SUPPLEMENTAL METHODS

Plasma Metabolite Quantification. Serum triacylglycerol (TAG), free fatty acids (FFAs), total ketone bodies (TKB), β-hydroxybutryate (βOHB), and acetoacetate (AcAc) were quantified using standard biochemical assays coupled to colorimetric substrates. Blood glucose was measured in duplicate using handheld glucometers (Aviva). Serum insulin was measured using an enzyme-linked immunosorbent assay (Millipore) and serum alanine aminotransferase (ALT) was measured using an assay from Teco Diagnostics, according to manufacturer's instructions.

Tissue metabolite quantification. For hepatic NAD^+ and NADH quantification, 5-10 mg of tissue was homogenized using a glass on glass dounce in 400 μl NADH/NAD extraction buffer. Homogenates were spun at 4^{o}C and 15,000 x g for 5 min. Supernatants were then cleared of protein by centrifugation at 4^{o}C at 15,000 x g for 1 hour using 10 kDa spin columns (Pall). NAD^+ was denatured from half of the filtrate by heating it at 70 ^{o}C for 1 hour. Using a 96 well plate, total NAD^+/NADH concentrations and NADH concentrations alone were measured using an enzyme cycling colorimetric assay (Biovision).

Histology. For hematoxylin and eosin stained sections, paraffin-embedded tissue was microtome-sectioned, stained and photographed using standard methods. For F4/80 immunostains, rat anti-F4/80 (Abcam, ab6640) was incubated for 1 h at room temperature on liver cryosections (diluted 1:200 in 1% bovine serum albumin/0.1% Triton X-100 in phosphate buffered saline), followed by Alexa Fluor 594 conjugated goat anti-rat IgG (Invitrogen, Carlsbad, CA; diluted 1:250 in 1% bovine serum albumin/0.1% Triton X-100 in phosphate-buffered saline) and coverslip application as previously described (1). For smooth muscle actin (SMA) immunostains, mouse monoclonal anti-SMA-Cy3 conjugate (Sigma C-6198) was incubated for 1 h at room temperature on liver cryosections (diluted 1:100 in 1% bovine serum albumin/0.1% Triton X-100 in phosphate buffered saline), followed by coverslip application. 4',6-Diamidino-2-phenylindole (DAPI) was used as a cell nuclear counterstain (40 ng/ml in phosphate buffered saline for 5 min). All immunofluorescence images were acquired at 0.5 μm slice thickness using a Zeiss LSM 700 confocal microscope and Zeiss Zen software. For F4/80 and SMA immunostains, five

random 20X fields and nineteen random 10X fields per liver, respectively (n = 3 animals/group), were quantified manually. In each case, only staining that was clearly associated with nuclei were counted, and for SMA, only cells outside of vessel walls were counted as activated stellate cells.

Immunoblotting. Lysates from liver were generated in a protein lysis buffer: 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% phosphatase inhibitor cocktail (Sigma), and protease inhibitor cocktail (complete mini EDTA-free, Roche), pH 7.5. Band intensities were quantified densitometrically using Quantity One software (Bio-Rad).

NMR-based quantitative substrate fate mapping. Neutralized perchloric acid tissue extracts were prepared and profiled using ^{13}C-edited proton nuclear magnetic resonance (NMR) measured at 11.75 T (Varian/Agilent Direct Drive-1) via first increment gradient heteronuclear single-quantum correlation (gHSQC). Signals were collected from extracts dissolved in 275 μL of D_2O + 1 mM trimethylsilyl propionate (TSP), loaded into high precision, thin walled 5-mm tubes (Shigemi). Tissue concentrations and fractional enrichments of metabolites were determined from quantification of signals by integration from the $^{1}H\{^{13}C\}$ and ^{13}C-edited (gHSQC) collections of carbon 2 for taurine, carbon 4 for βOHB, carbon 1 for glucose, carbon 4 for glutamate, and $^{1}H\{^{13}C\}$ of carbon 3 for α-ketoglutarate and carbon 2/3 for succinate.

Tandem mass spectrometry (MS^2) analysis of blood acylcarnitines. Blood was spotted onto 1.3-cm spots on Whatman 903 filter paper. Quantification was achieved in all cases using stable isotope ^{2}H-labeled internal standards using an electrospray ionization source coupled to an API 3200-Qtrap tandem mass spectrometer (Applied Biosystems).

Mitochondrial isolation and respiration. Largest liver lobe was excised, weighed, and rapidly homogenized on ice in mitochondrial isolation medium (MIM; 300 mM sucrose, 0.2 mM EDTA, 10 mM Na HEPES, pH 7.4) using a Glas-Col dounce homogenizer, and centrifuged at 600 x g for 10 min at 4°C. The supernatant, containing the mitochondria, was then centrifuged at 8,000 x g for 15 min at 4°C. After discarding the supernatant, the resulting mitochondrial pellet was resuspended in 5-10 mL chilled MIM buffer containing BSA (MIM-BSA; 1 mg/mL) and spun again at 8,000 x g for 15 min at 4°C. The pellet

was quickly washed in chilled MIM and each sample was resuspended in 75 uL ice-cold MIM (pH 7.2) per 100 mg of liver tissue. Immediately following mitochondrial isolation, total protein was quantified by Bradford assay (Bio-Rad). Respiration was quantified at 37°C using a water-jacketed Clark Electrode (Hansatech Instruments). Briefly, 0.5 mg of mitochondria were added to 1 mL of respiration buffer [125 mM KCl, 20 mM HEPES, 3 mM Mg-acetate, 0.4 mM EGTA, 0.3 mM dithiothreitol (DTT), 5 mM KH_2PO_4, 0.2% BSA, pH 7.1] containing 10 mM pyruvate or 10 mM pyruvate and 5 mM malate. The solubility of oxygen in the respiration buffer at 37°C was 235 nmol O_2 per mL. Following measurement of basal (state 2) respiration, ADP was added to the respiration chamber at a concentration of 1 mM in respiration buffer, and maximal (state 3) respiration quantified. Thereafter, state 4 (F_1F_0 ATPase-independent) respiration was measured by adding 1 μg/mL oligomycin (Sigma) to inhibit ATP synthase. Mitochondrial uncoupling was measured following the addition of 5 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP).

SUPPLEMENTAL FIGURES

Figure S1. Metabolic parameters of ketogenesis insufficient neonatal mice, specificity of HMGCS2 ASO treatment in adult mice, and quantification of ketogenesis in perfused livers. (A) Body weight (g) and **(B)** blood glucose levels (mg/dL) in neonatal mice treated with ASO daily for 11 days, beginning on the second day of life (P1), n > 4/group. **(C)** *Hmgcs2* transcript abundance in liver (n = 8-10/group) and **(D)** in subcutaneous adipose tissue (n = 4/group) of adult mice treated with control or HMGCS2 ASO biweekly for four weeks beginning at six weeks of age. **(E)** Cytoplasmic 3-hydroxymethylglutaryl-CoA synthase (HMGCS1) protein content in livers of adult mice treated with ASOs for four weeks. **(F)** ^{13}C-βOHB enrichment in perfused livers of ASO treated mice, demonstrating diminished production of ketone bodies from [^{13}C]octanoic acid. Decreased ^{13}C-βOHB enrichment in fasted control livers compared to fed control livers is likely due to increased competition from endogenous fatty acids, which

are mobilized to the liver from adipose stores during the fast, preceding the perfusion. n = 4-8/group. **, $p < 0.01$, ***, $p < 0.001$ by Student's *t* test or 2-way ANOVA as appropriate, as indicated.

Figure S2. Metabolic parameters of ketogenesis insufficient adult mice fed a standard low-fat chow diet. (A) Body weight (g), **(B)** average food intake (kCal/mouse/day), and **(C)** body composition (g) in ASO treated mice fed a low-fat diet (standard chow diet) for four weeks n > 8/group. **(D)** Serum free fatty acids (FFAs, mM; n = 20/group), **(E)** triacylglycerol (TAG, mg/dL; n = 15-19) **(F)** acetoacetate (AcAc) and βOHB (mM, n = 7 group for each ketone body), **(G)** blood glucose (mg/dL, n = 28-36/group), and **(H)** serum insulin (ng/mL, n = 20-23/group) concentrations in standard chow diet-fed ASO treated mice. **, $p < 0.01$ by Student's *t* test as indicated.

Figure S3. Serum metabolites of ASO-treated mice fed a 60% HFD. (A) Immunoblot for HMGCS2 and actin using protein lysates derived from livers of adult mice treated with control or HMGCS2 ASO biweekly for two weeks beginning at six weeks of age, during which time mice were maintained on a standard chow diet (quantification on right) and **(B)** following an additional eight weeks of 60% high-fat diet (HFD). **(C)** Serum [TAG] (mg/dL), **(D)** [FFA] (mM), and **(E)** [TKB] (mM) in ASO-treated mice fed a HFD for eight weeks, n = 8-10/group. ***, $p < 0.001$ by Student's *t* test as indicated.

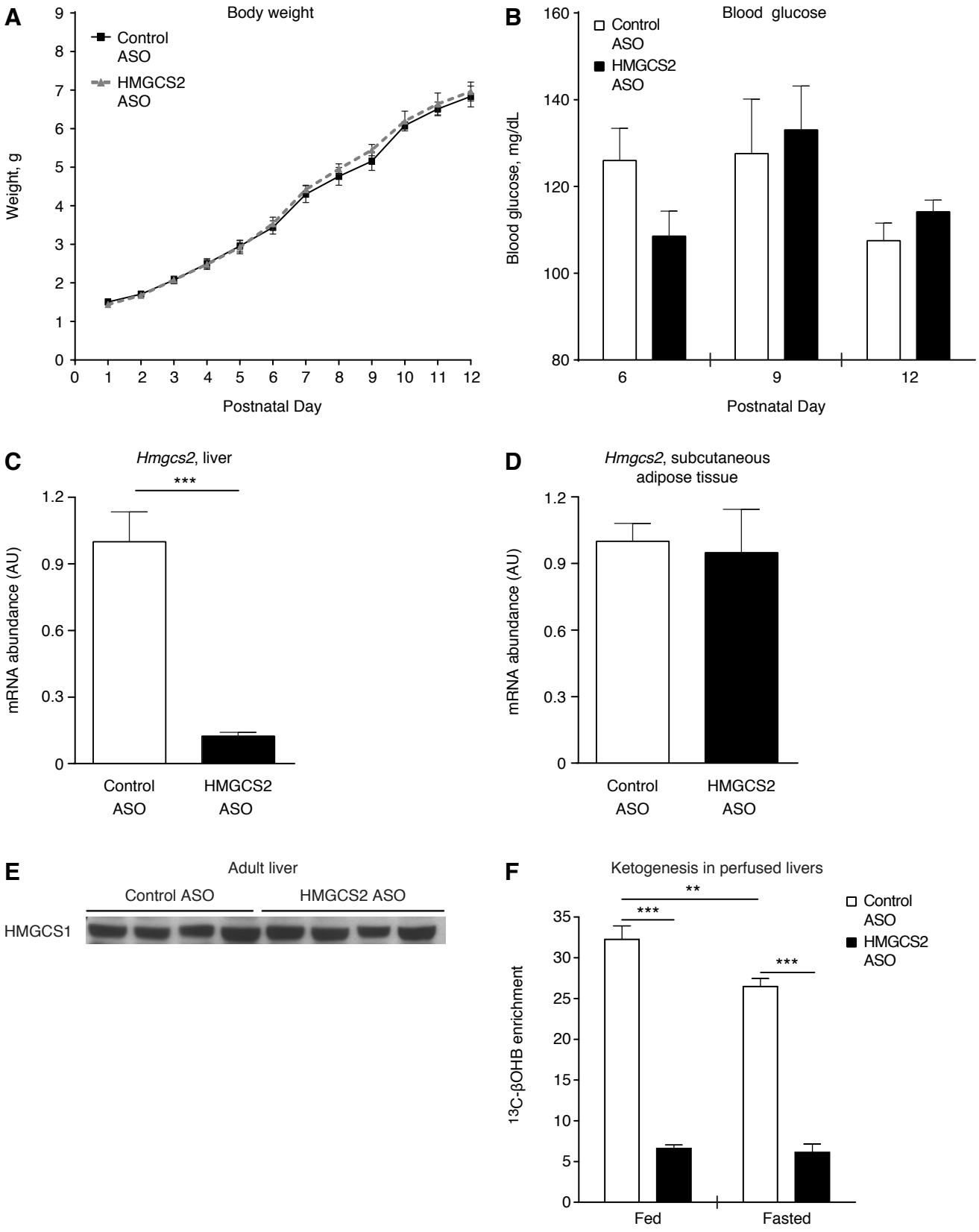
Figure S4. Body weight, energy intake, and body composition of ketogenesis insufficient mice fed a 40% fat diet. (A) Body weight (g) and **(B)** caloric intake (kCal/mouse/day) during 40% fat diet maintenance and ASO treatment, n = 8-10/group, ***, $p < 0.001$ by linear regression *t* test. **(C)** Body composition (g) pre- and post 40% fat diet feeding. n = 10/group, *, $p < 0.05$, **, $p < 0.01$ ***, $p < 0.001$ by 2-way ANOVA as indicated. **(D)** Blood glucose concentration (mg/dL) in mice fed a 40% fat diet for eight weeks. n =10-15, *, $p < 0.05$, by Student's *t* test.

Figure S5. Hepatic injury in ketogenesis insufficient mice fed a 40% HFD. (A) Hepatic [TAG] (mg/g tissue) and **(B)** serum alanine aminotransferase (ALT, U/L) activity in ASO-treated mice fed a 40% fat diet for eight weeks, n = 9/group for [TAG] and n = 4-5/group for ALT. **(C)** Representative hematoxylin and eosin stained sections of liver from ASO treated mice fed the indicated diet. Black arrowheads indicate microgranulomas comprised of lipid laden Kupffer cells and grey arrowheads indicate nests of inflammatory cells. *, $p < 0.05$, by Student's *t* test as indicated.

Figure S6. Metabolite concentrations in livers of HFD-fed ketogenesis insufficient mice. (A) [Glutamate] (nmol/mg tissue) and **(B)** [succinate] (nmol/mg tissue) in unperfused livers of 60% HFD-fed ASO-treated mice n = 4-5/group. **(C)** Free coenzyme A (CoASH, pmol/mg tissue) concentrations in unperfused livers of 60% HFD-fed ASO-treated mice, n = 8/group. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ by Student's *t* test or 2-way ANOVA as appropriate, as indicated.

Figure S7. Hepatic glucose pools in livers perfused with supplemental CoASH precursors. (A) Total hepatic glucose concentrations (pmol/mg tissue) in livers of standard chow diet-fed ASO-treated mice perfused with [^{13}C]lactate, [^{13}C]pyruvate, octanoic acid, cysteine, and pantothenic acid for 15 min, n = 6-7/group. **(B)** Total hepatic glucose concentrations (pmol/mg tissue) in livers of standard chow diet-fed ASO-treated mice perfused for 45 min with unlabeled lactate, pyruvate, cysteine, and pantothenic acid, followed by 15 min of perfusion with [^{13}C]lactate, [^{13}C]pyruvate, octanoic acid, cysteine, and pantothenic acid, n = 5-6/group.

Cotter et al., Fig. S1



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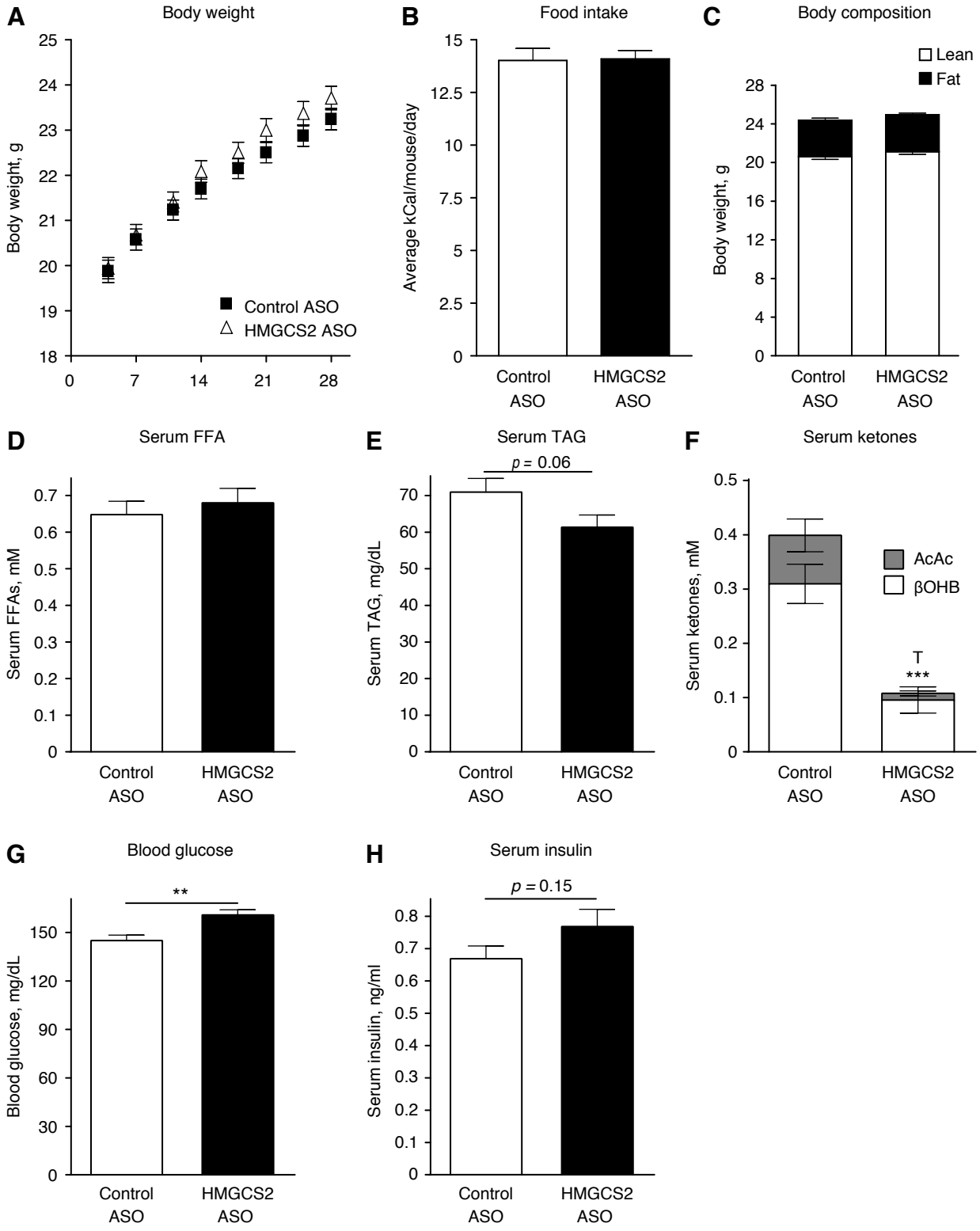

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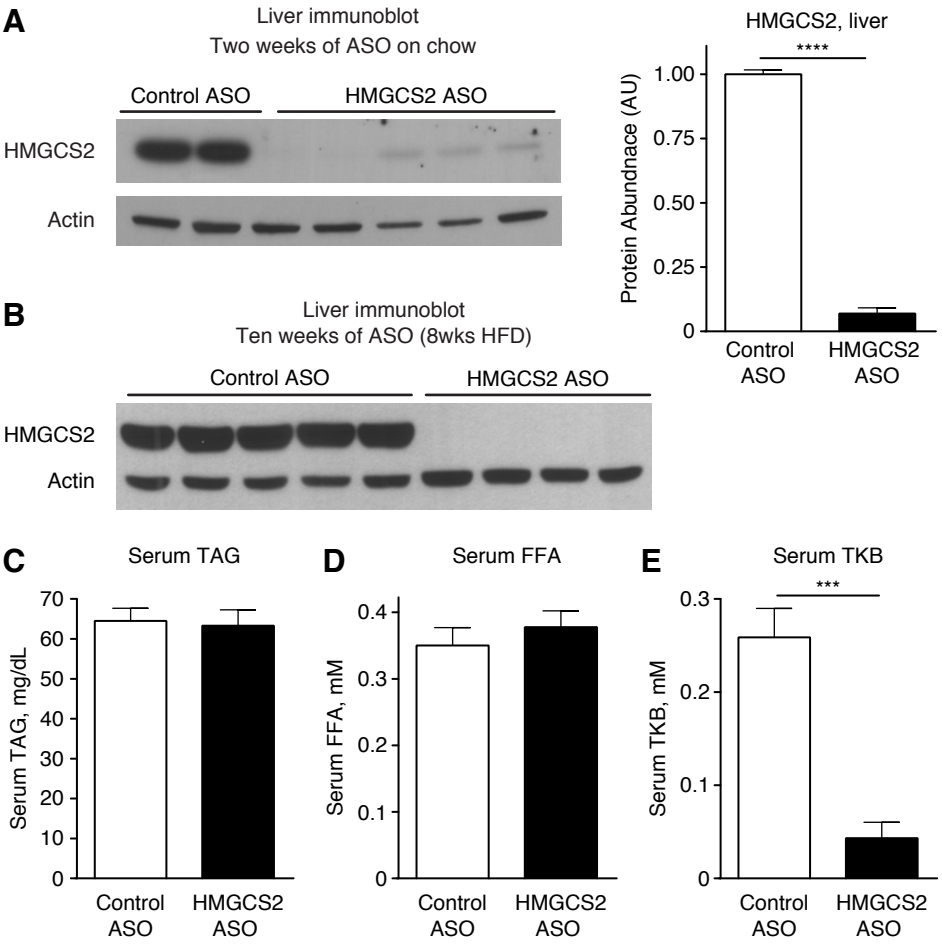

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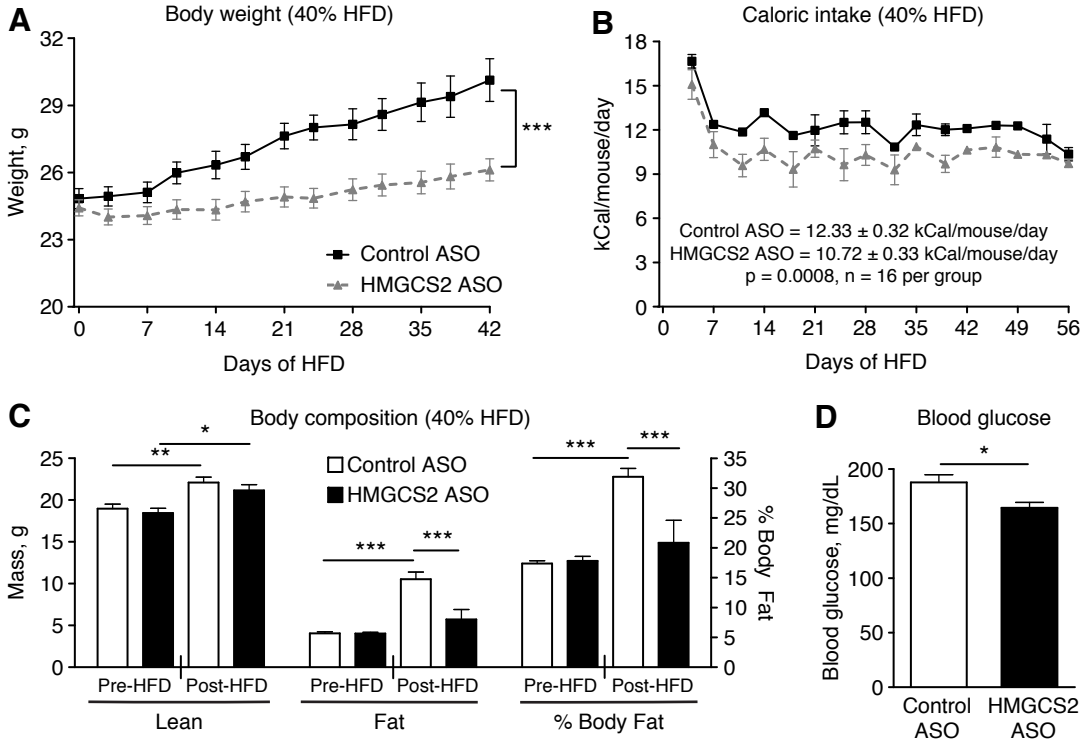

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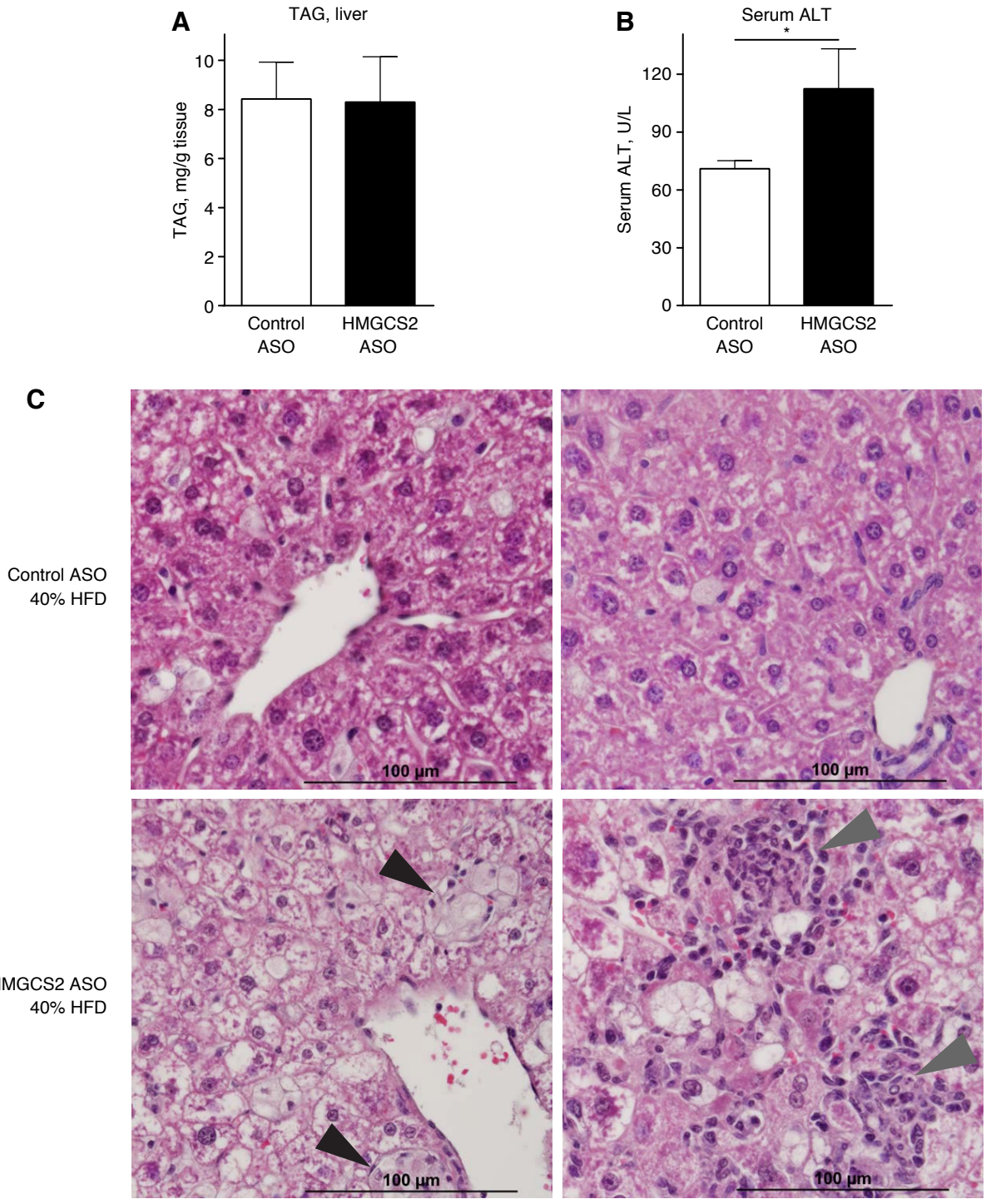

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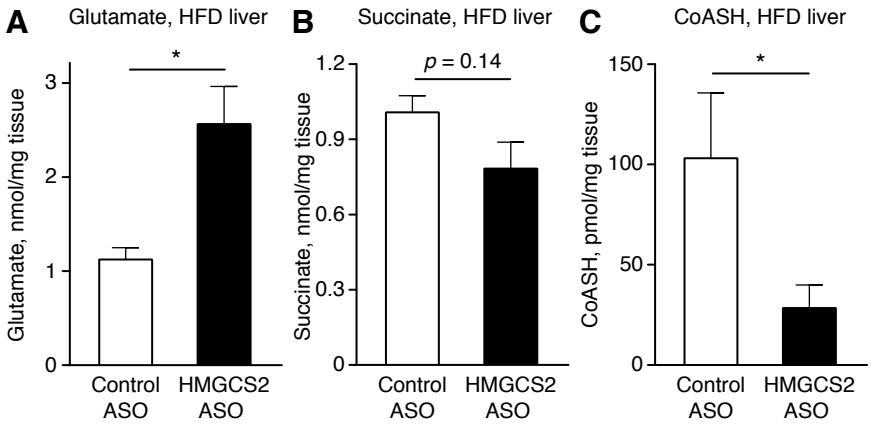

Cotter et al., Fig. S7