SUPPLEMENTAL INFORMATION

Supplemental Figure Legends

Figure S1 - Refers to Figure 1. Regulation of thermogenic and β-oxidation gene programs in BAT of WT and Myo-PGC1α4 mice, Related to Figure 1

(A) qPCR analysis of markers associated with thermogenic, mitochondrial and β-oxidation genes programs in BAT of WT and Myo-PGC1α4 (N=6). **(B)** qPCR analysis of PGC-1α4 transgene expression in the adipose tissue of WT and Myo-PGC1α4 mice. Transgene expression was measured primers targeting Flag epitope (forward) and Exon 2 of PGC1α4 (reverse).

Figure S2 – Refers to Figure 2. Regulation of Metrnl gene expression upon exercise and cold exposure, Related to Figure 2

(A) Cos 7 were transduced with Lac Z or Metrnl-Ad and 8h post-transduction, medium was changed to serum-free expression medium for overnight incubation. Medium was collected, concentrated using 10KD MW cut-off and 20ul was used for western blotting against a V5 antibody. **(B)** Analysis of PGC1α isoform gene expression in skeletal muscle biopsies from human volunteers. *Vastus lateralis* biopsies were obtained prior to commencement, 1hr and 4hr post-exercise and gene expression was analyzed by qPCR using human PGC-1α isoform specific primers. **(C)** Plasma obtained from C57/BL6 mice was subjected to ELISA to measure Metrnl levels. For the panel labeled “antibody + excess antigen” the antibody was incubated with 50ug of recombinant Metrnl for 1 hour before using it in ELISA (N=4). **(D)** Plasma obtained from mice expressing WT, heterozygous or KO allele for Metrnl were subjected to ELISA and western blotting analysis to measure Metrnl levels (N=1) **(E)** Analysis of Metrnl mRNA expression in subQ, epi and brown adipose tissue of mice chronically housed at 30^{o}C or acutely subjected to a 4^{o}C cold challenge for 2 weeks (N=5 per group). **(F)** Analysis of

Metrnl mRNA expression in quadriceps and back muscle of mice chronically housed at 30^{o}C or acutely subjected to a 4^{o}C cold challenge for 24 hrs (N=5 per group) **(G)** Metrnl mRNA expression in subQ and epi WAT after an acute bout of downhill running exercise. C57/BL6 mice were divided into groups: sedentary (N=9) and run (N=10). The subQ and Epi WAT were harvested 6 hours after run and processed for gene expression by qPCR *p<0.05, **p<0.001, ***p<0.0001. All data are presented as mean +/- s.e.m.

Figure S3 – Refers to Figure 3. Regulation of thermogenic and pro/anti-inflammatory gene programs by Metrnl in adipose tissue *in vivo,* Related to Figure 3

(A-B) C57/BL6 mice were injected with adenoviral vectors (Ad) expressing Lac Z or Metrnl intravenously (N=6) and **(A)** measurement of serum AST levels by calorimetric analysis at day 5 post-injection **(B)** Metrnl mRNA expression in liver at day 3, and **(C)** markers associated with thermogenesis and mitochondrial gene programs in BAT by qPCR analysis. **(D)** Under the same experimental conditions as in (A), kinetics of UCP-1 mRNA expression in the subQ WAT (N=5 per time-point) by qPCR **(E)** BALB/c mice were injected with Lac Z or Metrnl-Ad (i.v.) (N=6) and the subQ WAT depot was subjected to qPCR analysis of markers associated with thermogenesis and mitochondrial gene programs at day 7. **(F)** C57/BL6 mice fed a HFD for 20 weeks were injected with Lac Z or Metrnl adenovirus (i.v.) and the subQ WAT was analyzed for changes in pro/anti-inflammatory gene programs at day 6 (N=6). **(G)** C57/BL6 were injected i.p. with 9.5mg/mouse of Metrnl-Fc and blood was collected at the indicated time points (N=3). 2ul of serum were run on a non-reducing SDS-PAGE gel and immunoblotted against Metrnl Ab. The first two lanes represent 2.5 and 5ug/ml of Metrnl-Fc for reference standards. *p<0.05, **p<0.001, ***p<0.0001. All data are presented as mean +/- s.e.m.

Figure S4 – Refers to Figure 4. Analysis of food intake and locomotor activity in mice upon Metrnl expression *in vivo,* Related to Figure 4

(A-B) C57/BL6 mice fed a HFD for 20 weeks were injected with Lac Z or Metrnl adenovirus (i.v.) (N=7) and **(A)** food intake, and **(B)** locomotor activity were quantified.

Figure S5 – Refers to Figure 5. Metrnl promotes alternative activation of adipose tissue macrophages, Related to Figure 5

(A) Primary cortical neurons were treated with the indicated doses of recombinant Metrnl-Fc protein for 30 minutes and cell lysates were immuno-blotted with an antibody against phospho and total STAT3. **(B)** SVF from the inguinal fat depot was differentiated into adipocytes for 6 days and treated with recombinant Metrnl (R&D systems) during last two days of differentiation or transduced with adenovirus expressing Lac Z, Metrnl or Fgf21 during last four days of differentiation. qPCR analysis was performed for indicated genes at day 6 (N=4). **(C-E)** C57/BL6 mice were injected with Lac Z or Metrnl-Ad (i.v.) (N=6) and **(C)** analyzed for markers associated with alternative macrophage activation in the epi and brown adipose tissue at day 7, and **(D)** flow cytometric analysis of adipose tissue macrophages (defined as $CD11b^{+}$ and $F4/80^{+}$) in the SVF from subQ WAT at day 5. **(E)** analyzed for markers of classical (M1) macrophage activation in the subQ WAT at day 7. **(F-G)** SubQ WAT of C57/BL6 mice was injected with Lac Z and Metrnl-Ad (N=5) and the injected (left) and contralateral adipose tissue (right, un-injected) was harvested **(F)** at day 3 to analyze for changes in IL4/IL13 gene expression, and **(G)** at day 5 post-injection to assess for changes in markers associated with alternative macrophage activation, by qPCR. *$p<0.05$, **$p<0.001$. All data are presented as mean +/- s.e.m.

Figure S6 – Refers to Figure 6. Regulation of macrophage and eosinophil phenotype by Metrnl, Related to Figure 6

(A) Wild type (WT) and STAT6$^{-/-}$ mice (N=5) were injected with Lac Z or Metrnl-Ad (i.v.) and analyzed at day 7 post-injection for markers associated with classical (M1) macrophage activation in the subQ adipose tissue. **(B-C)** C57/BL6 mice were injected with adenoviral vectors expressing Lac Z or Metrnl (i.v) and administered with neutralizing Ab against IL-4Rα or isotype control (N=5). SubQ WAT was harvested at day 7 and analyzed for markers of **(B)** M2 macrophage activation, and **(C)** thermogenic and β-oxidation genes. **(D)** Under the same experimental setting as in (**B-C**) norepinephrine content of subQ WAT was assessed at day 7. **(E)** Primary bone marrow derived macrophages were treated with either saline, recombinant Metrnl-Fc (5ug/ml) or IL-4 (10ng/ml) for 48h and gene expression was analyzed by qPCR (N=4). **(F-G)** C57/BL6 mice were injected with adenoviral vectors expressing Lac Z or Metrnl (i.v.) (N=6) and **(F)** SVF was isolated at day 4. Eosinophils are Siglec-F^{+} within the CD11b^{+} population with a characteristic SSC/FSC profile. SVF cells were gated on side- and forward scatter (FSC) before analysis using CD11b and Siglec-F antibodies, and **(G)** eosinophil-specific gene markers by qPCR at day 4. **(H)** C57/BL6 mice fed a HFD for 20 weeks (N=8) were injected daily with saline or Metrnl-Fc protein (10mg/kg) (i.p.) and analyzed for eosinophil-specific gene markers by qPCR. **(I)** SubQ adipose tissue of C57/BL6 mice was injected with Lac Z and Metrnl adenovirus (N=5) and the injected (left) and contralateral adipose tissue (right. un-injected) was harvested at day 3 to analyze for changes in eosinophil-specific gene markers. **(J-L)** Under the same experimental setting as in (F) **(J)** kinetics of IL-4 and Siglec-F mRNA expression in subQ WAT by qPCR **(K-L)** flow cytometric analysis of **(K)** adipose tissue T cells (defined as CD3^{+} and CD4^{+}) and **(L)** basophils (defined as FceRI^{+} and CD117^{-}) in the SVF from subQ WAT at day 5. *p<0.05, **p<0.001, ***p<0.0001 comparison between WT mice injected with Lac Z and Metrnl-Ad or isotype Ab treatments of Lac Z and Metrnl-Ad injected mice. $^{\#}$p<0.05, $^{\#\#}$p<0.001, $^{\#\#\#}$p<0.0001 comparison between IL-4Rα Ab treatments of Lac Z and Metrnl-Ad injected mice. $^{+}$p<0.05, $^{++}$p<0.001, $^{+++}$p<0.0001 comparison

between Isotype and IL-4Rα Ab treatments of Metrnl-Ad injected mice. All data are presented as mean +/- s.e.m

Figure S7 – Refers to Figure 7. Metrnl is required for chronic cold-induced metabolic adaptations, Related to Figure 7

(A-B) Mice chronically housed at 30^{o}C were injected with isotype or Metrnl antibody (i.p.) and moved to cold 6 hrs later. Sub Q WAT was harvested and analyzed for markers of genes associated with thermogenic and M2 macrophage activation at **(A)** 24 hrs and **(B)** 48 hrs post cold-challenge. *p<0.05, **p<0.001, ***p<0.0001 comparison between mice at 30^{o}C and 4^{o}C or isotype Ab treatment of mice at 30^{o}C and 4^{o}C. $^{\#}$p<0.05comparison between Metrnl Ab treatment of mice at 30^{o}C and 4^{o}C. All data are presented as mean +/- s.e.m

Supplemental Table Legends

Table S1: List of short-listed genes from affymetrix gene array in quadriceps from Myo-PGC-1α4 mice, Related to Figure 2

qPCR analysis of quadriceps obtained from WT or Myo-PGC-1α4 mice (N=4). Genes with fold change (> 2) over WT control are shown.

Table S2: Quantitative Mass Spectrometric analysis of culture supernatants obtained from Lac Z or PGC-1α4 over-expressed primary myotubes, Related to Figure 2

Primary myotubes were transduced with Lac Z or PGC-1α4 adenovirus and supernatant was collected 48 hours post-transduction for Mass spectrometric analysis (N=3). Additional information on Mass Spectrometric analysis can be found in Extended Experimental Procedures.

Table S3: List of Shortlisted genes that were up-regulated (>2 fold) in both affymetrix and mass spectrometric analysis and qPCR analysis of genes in quadriceps from WT C57/BL6 mice, Related to Figure 2

Table S4: Primers sets used in this study, Related to Figure 1-7

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Reagents

Antibodies against UCP-1 and tubulin were from Abcam. Recombinant Metrnl, Fgf21 and neutralizing antibody against Metrnl (Cat # AF6679) and IgG control were purchased from R&D systems. Antibodies directed against CD16/32, CD11b, CD3, CD4, CD117, FceRI, CD11b, F4/80 (Biolegend) and Siglec F (BD Biosciences) were used for flow cytometric analysis. Insulin, Dexamathasone, 3-Isobutyl-1-methylxanthine (IBMX), Rosiglitazone was from Sigma. Recombinant mouse IL-4 was purchased from Peprotech. LipoD293 DNA transfection reagent was purchased from Signagen. Neutralizing antibody against IL-4Rα (Cat # 552288) and isotype IgG_{2a} control antibody (Cat # 554687) were purchased from BD Biosciences. Anti-V5-HRP antibody was purchased from Invitrogen.

Construction and use of adenoviral vectors

The vector used for Metrnl expression was purchased from OriGene. PCR primers were designed to remove the Stop codon and sub-clone it into the pENTR-D-TOPO vector (Invitrogen). The pENTR vector containing Metrnl cDNA was recombined into the pAd-CMV-DEST-V5 vector (Invitrogen) to obtain Metrnl with a C-terminal V5 epitope tag. Crude virus was produced using the Virapower Adenoviral Expression system (Invitrogen) as per manufacturer's protocol. Crude virus was amplified once (100ul used to infect one 15cm plate) before scaling up for purification. Amplified virus was purified and concentrated using the Vivapure adenopack 100 (Sartorius Stedim Biotech) and buffer exchanged to 10mM Tris-Cl at pH 8.0, 2mM MgCl2, 4% w/v Sucrose), reaching a concentration of ~3-5 x 10^11 ifu/mL. Scale-up and purification was always performed with crude virus and not with use of purified virus. Adenovirus titer was calculated using Adeno-X Rapid Titer kit (Clontech).

Additional Animals and in vivo experiments

For experiments involving lean mice, 8-12 wk old-male mice were used in all experiments. To render mice obese and diabetic, C57/BL6J mice were fed a high fat (60% kcal) diet (D12492, Research Diets) for ~20 wk, starting at 4 wk of age. For cold challenge experiments, C57/BL6J mice were first adapted to 30^{o}C in a laboratory incubator for 3 wks and then moved to 4^{o}C in individually housed cages for indicated time-points. For Metrnl neutralization in vivo, 200ug of Metrnl or control antibody were diluted in saline to a total volume of 300ul and injected intraperitoneally (i.p.) 6 hours before moving to 4^{o} C. For adenoviral injections, Lac Z or Metrnl adenovirus (1 x 10^10 ifu/mice) were diluted in saline and injected intravenously (i.v), via the tail vein (lean) or retro-orbital injections (obese), in a total volume of 150ul. For fat pad injections, Lac Z or Metrnl adenovirus (1x10^10 ifu/mice) were injected directly into the subQ WAT in a total volume of 50 µl. For recombinant protein injections, purified Metrnl-Fc protein was diluted with endotoxin-free TBS in a total volume of 300ul and injected i.p. at 10mg kg^{-1} into HFD-fed mice. For IL-4Rα neutralizing experiments, 125ul of 1 µg/µl of IL-4Rα or isotype control Ab were diluted in saline to a total volume of 300 µl and injected i.p. twice (Days 1 and 4) during the 7 day experiment. Cohorts of >5 mice per genotype or treatment, were assembled for all *in vivo* studies, and were repeated at least 2-3 independent times.

Primary cell cultures and recombinant protein treatments

For primary adipocyte cultures, SVF from inguinal fat depots of 8-12 wk old BALB/cJ mice was prepared and differentiated for 6 days as previously described (Kajimura et al., 2009). Briefly, the differentiation cocktail was used for the first two days, followed by Rosiglitazone and Insulin from day 2-4 and insulin only from day 4-6. Recombinant Metrnl and Fgf-21 were added to the culture media at indicated concentrations for last two days of differentiation. Primary myoblasts were cultured and differentiated as described previously (Rasbach et al., 2010).

Primary cortical neurons were isolated as described previously (Bartlett and Banker, 1984).

RT-PCR and Primers

qPCR was carried out after Trizol-based RNA extraction using RNAeasy (Invitrogen) and thereafter using SYBR green. All data were normalized to TBP and quantitative measurements were obtained using the $\Delta\Delta C_T$ method. All primers used are listed with their sequences in Table S1.

Metrnl ELISA

Mouse Metrnl DuoSet ELISA kit was purchased from R&D systems and the assay was performed as per manufacturer's protocol. 50ul of plasma was used for analysis.

Immunohistochemical staining for UCP-1

Slides were deparaffinized in 3 changes of xylene and hydrated in 95 %, 80 % and 70 % ethanol. Slides were rinsed in water before subjected to heat-mediated antigen retrieval in 10mM pH 6.0 sodium citrate buffer. Quencing of endogenous peroxidases were performed using peroxidase quencing solution (Invitrogen). Slides were blocked in 10 % goat serum and incubated with rabbit polyclonal UCP1 antibody (abcam, ab10983) at 2 µg/ml in PBS-T/1% BSA over night at 4°C. Slides were washed in PBS-T , incubated with 1:500 dilution of anti-rabbit IgG horseradish peroxidase-linked whole antibody from donkey (GE healthcare, NA934V) before developed according to manufacturers description using SuperPicture 3rd Gen IHC Detection Kit (Invitrogen). Hematoxylin was used as counterstain. Immunohistochemical stainings of different fat depots were observed with a Nikon 80i upright light microscope using a 10× objective lens.

Digital images were captured with a Nikon Digital Sight DS-Fi1 color camera and NIS-Elements acquisition software.

Flow cytometry and western blot analysis

Adipose tissues were washed, minced and digested for 60 minutes at 37^{o}C in PBS containing 10mM $CaCl_2$, 2.4 units/ml dispase II (Roche), and 1.5 units/ml Collagenase D (Roche). Digested tissue was then filtered through a 100μm cell strainer to remove undigested tissues. The flow-through was centrifuged for 5 minutes at 1000 x g to separate the stromal vascular fraction (SVF) from adipocytes. The SV cells, resuspended in PBS, were filtered through a 40μm cell strainer to remove clumps and larger adipocytes, and then pelleted again. Cell pellet was re-suspended in FACS buffer (PBS containing 2% FBS and 0.25% sodium azide) and used for staining. Stained cells were fixed with 4% paraformaldehyde and stored at 4^{o}C before analysis. Data was acquired on FACS Canto II (BD Biosciences) and data analysis was performed using FlowJo (Treestar). For immunoblot anaylsis, tissues were homogenized and lysed with metal beads in RIPA buffer (Thermo Scientific) containing protease inhibitor cocktail (Thermo Scientific) and phosphatase inhibitor cocktail (Thermo Scientific). For western blotting of plasma samples, 1 μl of plasma was prepared containing 2X sample buffer (Invitrogen) with reducing agent, boiled and analyzed using western blot against Metrnl or indicated antibody.

Metrnl-mFc construct, protein production and purification

Metrnl-Fc fusion protein was expressed and purified from Chinese hamster ovary (CHO) cells. Briefly, mouse Metrnl cDNA was PCR amplified and cloned into pLEV113 mammalian expression vector (LakePharma) downstream of a secretion signal peptide and upstream of an in-frame mouse IgG1 Fc domain. Metrnl-Fc was transiently transfected into CHO cells by electroporation (MaxCyte), and supernatant was collected after 8 days. After the supernatant

was clarified by centrifugation, Metrnl-Fc protein was purified with MabSelect SuRe Protein A medium (GE Healthcare) and flash frozen in 200 mM HEPES (pH 7.5), 0.2% NaOAc.

Comprehensive laboratory animal monitoring system (CLAMS)

C57/BL6J mice were fed a high fat (60% kcal) diet (D12492, Research Diets) for 20 weeks, starting at 4 weeks of age. Mice were then injected with adenovirus (1 x 10^10 ifu) expressing Lac Z or Metrnl, and CLAMS analysis was performed as described previously. Briefly, mice were acclimatized for 1 day before start of analysis and CO2 and O2 levels were then collected every 36 min for a period of 6 days. Data on activity, heat generation and food intake were measured at more frequent intervals and the data were normalized to body weights.

Norepinephrine Elisa

2-CAT (A-N) Research ELISA was purchased from Rocky Mountain Diagnostics and assay was performed was per manufacturer's protocol. Briefly, adipose tissues were homogenized and sonicated in homogenization buffer (1N HCL, 0.25 M EDTA, 1 M $Na_2S_2O_5$), and cellular debris was pelleted by centrifugation at 13000rpm for 10 min at 4^{o}C. The cleared homogenates were collected and stored at -80^{o}C before quantification. All samples were normalized to total protein concentration.

Mass Spectrometry Analysis

Protein extraction, digestion and tandem mass tagging (TMT) labeling: Secreted proteins from transfected Lac Z, or PGC1α4 expressing adenovirus primary myotubes (10 mL of serum free media, 12 hr incubation) were concentrated by methanol chloroform precipitation and homogenized with SDS lysis buffer (2.0 % SDS w/v, EDTA free protease inhibitor cocktail

(Promega, Madison, WI) and 50 mM HEPES, pH 8.8). Extracts were reduced with 5 mM DTT and cysteine residues were alkylated with iodoacetamide (14 mM) as previously described (Huttlin et al., 2010). Protein content was further separated from impurities by methanol-chloroform precipitation. Protein pellets were resuspended in 8 M Urea containing 50 mM HEPES (pH 8.5) and concentrations were measured by BCA assay (Thermo Scientific, Rockford, IL) prior to protease digestion. Protein lysates were diluted to 4 M urea and digested with LysC (Wako, Japan) in a 1/100-enzyme/protein ratio overnight (RT). Digests were acidified with 10% formic acid (FA) to a pH ~ 2 and subjected to C18 solid-phase extraction (SPE) (Sep-Pak, Waters, Milford, MA).

Isobaric labeling of the digested peptides was accomplished using 6-plex mass tag (TMT) reagents (Thermo Fisher Scientific, Rockford, IL). TMT reagents (0.8 mg), were resuspended in 40µl dry acetonitrile (ACN) and 10 µl was added to 100 µg of peptides dissolved in 100 µl of 200mM HEPES, pH 8.5. After 1hr (RT), the reaction was quenched by adding 4 µl of 5% hydroxylamine. Labeled peptides were combined, acidified with FA (pH ~2) and diluted to a final ~5% ACN concentration prior to C_{18} SPE on Sep-Pak cartridges (50 mg).

Strong cation exchange (SCX) chromatography: Secreted peptides were subjected to orthogonal SCX chromatography prior to proteomic analysis. Briefly, peptides were solubilized in SCX buffer A (7 mM KH_2PO_4, pH 2.7, 30% ACN) and separated via a 4.6 x 200 mm polysulfoethyl A HPLC column (particle size, 5µm; pore size, 200 Å; PolyLC, Columbia, MD). Peptides were separated by applying a dual two-buffer (SCX A and B) gradient from 10 to 55% SCX buffer B (7 mM KH_2PO_4, 350 mM KCl, pH 2.7, 30% ACN) in 50 min at a flow rate of 0.8 ml/min, followed by 50 to 100% SCX buffer A to buffer B in 4.5 min using an Agilent 1100 quaternary pump outfitted with a degasser (Agilent Santa Clara, CA) and a photodiode array detector (PDA) (Thermo Scientific, San Jose, CA). Samples were collected in ~40 sec increments into a 96-well plate, and dried under vacuum. Fractions were redissolved with 5% FA/5% ACN, combined into 16 samples, desalted by C18 StageTips (3M Empore, South Eagan, MN), dried

and reconstituted for LC-MS/MS analysis.

Mass spectrometry analysis: Liquid chromatography separation and tandem mass spectrometry (LC-MS/MS): All SCX fractions were subjected to LC-MS/MS analyses onto a LTQ Orbitrap Velos (Thermo Scientific San José, CA) instrument equipped with a Famos autosampler (LC Packings, Sunnyvale, CA) and an Agilent 1200 binary HPLC pump (Agilent Technologies, Santa Clara, CA). Peptides were separated onto a 100 µm I.D. microcapillary column packed first with approximately 1.5 cm of Magic C4 resin (5µm, 100 Å, Michrom Bioresources, Auburn, CA) followed by ~18 cm of Maccel C18AQ resin (1.8 µm, 200 Å, Nest Group, Southborough, MA). Peptides were separated by applying a gradient from 10 to 40% ACN in 0.5% FA over 160 min at ~250 nl/min flowrate. Electrospray ionization implemented through applying a voltage of 1.8 kV using an inert gold electrode via a PEEK junction at the end of the microcapillary column. The LTQ Orbitrap Velos was operated in data-dependent manner for the MS methods. The MS survey scan was performed in the Orbitrap in the range of 350-1500 m/z at a resolution of $3x10^4$, followed by the selection of the ten most intense ions (TOP10) for CID-MS^2 fragmentation in the ion trap using a precursor isolation width window of 2 m/z, AGC setting of 2000, and a maximum ion accumulation of 150ms. Singly charged ion species were excluded from CID fragmentation. Normalized collision energy was set to 35% and an activation time of 20ms. Ions within a 10-ppm m/z window around ions selected for MS^2 were excluded from further selection for fragmentation for 100s. Directly following each MS^2 event, 6-10 of most intense fragment ion in an m/z range between 110-160% of the precursor m/z was selected for HCD-MS^3 (McAlister et al., 2012; Ting et al., 2011). The fragment ion isolation width was set to 4 m/z, AGC was set to 20,000, the maximum ion time was 250ms, normalized collision energy was set to 60% and an activation time of 50ms for each MS^3 scan. For all MS^3 scans, Orbitrap resolving power was set to 15,000 (@ 400 *m/z*).

Mass spectrometry analysis: Data processing and spectra assignment: A

compilation of in-house software was used to convert mass spectrometric data (Raw file) to a mzXML format, as well as to correct monoisotopic m/z measurements and erroneous peptide charge state assignments. Assignment of MS/MS spectra was performed using the Sequest algorithm by searching the data against a protein sequence database including all entries from the mouse Uniprot database (December, 2011 download date) containing known contaminants such as human keratins and its reverse decoy components (Elias and Gygi, 2007). Sequest searches were performed using a 20-ppm precursor ion tolerance and requiring each peptides N-/C- termini to have a LysC protease specificity, while allowing up to two missed cleavages. TMT tags on peptide N termini/lysine residues (+229.162932 Da) and carbamidomethylation of cysteine residues (+57.02146 Da) were set as static modifications while methionine oxidation (+15.99492 Da) was set as variable modification. A MS^2 spectra assignment false discovery rate (FDR) of less than 1% was achieved by applying the target-decoy database search strategy (Elias and Gygi, 2007). Filtering was performed using an in-house linear discrimination analysis algorithm to create one combined filter parameter from the following peptide ion and MS^2 spectra metrics: Sequest parameters XCorr and ΔCn, peptide ion mass accuracy and charge state, peptide length and mis-cleavages. Linear discrimination scores were used to assign probabilities to each MS^2 spectrum for being assigned correctly and these probabilities were further used to filter the dataset to a 1% protein-level false discovery rate (Huttlin et al., 2010).

Determination of TMT reporter ion intensities and quantitative data analysis: For quantification, a 0.03 *m/z* window centered on the theoretical *m/z* value of each the six or ten reporter ions and the closest signal intensity from the theoretical *m/z* value was recorded. Reporter ion intensities were further de-normalized based on their ion accumulation time for each MS^3 spectrum and adjusted based on the overlap of isotopic envelopes of all reporter ions (manufacturer specifications). Total signal to noise values for all peptides were summed for each TMT channel, and all values were adjusted to account for

variance in sample handling. For each peptide, a total minimum signal to noise value of 60 and 100 was required for a 6-plex- and 10-plex- TMT experiment, respectively (McAlister et al., 2012; Ting et al., 2011).

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Figure S1
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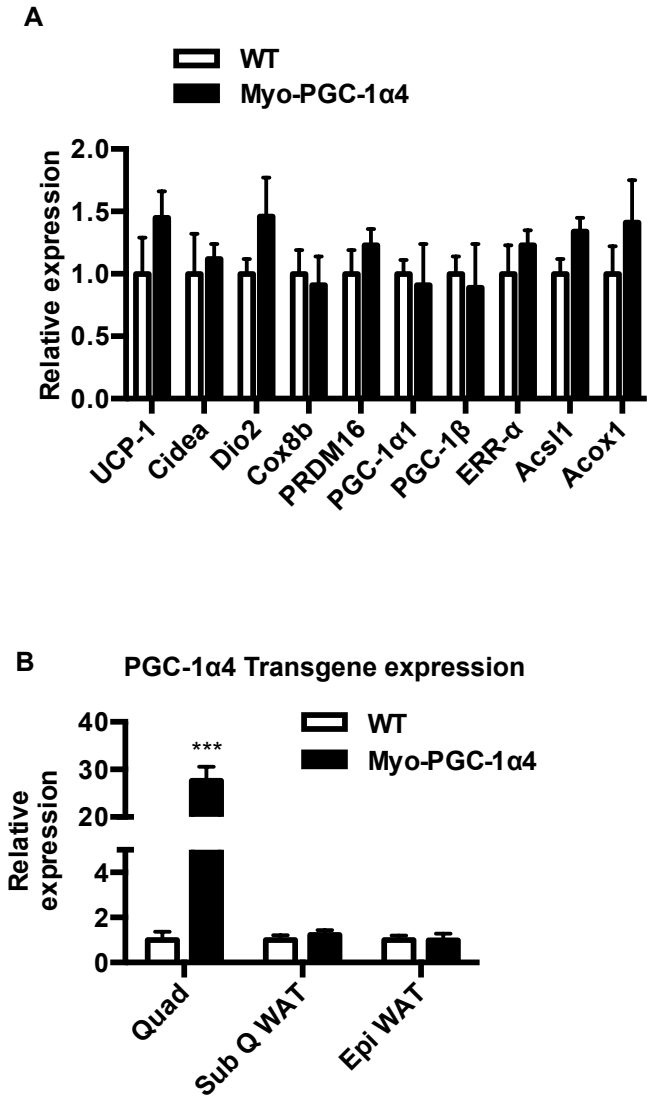
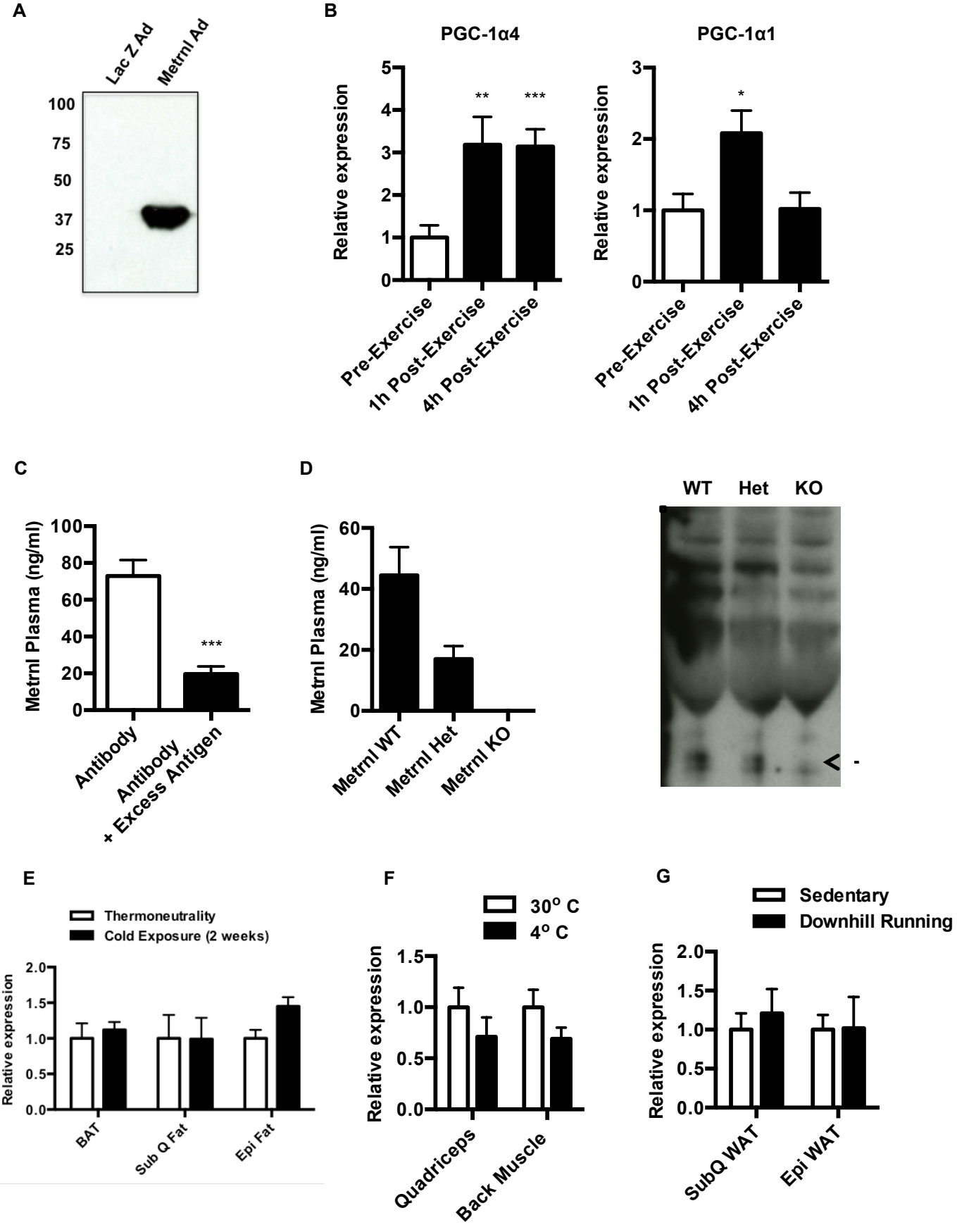


Figure S2
Rao et. al.



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Rao et. al.

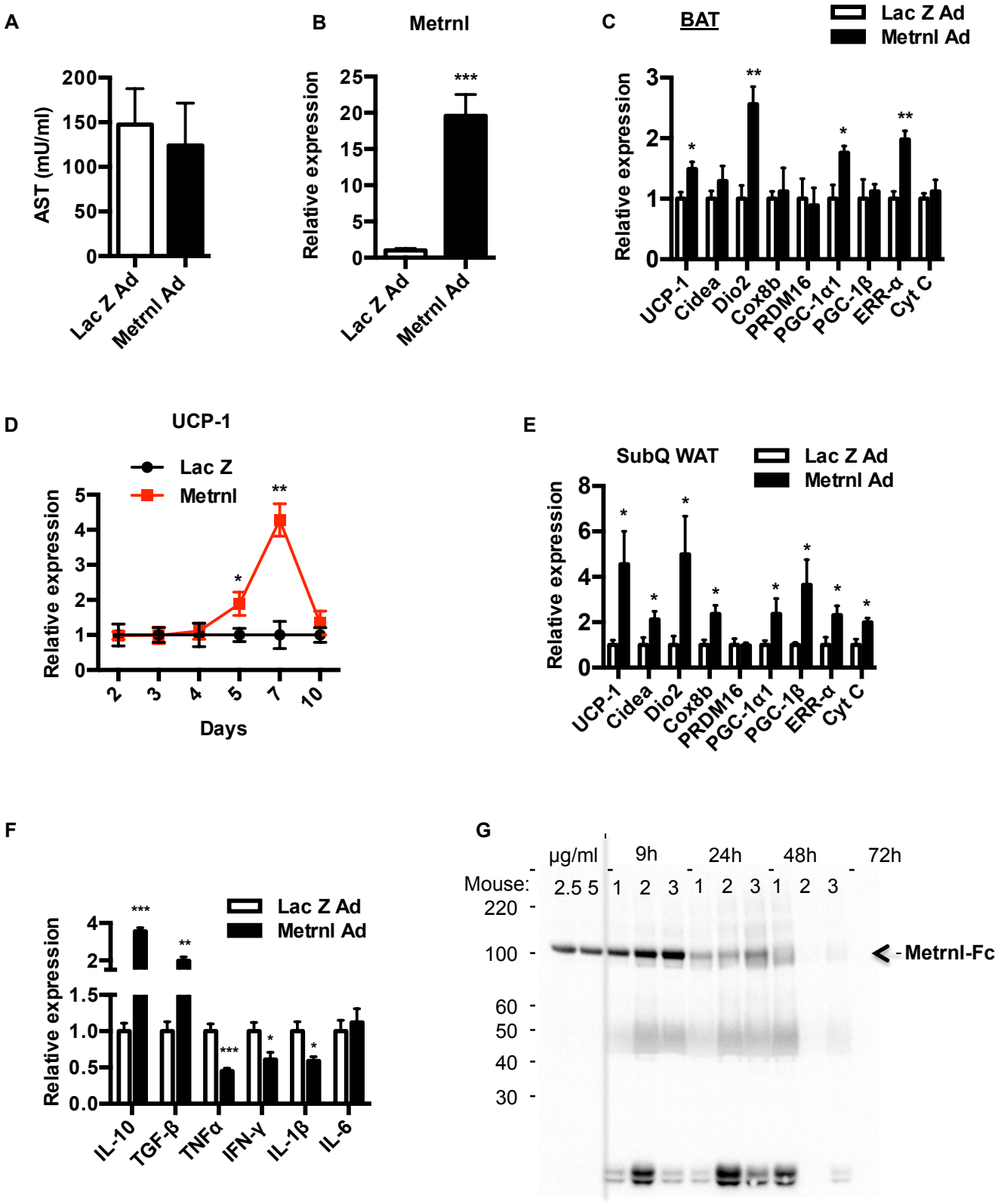
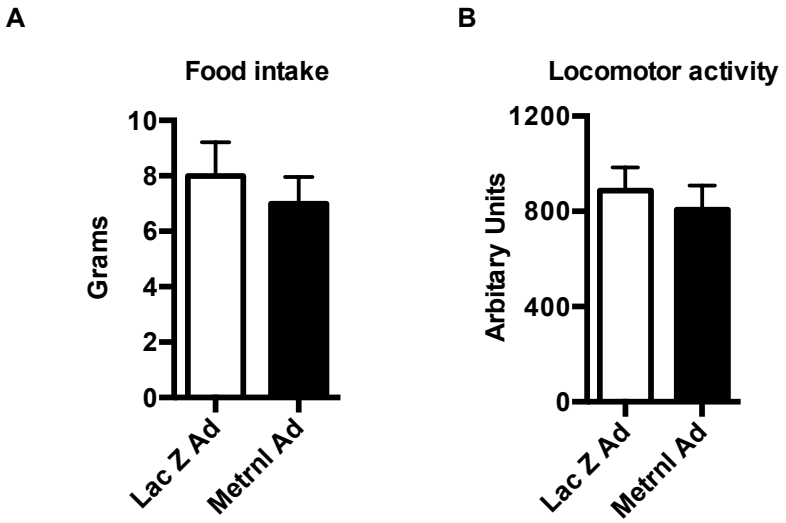
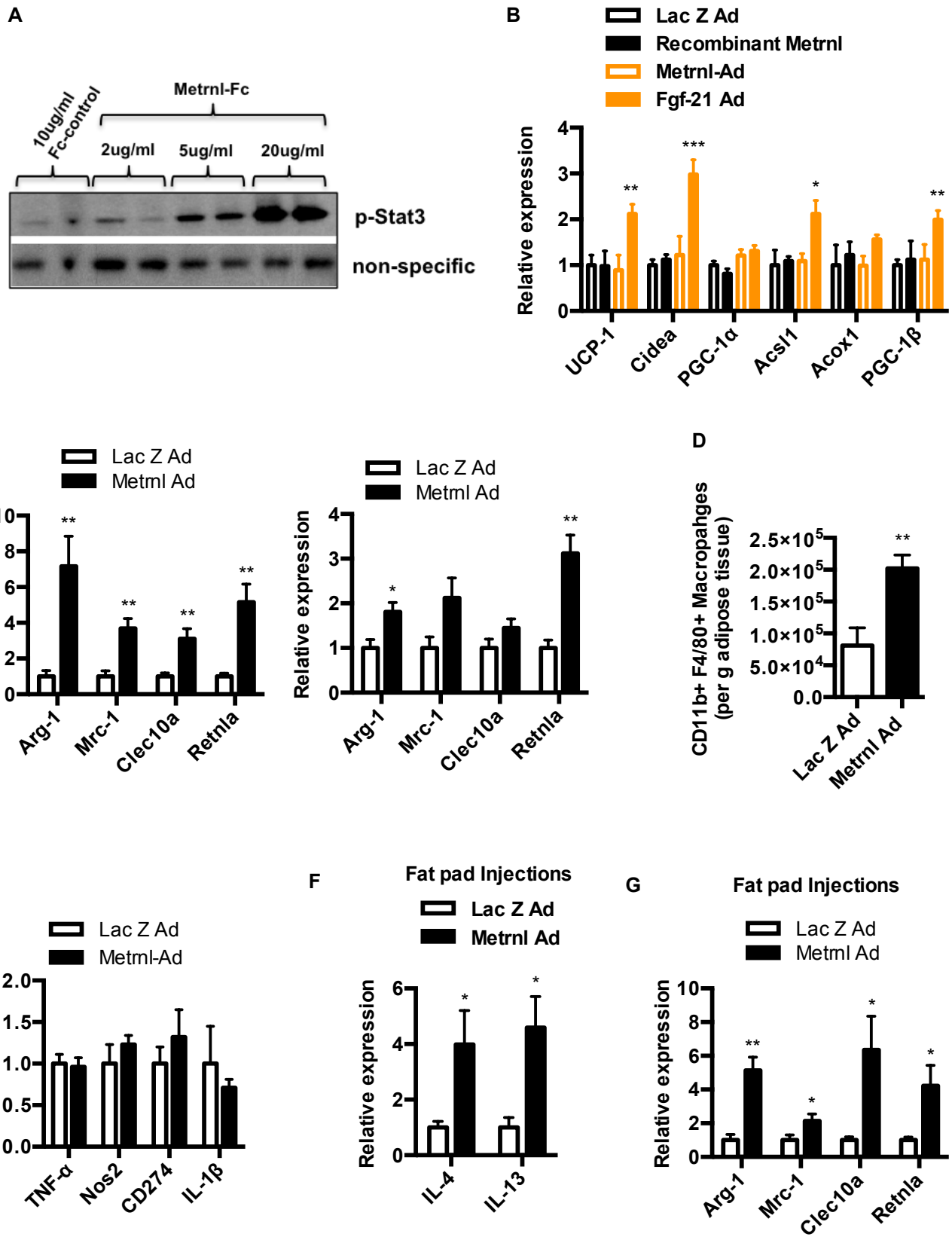


Figure S4
Rao et. al.



Supplemental Figure

Figure S5, Rao et. al.



Supplemental Figure

Figure S6
Rao et. al.

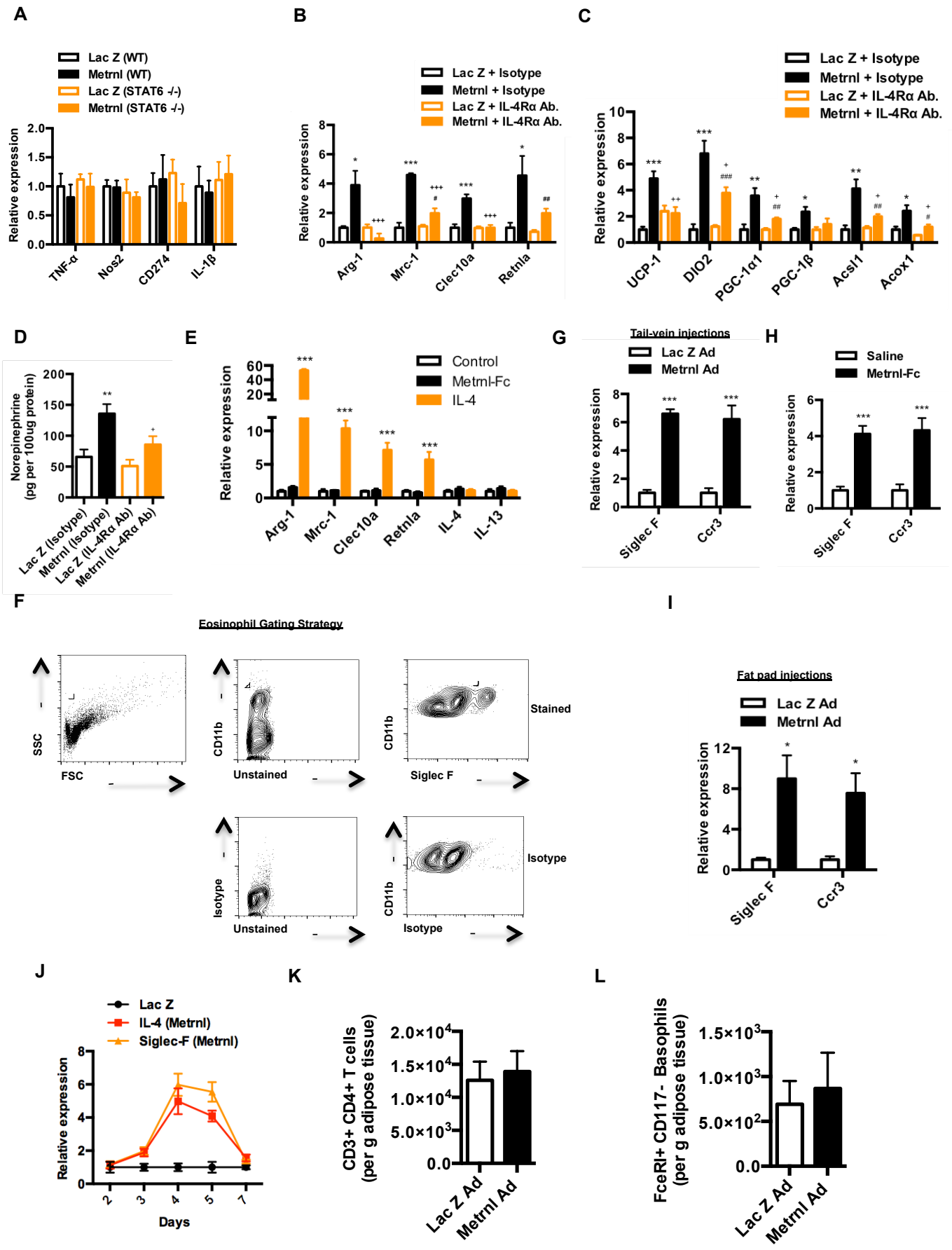


Figure S7
Rao et. al.