AGC GAC CAG ATG AAG CAG TG; R- TCC GCT CTC TGT CAA AGT GTG); *FAS* (F- GGA GGT GGT GAT AGC CGG TAT; R- TGG GTA ATC CAT AGA GCC CAG); *GST* (F- AAG AAT GGA GCC TAT CCG GTG; R- CCA TCA CTT CGT AAC CTT GCC); *IL-6* (F- ACA ACC ACG GCC TTC CCT ACT T; R- CAC GAT TTC CCA GAG AAC ATG TG); *MCP-1*

(F- CCA CTC ACC TGC TGC TAC TCA T; R- TGG TGA TCC TCT TGT AGC TCT CC); *NADPH oxidase 1* (F- GGT TGG GGC TGA ACA TTT TTC ; R- TCG ACA CAC AGG AAT CAG GAT); *SCD-1* (TTC TTG CGA TAC ACT CTG GTG C; R- CGG GAT TGA ATG TTC TTG TCG T); *SOCS-3* (F- CAC AGC AAG TTT CCC GCC GCC; R- GTG CAC CAG CTT GAG TAC ACA); *SOD-1* (F- AAC CAG TTG TGT TGT CAG GAC; R- CCA CCA TGT TTC TTA GAG TGA GG); *STAMP2* (F- TCA AAT GCG GAA TAC CTT GCT; R- GCA TCT AGT GTT CCT GAC TGG A); *STEAP* (F- GGT CGC CAT TAC CCT CTT GG; R- GGT ATG AGA GAC TGT AAA CAG CG); *STEAP3* (F- CCC GTC CAT TGC TAA TTC CCT; R- CAG AAA AGA GAC CCG AAC CCA); *TNF*α (F- CCC TCA CAC TCA GAT CAT CTT CT; R- GCT ACG ACG TGG GCT ACA G).

Knockdown of Flag-STAMP2. To express a tagged version of STAMP2 in adipocytes, stable doxycycline- responsive Flag-tagged STAMP2 expressing clones were generated in 3T3-F442A cells, using the pRev-TRE-TetOff system (Clontech), as recommended by manufacturer. Cells were maintained in medium containing doxycycline. 2 days after inducing differentiation, doxycycline was removed to allow expression of Flag-STAMP2. To knockdown Flag-STAMP2 in these cells, STAMP2 or non-specific siRNA were electroporated into the cells 5 days after inducing differentiation (3 days after removal of doxycycline). 48 hours after electroporation, lysate was harvested from cells and used for Western blot analysis.

Glucose uptake and Glut4 trafficking experiments. For experiments in cultured cells, adipocytes were electroporated with STAMP2 or control siRNA 5 days after induction of differentiation. The next day, cells were washed and switched into serum-free medium for overnight incubation. Cells were washed 3x in warm Krebs-Ringer Hepes (KRH) buffer, followed by treatment with 100 nM insulin in warm KRH buffer. In some wells, 50 μM cytochalasin B was added as a control to inhibit glucose transporter-mediated glucose uptake. Cells were incubated for 20 min at 37 degrees. 3H-2-deoxyglucose was then added to each well for an additional 10 minutes. Experiment was terminated by placing cells on ice and washing with ice-cold KRH buffer. After washing cells 3x in cold KRH buffer and removing final wash, cells were lysed in 0.1% SDS and uptake determined by scintillation counting. For Glut4 trafficking experiments 3T3-L1 adipocytes were co-electroporated with myc-Glut4-GFP construct (gift of M. Czech) and either control or STAMP2-specific siRNA, and cells were plated on coverslips. One day later, cells were placed into serum-free DMEM for overnight incubation. Cells were then treated with 160 nM insulin for 20 minutes and cells fixed in 2% paraformaldehyde. For immunofluorescence, non-permeabilized cells were incubated with mouse anti-myc antibody followed by anti-mouse Cy3. Confocal images were obtained using a Zeiss LSM-410 at 400x magnification. For quantitation, in 3 independent experiments, a total of 500 GFP positive cells were scored in each condition (plus or minus) for myc rim staining. For experiments in primary adipocytes, adipocytes were first isolated from either subcutaneous or epididymal fat pads. Mice were killed and fat pads removed. Fat pads were placed into isolation buffer (IB; KRP buffer containing 2.5% BSA and 200 μM adenosine) plus 1 mg/ml collagenase (Liberase RI, Roche), chopped thoroughly with

scissors, and incubated for 45 min with gentle shaking at 37 degrees. Fat cells were passed through nylon mesh and then washed 3x in IB, each time allowing fat cells to rise to the surface and then removing infranatant. 3 volumes of IB were added to fat cells and they were placed at 37 degrees. Cells were then treated with or without 100 nM insulin for 30 min. After insulin stimulation, 3H-2-deoxyglucose was added for 45 min. Glucose uptake was ended with the addition of 500 µl Fluka reagent (Sigma). Cells were spun for 2 min at 1000 rpm. Floating fat cakes were collected, transferred into scintillation vials, and counted.

Insulin signaling in FAO cells. For conditioned medium experiments, 3T3-L1 adipocytes were electroporated with siRNA. 48 hours after electroporation, medium was collected and filtered through a 0.2 µM syringe filter. Conditioned medium was combined in a 1:1 ratio with normal culture medium (RPMI 1640 + 10% FBS) and applied to FAO cells for another 44 hours. 4 hours prior to insulin stimulation, medium was changed to serum-free medium.

Reporter assays. A 2kb fragment upstream of the putative mouse STAMP2 transcription initiation start site was PCR amplified by Phusion proofreading polymerase (Finnzymes) using the BAC clone RP23-212F6 (Invitrogen) as a template. The PCR product was inserted into pCRR-Blunt II-TOPO (Invitrogen) and sequenced. The 2kb insert was excised out with HindIII and XhoI and ligated into the pGL2-Basic reporter plasmid (Promega) using the same sites. Empty pcDNA3, pBabe-C/EBPα (Tong et al. 2000), pBabe-PPARγ2 (Tong et al. 2000) or pcDNA3-LXRα (generous gift from H. Nebb) expression plasmids were used for co-transfection experiments. Hela cells

(ATCC) were cultured in DMEM supplemented with 10% fetal calf serum. Cells were seeded in 6-well plates, grown to ~50% confluency and transfected with plasmid DNA using FuGene (Roche) as transfection agent. After 8-10 h, media was changed and cells were grown for 24 h before addition of ligands. LXR ligand T0901317 (10μM) (Calbiochem), PPARγ ligand pioglitazone (1.5μM) (Alexis Biochemicals), or DMSO was then added and cells were incubated for 24 h before cell lysates were prepared. Luciferase assays were done as described previously (Slagsvold et al. 2001).

Histology and immunostaining. 5-6 month old wild type and *STAMP2-/-* mice were sacrificed. Tissue samples were fixed in formalin immediately upon dissection from animals. Paraffin embedding, mounting, and hemotoxylin and eosin staining were carried out by Harvard Medical School Rodent Histopathology core. For F4/80 staining, samples were first paraffin embedded and 5-micron slices mounted on slides. Samples were deparafinized and immunoperoxidase staining conducted using anti-F4/80 primary antibody and VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA). For detection of STAMP2 in cells, cells were fixed in 2% paraformaldehyde for 20 minutes at room temperature and permeabilized in 0.5% Triton-X for 5 minutes on ice. Slides were blocked in 4% donkey serum plus 2% BSA and STAMP2 was detected using the anti-TIARP antibody (gift of A.M. Chambaut-Guerin) followed by anti-rabbit Alexa-488. For STAMP2 staining in tissue sections, nonspecific antigenic sites were blocked by 5% goat serum and 2% bovine serum albumin in phosphate buffered saline at room temperature for 1 hour. Slides were incubated in primary antibody (anti-TIARP, 1:200) at 4°C overnight. The sections were then incubated at room temperature with a biotinylated

secondary antibody (Vector) and with the ABC-complex for 30 minutes respectively. Staining was done using the Vector Red Alkaline Phospatase Substrate Kit.

Hyperinsulinemic-euglycemic clamp studies. Surgery was performed to catheterize the jugular vein of 4-month-old wild type (n = 8) and *STAMP2-/-* (n = 7) mice, as described previously (Ozcan et al. 2006). After four days recovery period, clamp experiments were performed as previously described (Ozcan et al. 2006). Details may be found in the supplement. Briefly, for the basal infusion period, [3-^{3}H]-glucose (0.05 μCi/min) was infused for two hours using micro-dialysis pumps. Blood samples were collected from the tail vein at the end of this period and used to calculate the rate of basal hepatic glucose production. Following the basal infusion period, a 120 min hyperinsulinemic-euglycemic clamp period was initiated with an insulin prime of 100 mU/kg/min for 3 min, followed by continuous infusion of human insulin (Humulin R) at a rate of 2.5 mU/kg/min rate. Blood samples were collected at 20 min intervals for the measurement of plasma glucose concentration. During the clamp, 25% glucose solution was infused at variable rates to maintain euglycemia. To analyze insulin-stimulated glucose uptake in tissues (muscle and white adipose tissue), 2-deoxy-D-[1-^{14}C]-glucose (2-[^{14}C]-DG) was administered as a bolus (1 μCi) 75 min after the start of insulin administration. Glucose uptake calculated by determining the tissue content of 2-deoxyglucose-6-phosphate. Blood samples were collected at 80, 85, 90, 100, 110, and 120 min after the initiation of the clamps to determine plasma [^{3}H]-glucose, 3H2O, and 2-[^{14}C]-DG concentrations. At the end of the experiment, animals were sacrificed and muscle and white adipose tissues removed and frozen in liquid nitrogen, and kept at –80°C until processing.

Calculations for *in vivo* glucose uptake. Rates of basal hepatic glucose production and insulin-stimulated whole-body glucose uptake were determined as the ratio of [^{3}H] glucose infusion rate to the specific activity of plasma glucose at the end of basal period and during the final 30 min of clamps, respectively. Hepatic glucose production during the hyperinsulinemic-euglycemic clamps was determined by subtracting the glucose infusion rate from whole-body glucose uptake.

Supplementary Figure Legends

Supplementary Figure 1: STAMP2 expression during feeding and fasting in mice fed regular or high fat diet. Expression of STAMP2 in mice fed regular or high fat diet for 16 weeks was examined by quantitative PCR and normalized to 18S in adipose tissue. (n=2 for each condition).

Supplementary Figure 2: Impaired insulin action in the absence of STAMP2. (A) Signaling in response to insulin was examined after transfection of control and STAMP2 siRNA. Quantitation of pAkt/Akt represents combined data from 2 independent experiments, represented as mean ± s.e.m. * indicates $p< 0.05$. Open bars- basal conditions, closed bars- insulin stimulated. (B) Conditioned media collected from STAMP2 knockdown adipocytes was applied to Fao liver cells. Fao cells were treated with insulin and phosphorylation of Akt was examined.

Supplementary Figure 3: Expression of oxidative stress-related genes in subcutaneous (SWAT) of *STAMP2-/-* mice and in vivo insulin action. (A) Oxidative stress-related gene expression levels in SWAT were measured by qPCR in 6-month-old male mice. Open bars represent WT; closed bars represent *STAMP2-/-* (KO). * indicates $p< 0.05$. (B) Absolute quantification of *in vivo* insulin signaling shown in Figure 4. Data combined from 2 experiments represents mean ± s.e.m.

Supplementary Figure 4: Metabolic analysis of wild type and *STAMP2-/-* mice. Mice were evaluated in metabolic cages for 48 hours. (A) Food intake, (B) VO_2 and VCO_2, and (C) movement along X, Y, and Z axes were measured throughout the duration

of the experiment. (D) Differences in movement between genotypes were particularly noticeable during the dark periods. * represents $p < 0.05$. For parts A, B, and C, open bars represent wt and closed bars represent KO. For part D, closed squares represent WT and open triangles represent KO. Data represents mean ± s.e.m.

Supplementary Figure 5: Hyperinsulinemic-euglycemic clamp studies. (A) Body weight was equal in both genotypes at time of catheterization surgery and at time of clamp. (B) Blood glucose before and during the clamp studies. Data represents mean ± s.e.m.

Supplementary References

Maeda, K., Cao, H., Kono, K., Gorgun, C.Z., Furuhashi, M., Uysal, K.T., Cao, Q., Atsumi, G., Malone, H., Krishnan, B., Minokoshi, Y., Kahn, B.B., Parker, R.A., and Hotamisligil, G.S. (2005). "Adipocyte/macrophage fatty acid binding proteins control integrated metabolic responses in obesity and diabetes." Cell Metabolism **1**: 107-119.

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SUPPLEMENTARY FIGURE LEGENDS

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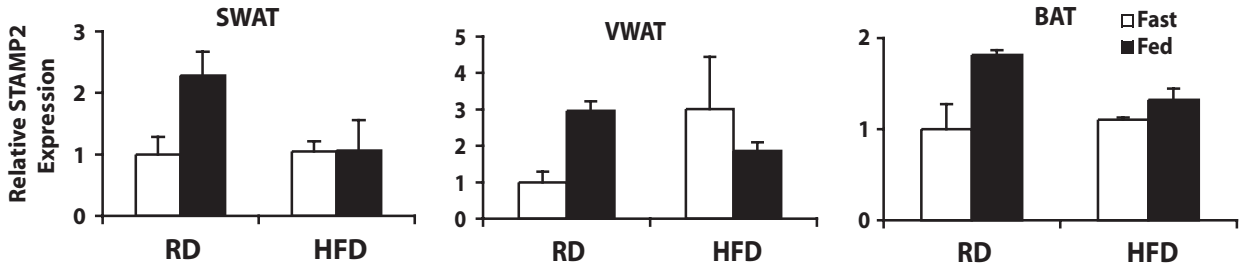
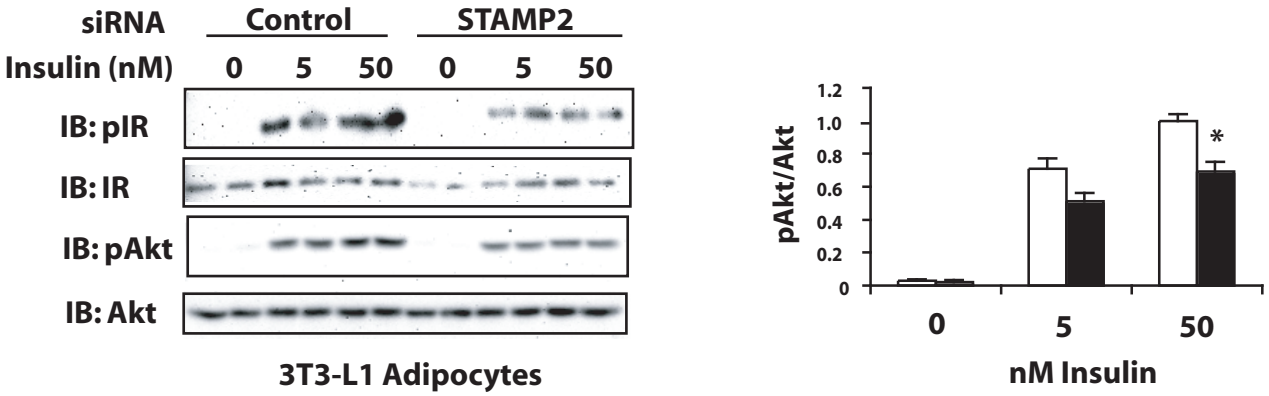
[G] Supplemental Text and Figures

Supplementary Figure 1



Supplementary Figure 2

A.



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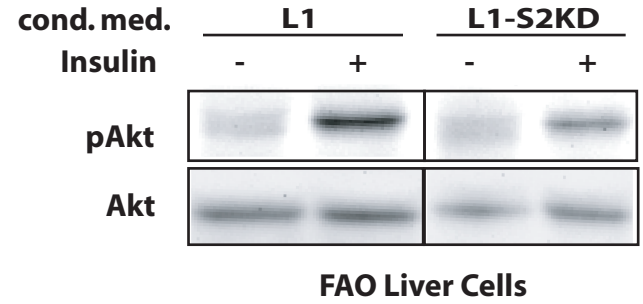
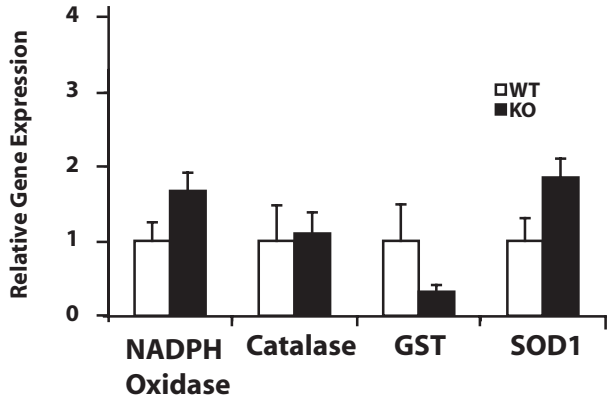


Supplementary Figure 3

A.



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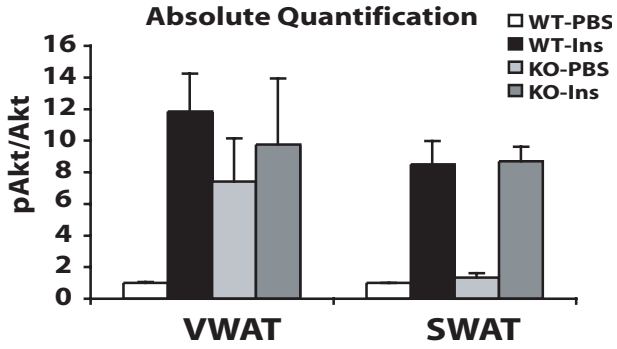
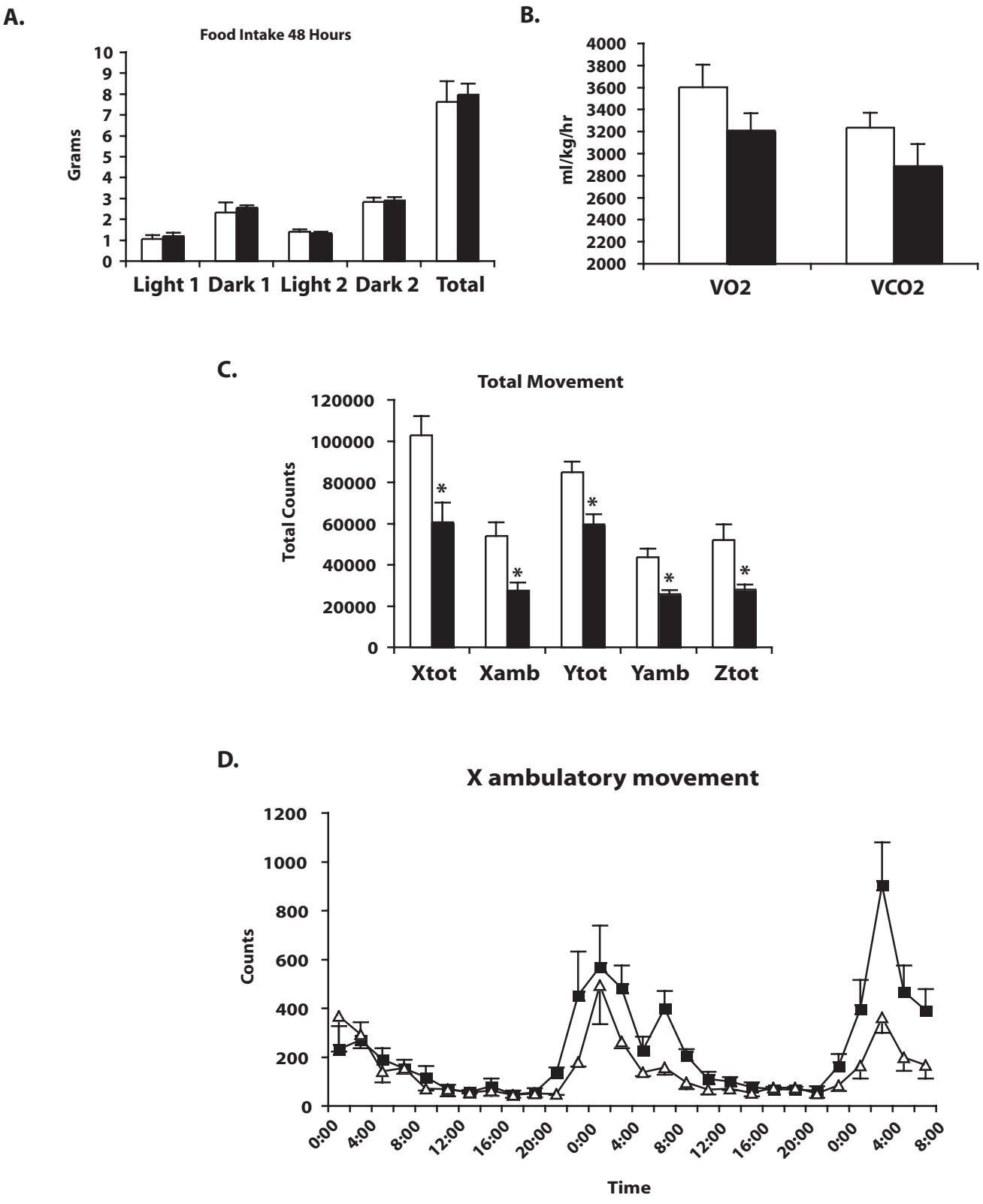


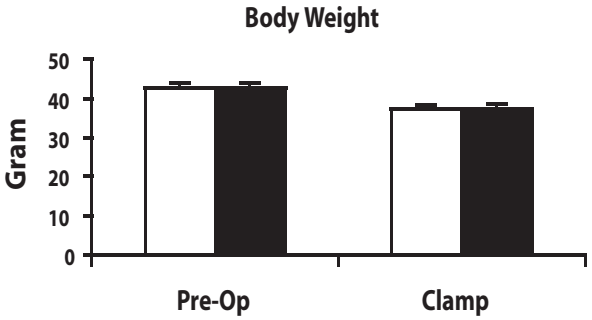
Supplementary Figure 4



[G] Supplemental Text and Figures

Supplementary Figure 5

A.



B.