Fatty acid synthase downregulation contributes to acute lung injury in murine diet-induced obesity

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Supplemental Material

Methods

Experimental Animals, Obesity Model and Exposure to Hyperoxia

Four-week-old male C57BL/6J mice and 13 week old male mice homozygous for the obese spontaneous mutation *Lep*^{ob} (B6.Cg-*Lep*^{ob}/J, *ob/ob*) and heterozygous controls (*ob/+*) were purchased from Jackson Laboratory (Bar Harbor, ME). *FASN*$^{loxp/loxp}$ mice were kindly provided by Dr Clay F Semenkovich, Washington University School of Medicine (1) and *Sftpc*$^{CreERT2+/+}$ mice by Dr Brigid Hogan (2). To generate male mice with tamoxifen inducible fatty acid synthase (FASN) deletion specifically in alveolar epithelial type II cells, *FASN*$^{loxp/loxp}$ were crossed to *Sftpc*$^{CreERT2+/+}$ mice. Six daily intraperitoneal tamoxifen (100 mg/kg/dose; Sigma-Aldrich) injections were given at 5 weeks of age to induce recombination by CreERT2 (3). *Sftpc*$^{CreERT2+/-}$ mice were used as controls for all experiments except one, in which tamoxifen injected *FASN*$^{loxp/loxp}$ served as controls. At the age of 6 weeks, mice were divided into two groups. One group (obese group) received a diet in which 60% of the calories are derived from fat in the form of lard (D12492, OpenSource Diets, NJ USA) and the other group (control group) an ingredient matched diet in which 10% of the calories are derived from fat (D12450B) for 12-14 weeks. All animals were maintained under identical conditions of temperature, humidity and dark/light cycle in a specific pathogen-free animal facility at Weill Cornell Medicine. Mice were exposed to hyperoxia in an air-tight custom built plexiglass exposure chamber (27 x 27 x 12 inches) with constant

temperature and humidity maintained with 18 L/min oxygen (O_2) (>95% O_2) equipped with a slotted floor containing carbon dioxide (CO_2) absorbent. The oxygen concentration in the chamber was monitored with a VTI Oxygen Analyzer (Vascular Technology Inc, Nashua, NH). Animals were supplied food and water throughout exposure.

Sample Collection

Mice were anesthetized with intraperitoneal injection of sodium pentobarbital (8 mg). The trachea was exposed and a 19-gauge cannula was inserted and sutured around the tracheal lumen. Bronchoalveolar lavage fluid (BALF) was collected by slowly washing the lungs with Phosphate Buffered Saline (PBS) (700 µl) via the endotracheal tube three times. BALF was centrifuged at 161 x g for 5 minutes at 4^{o}C to separate cellular components and supernatant stored at -80 ^{o}C. BALF supernatant was used for measurement of total protein (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Rockford, IL), interleukin-6 (IL-6, IL-6 mouse uncoated ELISA kit, Thermo Fisher Scientific, Rockford, IL), tumor necrosis factor-α (TNFA, TNFA mouse uncoated ELISA kit, Thermo Fisher Scientific, Rockford, IL) and immunoglobulin M (IgM, IgM mouse uncoated ELISA kit, Thermo Fisher Scientific, Rockford, IL) concentration, and free fatty acid quantification (Free Fatty Acid Quantification Colorimetric/Fluorometric Kit, Biovision, Milpitas, CA) as per manufacturer's instructions. The pellet was resuspended in PBS (500 µl), and total concentration of cells was determined. BALF cells were spun onto glass slides using a cytocentrifuge and differential

counts of inflammatory cells performed after staining cells with the Hema 3 Stain Set (Fisher Scientific, Kalamazoo, MI). At least 200 cells were counted for each sample from >3 independent fields of view (x100) in a blinded fashion. Animals were euthanized by exsanguination from the heart. Blood was allowed to clot, centrifuged at 2,000 x g, and serum was stored at -20 ^{o}C. The lungs were perfused with PBS and resected for immunoblotting. For histology, in a different set of animals, to maintain architecture the lung was distended through the trachea with 0.5% low melting agarose and placed into cold 4% paraformaldehyde (PFA) (4). For RNA sequencing, the lungs were resected and placed in RNA*later* (Sigma-Aldrich, St. Louis, MO).

Serum Cholesterol and Fasting Glucose Measurements

Concentration of total serum cholesterol was determined spectrophotometrically on a Beckman Coulter AU analyzer (OSR6116, Beckman Coulter, Atlanta, GA). Mice were fasted for 5 hours by placing them in new cages without food but with access to water. Blood glucose levels were measured by placing a drop of blood obtained from the tip of the tail onto a glucose strip (Accu-Chek, Roche, Indianapolis, IN) into a glucometer (Accu-Chek Aviva, Roche, Indianapolis, IN).

Cell Death Assays

Cell death was assessed by measuring lactate dehydrogenase (LDH) in BAL fluid supernatant using a commercially available assay (LDH-Cytotoxicity

Colorimetric Assay Kit II, BioVision, Milpitas, CA) and by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining on histology slides as previously described (5). The degree of cell death was expressed as a ratio of the number of TUNEL positive cells per total number of alveoli in the field from 10 random images from each mouse.

Measurement of Lung Tissue Free Fatty Acids

Lipids were extracted from lung tissue and dissolved in 3% Triton-X (6). Concentrations of free fatty acids were measured enzymatically in total lung lipid extracts (Wako Diagnostics, CA, USA).

RNA Isolation and RNA-seq Analysis

RNA samples were obtained from lung tissue of C57BL/6J mice fed high fat and control diet exposed to room air and hyperoxia. RNA was extracted using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) and submitted to the Genomic Resource Core Facility of Weill Cornell Medical College. The RNA quality was determined by 260:280 ratio and the RNA integrity number (RIN) determined by an Agilent Technologies 2100 Bioanalyzer. Only high quality RNA samples with a 260:280 ratio > 1.6 and a RIN > 7 were used for the library construction using the TruSeq Stranded mRNA Library Preparation kit (Illumina), according to manufacturer's instructions. The cBot fluidic device (Illumina) was used to hybridize samples onto a flow cell and to generate clonal clusters of the DNA fragments. The sequencing was performed on the HiSeq4000 sequencer

(Illumina). RNA reads were aligned and mapped to the mm9 mouse reference genome by TopHat2 (version 2.0.11) (7), and transcriptome reconstruction was performed by Cufflinks (version 2.1.1). The abundance of transcripts was measured as fragments per kilobase of transcript per million fragments sequenced (FPKM) (8, 9). Differential expression, cluster, and principle component analyses were performed using *DESeq2* package (10). Adjusted p-values for multiple testing were calculated based on the Benjamini-Hochberg method. We compared the genes that were significantly different between high fat and control diet mice with mouse MitoCarta2.0, an inventory of mouse genes encoding proteins with strong support of mitochondrial localization based on integrated proteomics, computation, and microscopy (https://www.broadinstitute.org/scientific-community/science/programs/metabolic-disease-program/publications/mitocarta/mitocarta-in-0) (11). Heat maps were plotted using *pheatmap* R package (12). RNA seq data are available under the accession code GSE123938 in Gene Expression Omnibus.

Isolation of Alveolar Epithelial Type II (AEC2) Cells from Mouse Lungs through MACS Separation

The isolation of AEC2 cells from mouse lungs was performed as previously described (13). Briefly, mice were euthanized by intraperitoneal injection of 8 mg pentobarbital, and thoracotomy was performed. The mouse lungs were perfused through the right ventricle using PBS, and then inflated by 1.5 mL dispase (BD Biosciences, Franklin Lakes, NJ) and 0.5mL 1% low-melting point agarose (Invitrogen, Carslbad, CA). After cooling by ice for 2 minutes, the lungs were

excised and were transferred to a 50 ml polypropylene tube containing 2 mL dispase. After digestion for 45 minutes at ambient temperature, the lungs were homogenized manually using the plunger of a 1 mL syringe in a 10 cm petri dish with Dulbecco's modified Eagle's medium (DMEM) containing 200 U/mL DNase (Sigma-Aldrich, St Louis, MO). After filtration sequentially through 100 μm, 40 μm (BD Biosciences, Franklin Lakes, NJ), and 0.22 μm (EMD Millipore, Burlington, MA) strainers, and centrifugation, the whole lung cell suspension was obtained, and was negatively selected by CD45 microbeads (Miltenyi Biotec, Auburn, CA), followed by positive selection by streptavidin microbeads (Miltenyi Biotec, Auburn, CA) and biotin-conjugated anti-EpCAM antibody (eBioscience, San Diego, CA), through MACS separation columns. The CD45(-)EpCAM(+) population mainly composed of AEC2 cells (purity ~94% quantification by immunofluorescence staining; please refer to the methods about immunofluorescence staining for details about purity examination).

Immunofluorescent Staining for the Purity of Isolated AEC2 Cells

To quantify the AEC2 purity in MACS-isolated CD45(-)EpCAM(+) populations, we performed immunofluorescent staining of surfactant protein C (SP-C) using cytospin slides. After isolation, the CD45(-)EpCAM(+) cells were fixed by 4% PFA in a flow cytometry tube for 12 minutes under ambient temperature, and were transferred to slides by cytospin centrifugation at 350 rpm for 3 minutes. The CD45(-)EpCAM(-) population was used to prepare cytospin slides for negative control. The blocking and permeation were performed at

ambient temperature for 1 hour, using the buffer containing 5% normal goat serum (Vector Laboratories, Burlingame, CA) and 0.3% Triton X-100 (Sigma-Aldrich, St Louis, MO) in tris-buffered saline (TBS). The cells were incubated overnight with primary antibody against SP-C (1: 1000 in blocking buffer, EMD Millipore ABC99, Burlington, MA) in a humidified chamber at 4^{o}C. 16-24hours later, the cells were incubated with the Alexa Fluor-488-conjugated secondary antibody (Thermo Fisher, Waltham, MA) for 1 hour under ambient temperature. Hoechst 33342 (1:1000 dilution in TBS) was used to stain the nucleus. The slides were mounted using Prolong Gold antifade solution (Invitrogen, Carslbad, CA), and the images of the slides were obtained by confocal microscopy (Zeiss LSM 880 laser scanning microscope).

Western Immunoblot Analysis

Proteins were isolated from lung tissue samples and analyzed by Western blot using rabbit antimouse FASN (1:1000, Cell Signaling Technology 3180s, Danvers, MA), rabbit anti-Claudin 18 (1:1000, Thermo Fisher 38-8100, Waltham, MA), and mouse anti-β-actin (1:5000, Sigma-Aldrich A2228, St. Louis, MO). Immunoblotting was also performed with lysates of MACS-isolated AEC2 cells using total OXPHOS rodent WB antibody cocktail (1:1000, Abcam 110413, Cambridge, MA), mitochondrial membrane integrity WB antibody cocktail (1:1000, Abcam 110414, Cambridge, MA), and TOM20 (1:2000, Santa Cruz 11415, Dallas, TX). Briefly, Tissue Extraction Reagent (Invitrogen, Vienna, Austria) with protease (Complete Mini, Roche Diagnostics, Indianapolis, IN) and phosphatase

(PhosStop, Roche Diagnostics, Indianapolis, IN) inhibitor cocktail were used to prepare the tissue lysates, and RIPA buffer with protease inhibitor cocktail to prepare the cell lysates. Protein concentrations were measured with the BCA protein assay (ThermoFisher, Kalamazoo, MI). The proteins were then resolved by NuPAGE 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL). The horseradish peroxidase (HRP)-conjugated secondary antibodies, anti-rabbit IgG (7074s) and anti-mouse IgG (7076s), were from Cell Signaling. The densitometry of the bands was measured using FIJI running ImageJ software (version 1.52b) (https://fiji.sc/) and were normalized to β-actin as a loading control.

Histology and Immunohistochemistry (IHC)

For histological examination, after 24 hours of fixation in 4% PFA at 4ºC, murine lungs were transferred to 70% ethanol and submitted to the core lab at Weill Cornell Pathology and Laboratory Medicine for tissue processing, paraffin embedding, hematoxylin and eosin, and IHC staining. The primary antibodies against FASN (1:500, Cell Signaling Technology 3180s, Danvers, MA) and PCNA (1:2500, Abcam ab29, Cambridge, MA) were used for IHC. The paraffin-embedded lung sections were first baked and deparaffinized. For antigen retrieval, the slides were heated on the Bond III Autostainer at 99-100ºC, and the sections subjected to sequential incubation with an endogenous peroxidase block, primary antibody, secondary antibody, polymer, diaminobezidine, and hematoxylin. Finally, the sections were dehydrated in 100% ethanol, and mounted in Cytoseal XYL (Richard

Allan Scientific, San Diego, CA). Appropriate positive and negative controls were included.

Seahorse Analysis

Extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) were determined by the Agilent Seahorse XFe96 flux analyzer (Seahorse Bioscience, Agilent Technologies, Billerica, MA). AEC2 cells were isolated as described above and plated overnight onto cell culture microplates (Seahorse Bioscience, Agilent Technologies, Billerica, MA) coated with 50 ng/µl Laminin 1 (Trevigen, Gaithersburg, MD). Cells were incubated in XF RPMI assay medium (Seahorse Bioscience, Agilent Technologies, Billerica, MA), supplemented with 5 mM glucose, 4 mM glutamine and 1 mM pyruvate (Sigma, St Louis, MO) for one hour prior to the measurement. After the recording of the basal rates of ECAR and OCR, final concentrations of 1 µM oligomycin, 2 µM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) and 0.5-0.5 µM rotenone and antimycin A (Seahorse Bioscience, Agilent Technologies, Billerica, MA) were serially injected and corresponding metabolic rates measured. Components of cellular oxygen consumption were then calculated including proton leak, maximal respiration, spare respiratory capacity and non-mitochondrial respiration respectively.

Statistics

Survival analysis between groups was calculated using the log-rank test.

Continuous variables are presented as mean ± SEM and compared with the nonparametric Mann Whitney *U* test (for comparison of two groups) or the analysis of variance with Tukey post hoc correction (for comparisons of more than two groups). A 2-tailed *P* value of less than 0.05 was considered to denote statistical significance. The detailed statistical analyses for RNA-seq data were described in the method details of RNA-seq. All analyses were performed using GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, CA).

Study Approval

All experiments were approved by the Weill Cornell Medicine Institutional Animal Care and Use Committee.

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Supplemental Figure Legends:

Figure 1s. A) Western blot analysis for Claudin-18 in lungs with β-actin loading control. Densitometry analysis shown on right (n=3 per group for room air and n=4 per group for hyperoxia, ANOVA with Tukey post hoc correction: **p<0.01, *p<0.05, ns=non significant), B) Bronchoalveolar lavage fluid (BALF) total cell count after 48 hours of hyperoxia or room air (cells/ml, n=3 per group for room air and n=8 per group for hyperoxia, ANOVA with Tukey post hoc correction non significant), C) BALF macrophage count after 48 hours of hyperoxia or room air (macrophages/ml, n=3 per group for room air and n=8 per group for hyperoxia, ANOVA with Tukey post hoc correction non significant), D) BALF interleukin 6 (IL-6) levels after 48 hours of hyperoxia or room air (pg/ml, n=3 per group for room air and n=8 per group for hyperoxia, ANOVA with Tukey post hoc correction non significant), E) Representative section of proliferating cell nuclear antigen (PCNA) stained lungs (n=2 per group for room air and n=7 per group for hyperoxia, original magnification x40). Data are expressed as mean ± SEM.

Figure 2s. A) Weight of B6.Cg-*Lep*^{ob}/J (*ob/ob*) mice and heterozygous controls (*ob/+*) (13 weeks old) receiving regular diet. (gr, n=11 per group, Mann-Whitney test: ***p<0.0001), B) Bronchoalveolar lavage fluid (BALF) total cell count after 48 hours of hyperoxia or room air (cells/ml, n=3 per group for room air and n=8 per group for hyperoxia, ANOVA with Tukey post hoc correction: *p<0.05). C) Western blot analysis for fatty acid synthase (FASN) in lungs of *ob/ob* mice and controls with β-actin loading control. Densitometry analysis shown on right (n=3 per group

for room air and n=4 per group for hyperoxia). Data are expressed as mean ± SEM.

Figure 3s. A) Bronchoalveolar lavage fluid (BALF) total cell count in *FASN*$^{loxp/loxp}$*Sftpc*$^{CreERT2+/-}$ (*Fasn*$^{i\Delta AEC2}$) and *Sftpc*$^{CreERT2+/-}$ (Control) mice after 48 hours of hyperoxia or room air (cells/ml, n=8 per group for room air and n=15 per group for hyperoxia, ANOVA with Tukey post hoc correction: ns=non significant), B) BALF macrophage count after 48 hours of hyperoxia or room air (macrophages/ml, n=8 per group for room air and n=14 for control and n=15 for *Fasn*$^{i\Delta AEC2}$ mice for hyperoxia, ANOVA with Tukey post hoc correction: ns=non significant), C) BALF interleukin 6 (IL-6) levels after 48 hours of hyperoxia or room air (pg/ml, n=8 per group for room air and n=15 per group for hyperoxia, ANOVA with Tukey post hoc correction: ***p<0.001, **p<0.01, ns=non significant), D) BALF protein levels from *Fasn*$^{i\Delta AEC2}$ and *FASN*$^{loxp/loxp}$*Sftpc*$^{CreERT2-/-}$ mice after 48 hours of exposure to >95% oxygen or room air (mg/ml, n=3 per group for room air and n=5 for *FASN*$^{loxp/loxp}$*Sftpc*$^{CreERT2-/-}$ and n=7 for *Fasn*$^{i\Delta AEC2}$ mice for hyperoxia, ANOVA with Tukey post hoc correction: **p<0.01, *p<0.05). E) Alveolar epithelial type II cells were isolated from *Fasn*$^{i\Delta AEC2}$ and control mice and protein expression was assessed using the Total OXPHOS (complex I- ubiquinone oxidoreductase subunit B8 (NDUFB8), complex II- succinate dehydrogenase complex iron sulfur subunit B (SDHB), complex III- ubiquinol-cytochrome c reductase core protein 2 (UQCRC2), complex IV- mitochondrially encoded cytochrome c oxidase 1 (MTCO1), complex V- ATP synthase subunit alpha (ATP5A)) (left) and the mitochondrial Membrane Integrity antibody cocktail (outer membrane-porin,

intermembrane space-cytochrome c, inner membrane-complex VA and complex III core 1, matrix space-cyclophilin F) (right). TOM20 expression was used to confirm equivalent protein input. Densitometry analysis shown (n=3 per group). F). Energy phenotype profile, basal and maximal respiration, and spare respiratory capacity of isolated AEC2 cells from *Fasn*$^{i\Delta AEC2}$ and control mice exposed to room air or hyperoxia for 48 hrs. All data are raw values (similar results were obtained from two independent experiments). Data are expressed as mean ± SEM.

Figure 1s

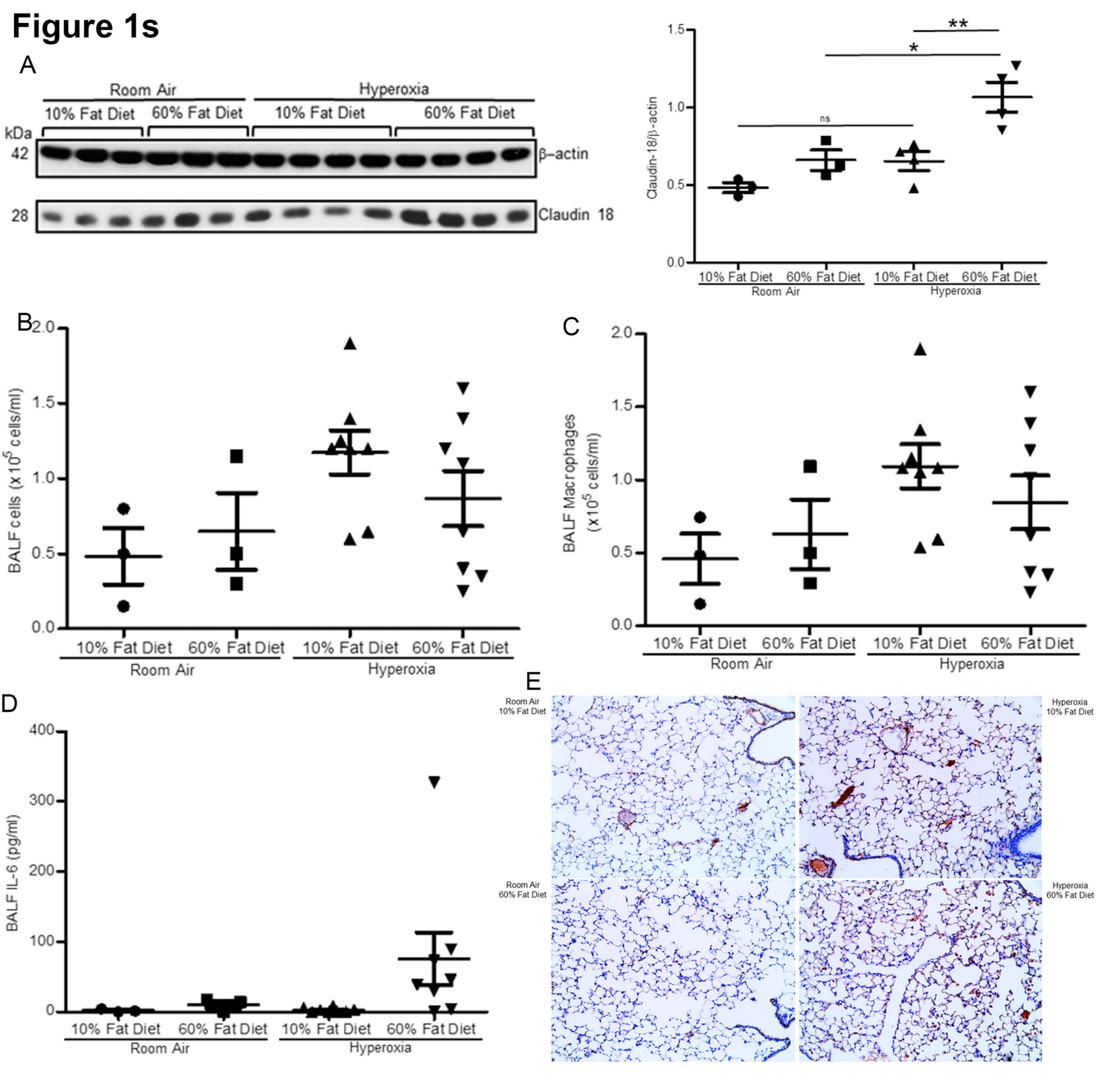
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Figure 2s

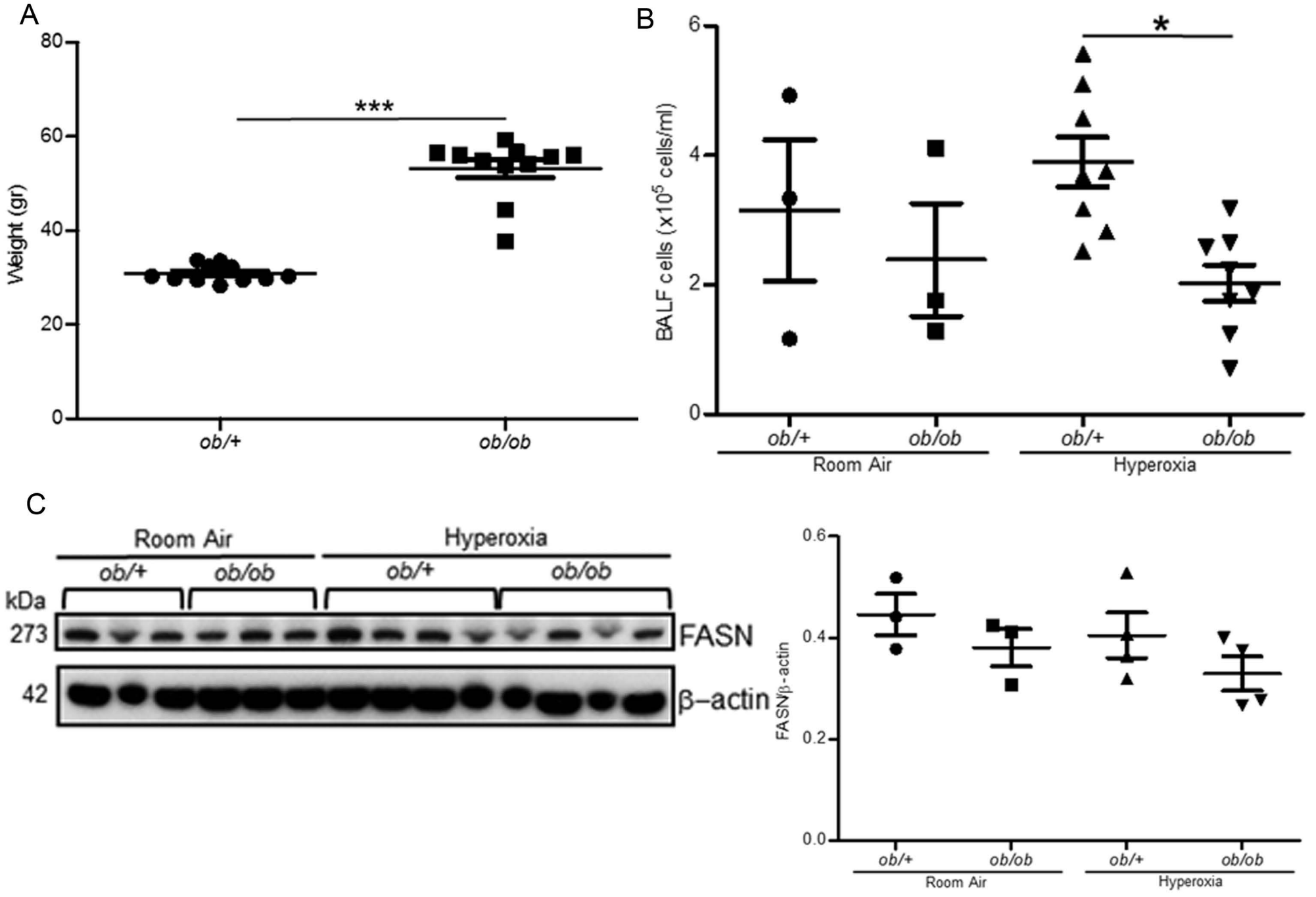
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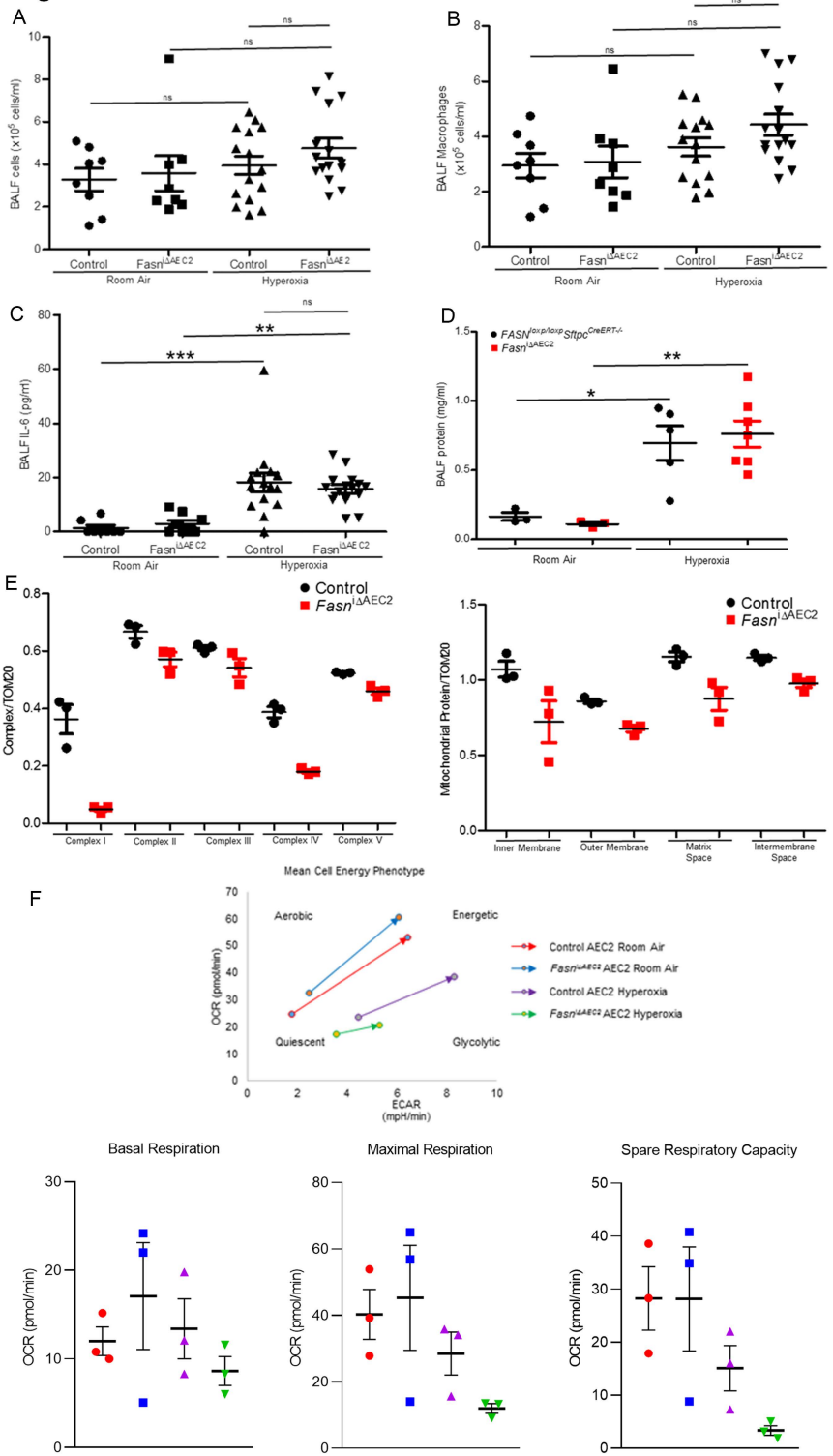
Figure 3s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