Metformin improves healthspan and lifespan in mice

Authors and affiliations

Alejandro Martin-Montalvo[1*], Evi M. Mercken[1*], Sarah J. Mitchell[1,2,3], Hector H. Palacios[1], Patricia L. Mote[4], Morten Scheibye-Knudsen[5], Ana P. Gomes[6], Theresa M. Ward[1], Robin K. Minor[1], Marie-José Blouin[7], Matthias Schwab[8], Michael Pollak[7], Yongqing Zhang[9], Yinbing Yu[10], Kevin G. Becker[9], Vilhelm A. Bohr[5], Donald K. Ingram[11], David A. Sinclair[6], Norman S. Wolf[12], Stephen R. Spindler[4], Michel Bernier[1] and Rafael de Cabo[1].

[1]Translational Gerontology Branch, National Institute on Aging, National Institutes of Health, 251 Bayview Boulevard, Baltimore, MD 21224, USA.

[2]Kolling Institute of Medical Research, Royal North Shore Hospital, St Leonards, NSW 2065, Australia.

[3]Sydney Medical School, University of Sydney, Sydney, NSW 2006, Australia.

[4]Department of Biochemistry, University of California Riverside, 3401 Watkins Drive, Riverside, CA 92521, USA.

[5]Laboratory of Molecular Gerontology, National Institute on Aging, National Institutes of Health, 251 Bayview Boulevard, Baltimore, MD 21224, USA.

[6]Department of Genetics, Paul F. Glenn Laboratories for the Biological Mechanisms of Aging, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA.

[7]Department of Medicine and Oncology, McGill University, Montreal, QC, Canada, H3A 2B3.

[8]Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart and University of Tübingen , Auerbachstrasse 112, Stuttgart 70376, Germany

[9]Gene Expression and Genomics Unit, National Institute on Aging, National Institutes of Health, 251 Bayview Boulevard, Baltimore, MD 21224, USA.

[10]Laboratory of Epidemiology, Demography and Biometry, National Institute on Aging, Bethesda, Maryland, 20892, U.S.A

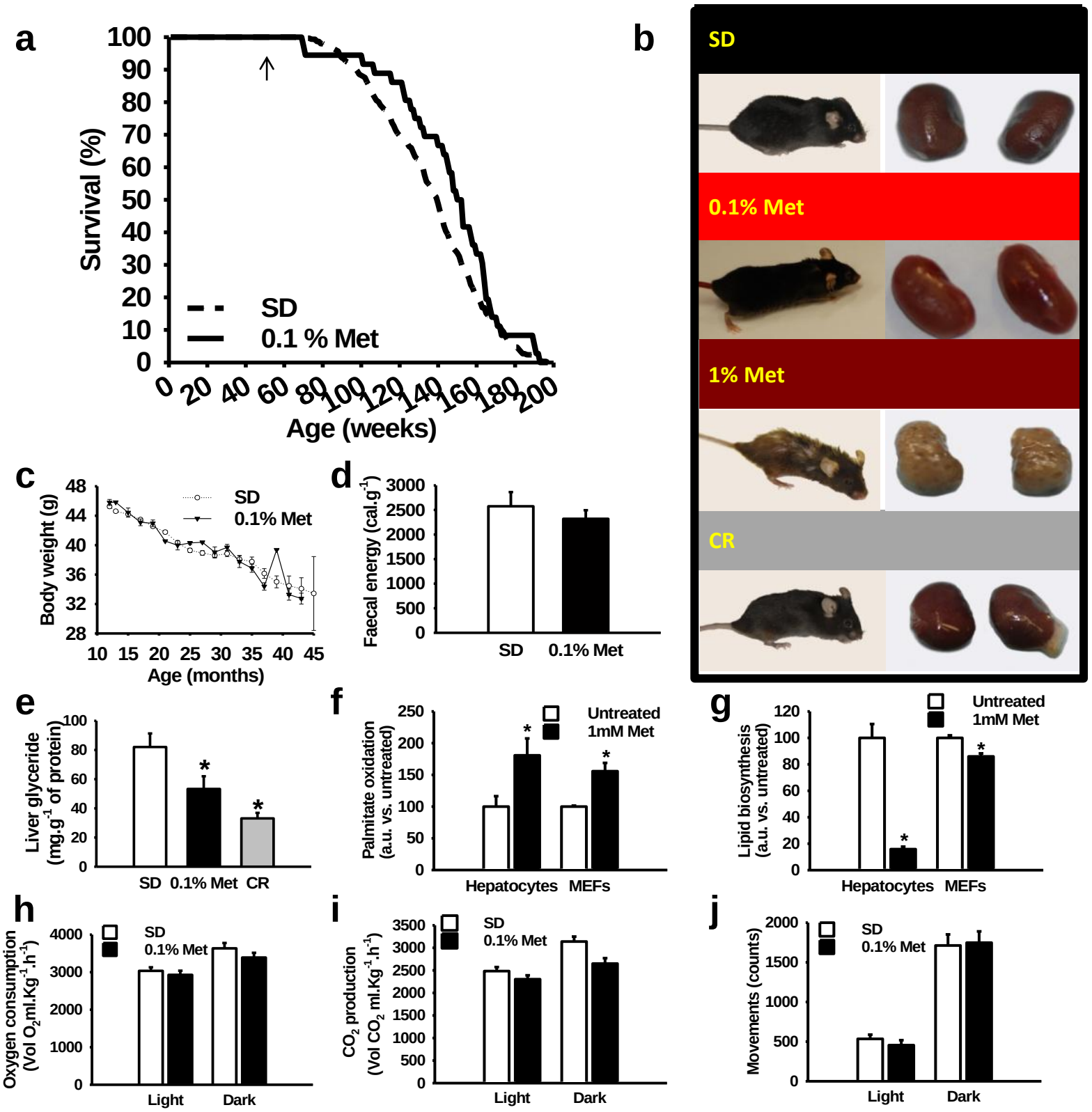
[11]Nutritional Neuroscience and Aging Laboratory, Pennington Biomedical Research Center, Louisiana State University System, 6400 Perkins Road, Baton Rouge, LA 70808, USA.

[12]Department of Pathology, University of Washington, Seattle, WA 98195-7470, USA

To whom correspondence should be sent: Rafael de Cabo, Phone: 410-558-8510; Fax: 410-558-8302; Email: decabora@grc.nia.nih.gov

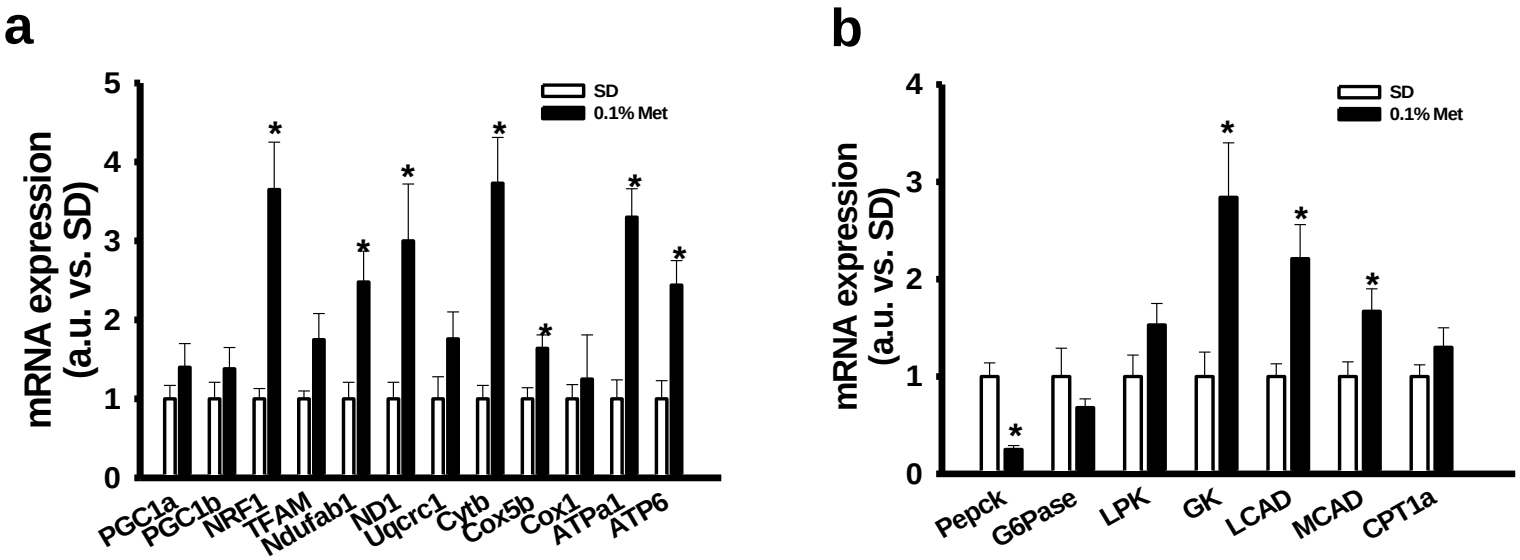
* These authors equally contributed to this work.

Supplementary Figure S1. Effects on longevity and metabolic and physical activity responses to metformin.



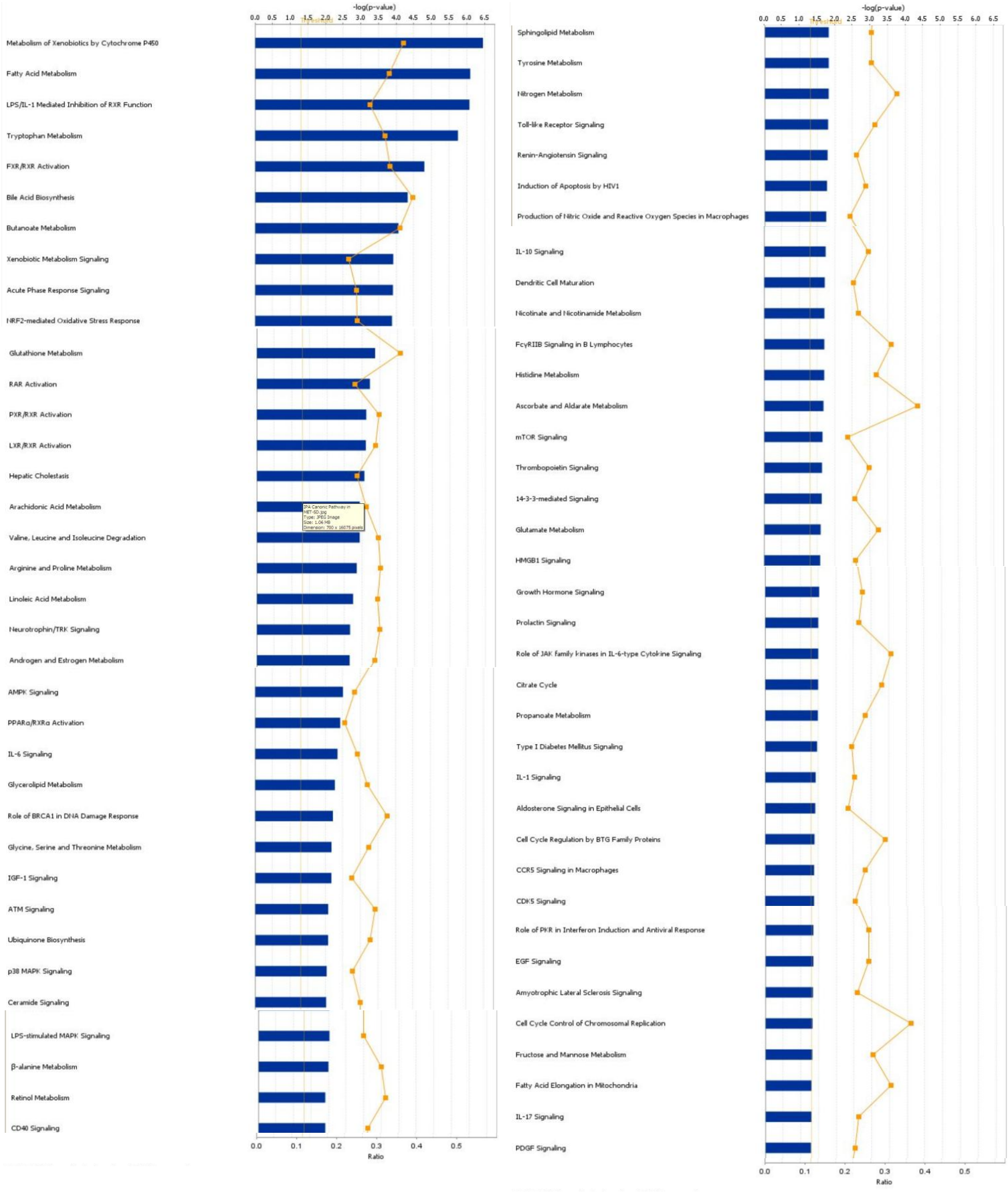
(**a**), Kaplan–Meier survival curve for B6C3F1 mice treated either with 0.1% metformin. n= 36 for metformin 0.1% group and n= 297 for their untreated counterparts. The arrows at 54 weeks indicate the age at which metformin treatment was initiated. (**b**), Representative images depicting animals and kidney appearance. (**c**), Body weights of B6C3F1 mice. n = all live animals at each point. (**d**), energy content in faeces. n = 3-5 cages per group. (**e**), Liver glyceride levels in liver. n = 6-8 per group. (**f**) Palmitate oxidation in rat primary hepatocytes and MEFs. n = 3-10 per group. (**g**) Lipid biosynthesis in primary hepatocytes and MEFs. n = 3-10 per group. (**h**), Oxygen consumption in metabolic cages, (**i**), CO_2 production in metabolic cages, or (**j**), X-axis movement, an indicator of spontaneous locomotor activity, in metabolic cages. n = 9 per group. Data are represented as the mean ± SEM. *P < 0.05 versus untreated cells or standard diet (SD)-fed mice (t-test two tailed).

Supplementary Figure S2. Expression of genes related to energy metabolism and mitochondrial function.



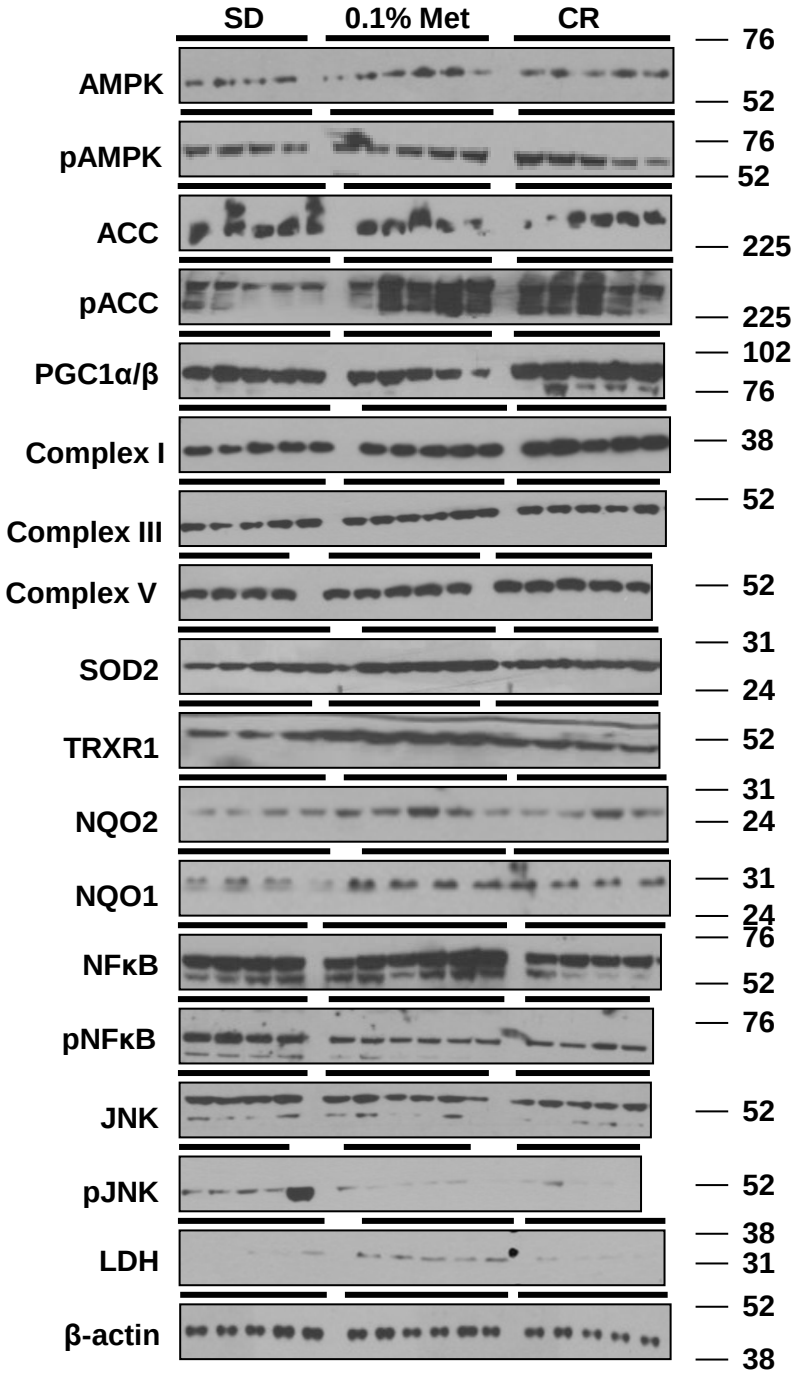
(**a)**, RT-PCR determining the expression of some transcript levels of the electron transport chain subunits in 0.1% metformin mice. (**b)**, RT-PCR determining the expression of gluconeogenesis-related and glycolysis-related and fatty acid metabolism-related genes in 0.1% metformin mice. n = 5 per group. Data are represented as the mean ± SEM. * $p < 0.05$ versus standard diet (SD)-fed mice (*t*-test two tailed).

Supplementary Figure S3. Ingenuity pathway analysis in response to metformin treatment.



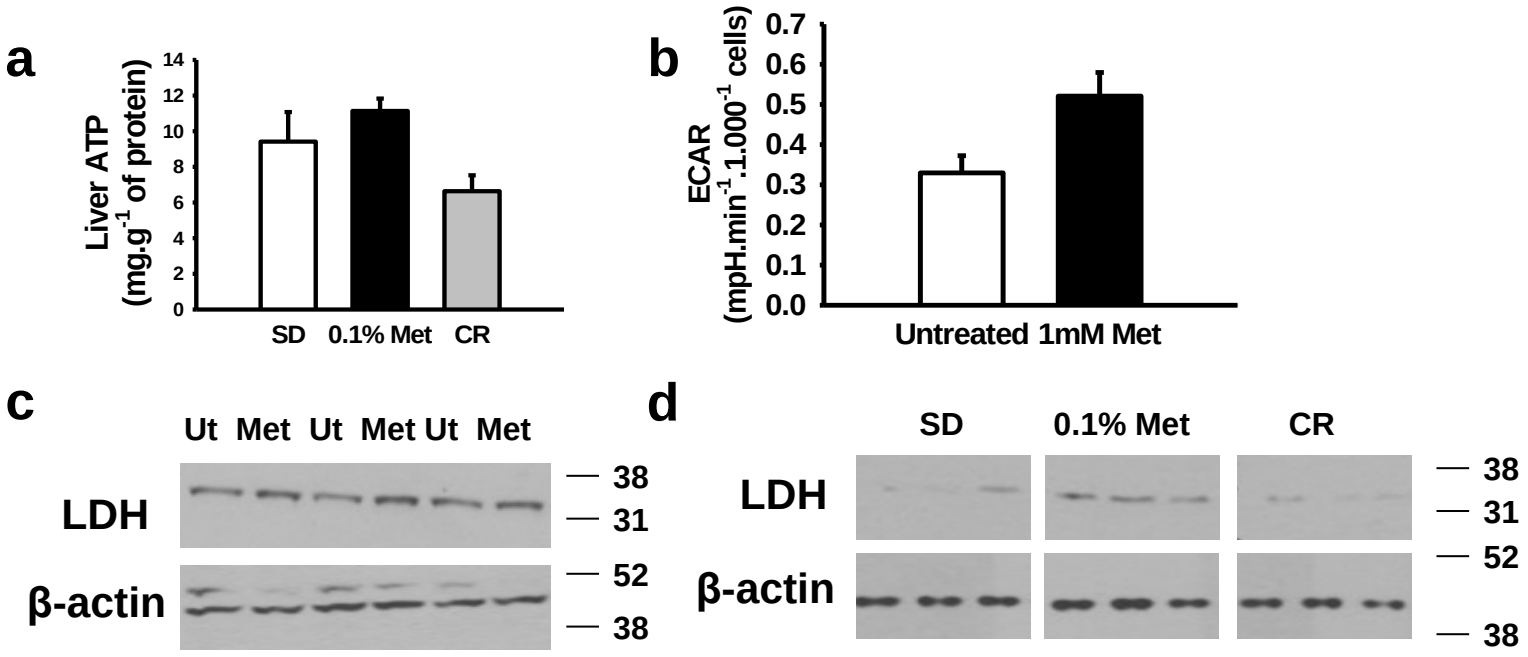
Significant canonical pathways based on ingenuity pathways analysis in the livers of 0.1% metformin-treated mice. Blue bars indicate the –log(p value); Yellow line indicate the proportion of genes that are significantly modified in each specific pathway (ratio) calculated by Fisher's exact test right-tailed.

Supplementary Figure S4. Entire images of the western blots.



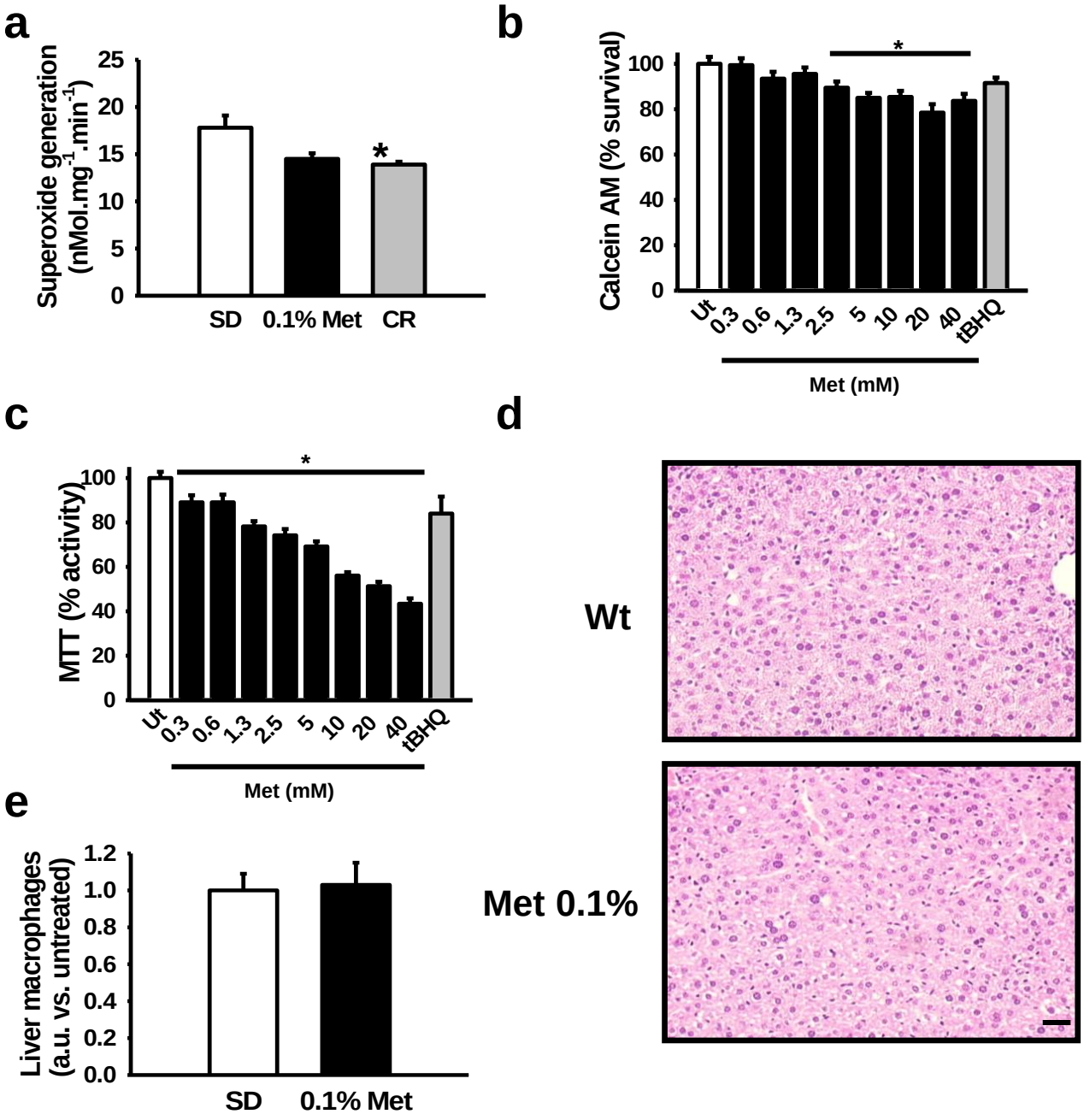
The whole images from the western blots that were included in the manuscript are shown.

Supplementary Figure S5. Induction of glycolysis by metformin.



(**a)**, ATP liver content. Liver. n = 4-5 per group. (**b**), Extracellular acidification rate (ECAR) in MEFs treated with metformin. n = 3 per group. (**c)**, Western blots showing levels of lactate dehydrogenase in metformin-treated MEFs. n = 3 per group. (**d)**, Western blots showing levels of lactate dehydrogenase in the livers of 0.1% metformin-fed mice. n = 4-6 per group. Ut; untreated. Data are represented as the mean ± SEM. * $p < 0.05$ versus untreated cells or standard diet (SD)-fed mice (*t*-test two tailed).

Supplementary Figure S6. Reduction in oxidative damage by metformin.



(a), Superoxide production in mitochondrial complexes I+II to III in the livers of 0.1% metformin-treated mice. Data is expressed as nmoles.mg prot^{-1}.min^{-1}. n = 5-6 per group. (**b)**, Cell survival was determined by calcein AM. n = 3 per group. (**c)**, Total reductase activity by using MTT assay. n = 3 per group. (**d**), Liver haematoxylin and eosin staining. The bar indicates 50 μm (**e**) Macrophage infiltration determined as the number of liver-infiltrated macrophages is shown relative to untreated livers. For quantifications, at least five microscope fields at x 20 magnifications were scored. n = 5 per group. Data are represented as the mean ± SEM. Ut; untreated. * $p < 0.05$ versus untreated cells or standard diet (SD)-fed mice (*t*-test two tailed).

Supplementary Tables

Supplementary Table S1. List of major pathologies identified at necropsy in C57BL/6 mice.

Organ	Pathology	Diet Treatment (n)			
		SD (83)	1% Met (90)	0.1%Met (63)	CR (74)
Heart	Enlarged	9	0	10	1
	Ischemic foci	7	1	3	0
Kidney	Enlarged	3	68	6	0
	Lumpy	1	71	0	0
	Discolored	6	59	2	0
Liver	Cancer	22	3	18	7
Lung	Cancer	3	1	2	0

The number of mice per group is presented between brackets. Metformin: Met, Calorie restriction: CR.

Supplementary Table S2. List of major pathologies at 115 weeks of age as determined by blinded histopathological analysis in C57BL/6 mice. Metformin: Met, Calorie restriction: CR.

Tissue	Pathology	Diet Treatment (n)		
		SD (4-5)	0.1% Met (5)	CR (4-5)
Heart	Lymphocyte infiltration	0%	0%	75%
	Pericarditis	50%	40%	0%
	Fibrosis (endocardial or interstitial)	25%	20%	25%
Lung	Lymphocyte infiltration	75%	60%	75%
	Lymphoma/sarcoma/adenoma	0%	40%	0%
Liver	Lymphocyte infiltration	80%	60%	20%
	Lymphoma/sarcoma/adenoma	0%	40%	0%
Kidney	Lymphocyte infiltration	100%	100%	0%
	Glomerulonephritis	60%	100%	40%

Major pathologies identified in 4% PFA fixed tissues from mice aged 115 weeks (61 weeks on the diet). Data is presented as the percentage of mice that developed a specific pathology. (n = 4-5 per group).

Supplementary Table S3. List of major pathologies identified at necropsy in B6C3F1 mice.

Organ	Pathology	Diet Treatment (n)	
		SD (n)	0.1% Met (n)
Spleen	Enlarged/tumorous	18	18
Liver	Tumor	11	8
	Enlarged/tumorous	3	3
	Hemangioma	3	4
Intestinal	Tumor	3	6
Lung	Tumor	8	10
Penis	Necrosed/inflamed	5	1
Bladder	Distended	5	3
Body cavity	Hemorrhage	7	11

n = 36 per group**.** Metformin: Met**.**

Supplementary Table S4. Body composition.

Diet (age and duration, weeks)	Fat Mass	Lean Mass	% fat	% lean	lean:fat ratio
SD (70, 16)	10.11±0.27	24.12±0.31	23.82±0.41	57.83±0.18	2.53±0.07
0.1% Met (70, 16)	9.96±0.25	23.99±0.34	23.79±0.31	57.94±0.23	2.46±0.03
SD (92, 38)	9.21±0.31	23.21±0.38	22.37±0.46	57.83±0.24	2.71±0.08
0.1% Met (92, 38)	8.73±0.32	22.01±0.38*	22.12± 0.54	56.84± 0.49	2.69±0.07
SD (114, 50)	7.53±0.38	23.08±0.45	18.94±0.69	60.60±0.51	3.64±0.20
0.1% Met (114, 50)	7.61±0.40	22.70±0.50	19.32±0.61	59.86±0.27	3.40±0.20

The NMR method was used in body composition assessment several times during the mice lifetime. All live animals were included at the time of the assay. Metformin: Met. n = all live animals at each time point. Data are represented as the mean ± SEM. * $p < 0.05$ standard diet (SD) (*t*-test two tailed).

Supplementary Table S5. List of the most highly up- and down-regulated genes in livers and muscles of 0.1% metformin- and CR-fed mice.

Symbol	Liver (zratio) 0.1% Met -SD	Symbol	Liver (zratio) CR -SD	Symbol	Muscle (zratio) 0.1% Met -SD	Symbol	Muscle (zratio) CR -SD
Fos	12.82	*Acot3*	14.5	*Cfd*	12.21	*G0s2*	13.61
Egr1	11.66	*D0H4S114*	11.5	*Adipoq*	10.6	***Cish***	**11.58**
Cish	**11.66**	*Fos*	10.51	***Cish***	**10.48**	*Egr1*	9.34
Chrna4	10.39	*Per2*	9.84	*Tmem45b*	10.15	*Ier3*	8.42
Hhex	8.33	*EG624219*	9.7	*Lep*	10.07	*Per2*	7.5
EG624219	7.47	***Cish***	**9.44**	*Scd1*	9.59	*LOC100043257*	6.41
Aacs	7.2	*Alas1*	9.36	*Cdo1*	8.74	*Clec2d*	6.18
Socs2	6.96	*Cyp2a5*	9.14	*Cidec*	8.61	*Zfp36l1*	5.91
Hba-a1	6.74	*Tnnc1*	8.44	*Ifi27*	8.4	*Mid1ip1*	5.9
Egr2	6.7	*Cyp7a1*	8.41	*Trf*	8.28	*Inmt*	5.85
Etnk2	-6.67	*Cdkn1a*	-8.75	*Angptl7*	-8.16	*8430408G22Rik*	-8.42
Usp2	-6.76	***Saa2***	**-9.1**	*Irs2*	-8.2	*Cdkn1a*	-8.58
Lpin1	-7.02	*Spon2*	-9.53	*Srxn1*	-8.24	*Nr1d1*	-8.79
Pim3	-7.17	*Elovl3*	-9.58	*Arrdc2*	-8.3	*Fbxo32*	-9.17
Angptl4	-7.8	*Chac1*	-9.67	*Pde4d*	-8.31	*Irs2*	-9.27
Upp2	-9.36	*Rnf125*	-10.51	*8430408G22Rik*	-8.76	*Mt1*	-9.3
Saa2	**-11.2**	***Saa1***	**-10.57**	*Ankrd1*	-9.04	*Mef2c*	-10.21

Saa1	**-13.27**	*Insig2*	-12.43	*Tob2*	-9.43	*Tob2*	-10.78
Dbp	-17.21	*Cfd*	-12.76	*Mt1*	-9.94	*LOC100047427*	-12.02
Lcn2	-17.64	*Lcn2*	-17.14	*Ddit4*	-12.94	*Ddit4*	-13.56

Genes that are shared between CR and 0.1% metformin are marked in bold. Metformin: Met. All genes were significantly regulated with $p < 0.05$ one way-ANOVA

Supplementary Table S6. List of significant GO terms in liver of metformin-treated mice.

Gene Ontology Term	Zscore 0.1%Met-SD	Gene Ontology Term	Zscore 0.1%Met-SD
GO0006937 REGULATION OF MUSCLE CONTRACTION	15.11	GO0005550 PHEROMONE BINDING	1.82
GO0005861 TROPONIN COMPLEX	14.79	GO0015057 THROMBIN RECEPTOR ACTIVITY	1.74
GO0005200 STRUCTURAL CONSTITUENT OF CYTOSKELETON	10.31	GO0001708 CELL FATE SPECIFICATION	1.73
GO0016459 MYOSIN COMPLEX	10.15	GO0045261 PROTON TRANSPORTING ATP SYNTHASE COMPLEX	1.73
GO0016126 STEROL BIOSYNTHETIC PROCESS	9.33	GO0006952 DEFENSE RESPONSE	1.72
GO0006695 CHOLESTEROL BIOSYNTHETIC PROCESS	9.27	GO0060113 INNER EAR RECEPTOR CELL DIFFERENTIATION	1.69
GO0005859 MUSCLE MYOSIN COMPLEX	8.39	GO0007218 NEUROPEPTIDE SIGNALING PATHWAY	1.64
GO0008307 STRUCTURAL CONSTITUENT OF MUSCLE	8.37	GO0050766 POSITIVE REGULATION OF PHAGOCYTOSIS	1.61
GO0005863 STRIATED MUSCLE THICK FILAMENT	8.31	GO0045019 NEGATIVE REGULATION OF NITRIC OXIDE BIOS	1.56
GO0007010 CYTOSKELETON ORGANIZATION AND BIOGENESIS	8.11	GO0004888 TRANSMEMBRANE RECEPTOR ACTIVITY	1.55
GO0055003 CARDIAC MYOFIBRIL ASSEMBLY	8.07	GO0048489 SYNAPTIC VESICLE TRANSPORT	1.55
GO0006936 MUSCLE CONTRACTION	7.96	GO0042359 VITAMIN D METABOLIC PROCESS	1.54
GO0007517 MUSCLE DEVELOPMENT	7.88	GO0006836 NEUROTRANSMITTER TRANSPORT	1.53
GO0006694 STEROID BIOSYNTHETIC PROCESS	7.72	GO0007202 PHOSPHOLIPASE C ACTIVATION	1.52
GO0006941 STRIATED MUSCLE CONTRACTION	7.68	GO0004022 ALCOHOL DEHYDROGENASE ACTIVITY	1.51
GO0003774 MOTOR ACTIVITY	7.34	GO0044265 CELLULAR MACROMOLECULE CATABOLIC PROCESS	-1.50
GO0030018 Z DISC	7.33	GO0004046 AMINOACYLASE ACTIVITY	-1.51
GO0008610 LIPID BIOSYNTHETIC PROCESS	6.86	GO0004301 EPOXIDE HYDROLASE ACTIVITY	-1.55
GO0016529 SARCOPLASMIC RETICULUM	6.48	GO0045046 PROTEIN IMPORT INTO PEROXISOME MEMBRANE	-1.55
GO0005830 CYTOSOLIC RIBOSOME (SENSU EUKARYOTA)	5.89	GO0007175 NEGATIVE REGULATION OF EPIDERMAL GROWTH	-1.56
GO0005856 CYTOSKELETON	5.70	GO0006829 ZINC ION TRANSPORT	-1.57
GO0005520 INSULIN LIKE GROWTH FACTOR BINDING	5.62	GO0004571 MANNOSYL OLIGOSACCHARIDE 1 2 ALPHA MANNO	-1.57
GO0006096 GLYCOLYSIS	5.42	GO0004722 PROTEIN SERINE OR THREONINE PHOSPHATASE	-1.60
GO0009605 RESPONSE TO EXTERNAL STIMULUS	5.30	GO0005802 TRANS GOLGI NETWORK	-1.61
GO0005509 CALCIUM ION BINDING	5.22	GO0016944 RNA POLYMERASE II TRANSCRIPTION ELONGATI	-1.63
GO0003735 STRUCTURAL CONSTITUENT OF RIBOSOME	4.74	GO0007628 ADULT WALKING BEHAVIOR	-1.65
GO0006085 ACETYL COA BIOSYNTHETIC PROCESS	4.70	GO0006996 ORGANELLE ORGANIZATION AND BIOGENESIS	-1.65
GO0005840 RIBOSOME	4.63	GO0006268 DNA UNWINDING DURING REPLICATION	-1.66
GO0001558 REGULATION OF CELL GROWTH	4.59	GO0002467 GERMINAL CENTER FORMATION	-1.68
GO0043433 NEGATIVE REGULATION OF TRANSCRIPTION FAC	4.49	GO0005779 INTEGRAL TO PEROXISOMAL MEMBRANE	-1.69
GO0008201 HEPARIN BINDING	4.35	GO0007266 RHO PROTEIN SIGNAL TRANSDUCTION	-1.70
GO0005178 INTEGRIN BINDING	4.22	GO0008601 PROTEIN PHOSPHATASE TYPE 2A REGULATOR AC	-1.72

GO0042254 RIBOSOME BIOGENESIS AND ASSEMBLY	4.21	GO0004672 PROTEIN KINASE ACTIVITY	-1.74
GO0005576 EXTRACELLULAR REGION	4.19	GO0004386 HELICASE ACTIVITY	-1.74
GO0030239 MYOFIBRIL ASSEMBLY	4.17	GO0016558 PROTEIN IMPORT INTO PEROXISOME MATRIX	-1.78
GO0006000 FRUCTOSE METABOLIC PROCESS	4.14	GO0017110 NUCLEOSIDE DIPHOSPHATASE ACTIVITY	-1.79
GO0042632 CHOLESTEROL HOMEOSTASIS	4.13	GO0005768 ENDOSOME	-1.80
GO0004871 SIGNAL TRANSDUCER ACTIVITY	4.07	GO0008135 TRANSLATION FACTOR ACTIVITY NUCLEIC ACI	-1.82
GO0004930 G PROTEIN COUPLED RECEPTOR ACTIVITY	4.04	GO0004003 ATP DEPENDENT DNA HELICASE ACTIVITY	-1.85
GO0003779 ACTIN BINDING	4.01	GO0004693 CYCLIN DEPENDENT PROTEIN KINASE ACTIVITY	-1.86
GO0006641 TRIACYLGLYCEROL METABOLIC PROCESS	3.94	GO0015914 PHOSPHOLIPID TRANSPORT	-1.87
GO0015629 ACTIN CYTOSKELETON	3.93	GO0005793 ER GOLGI INTERMEDIATE COMPARTMENT	-1.87
GO0004872 RECEPTOR ACTIVITY	3.91	GO0005385 ZINC ION TRANSMEMBRANE TRANSPORTER ACTIV	-1.88
GO0007186 G PROTEIN COUPLED RECEPTOR PROTEIN SIGNA	3.87	GO0006511 UBIQUITIN DEPENDENT PROTEIN CATABOLIC PR	-1.88
GO0019838 GROWTH FACTOR BINDING	3.86	GO0035035 HISTONE ACETYLTRANSFERASE BINDING	-1.89
GO0007507 HEART DEVELOPMENT	3.80	GO0003960 NADPH QUINONE REDUCTASE ACTIVITY	-1.90
GO0005615 EXTRACELLULAR SPACE	3.79	GO0000320 RE ENTRY INTO MITOTIC CELL CYCLE	-1.92
GO0005843 CYTOSOLIC SMALL RIBOSOMAL SUBUNIT (SENSU	3.71	GO0006556 S ADENOSYLMETHIONINE BIOSYNTHETIC PROCES	-1.94
GO0005667 TRANSCRIPTION FACTOR COMPLEX	3.63	GO0016568 CHROMATIN MODIFICATION	-1.95
GO0030529 RIBONUCLEOPROTEIN COMPLEX	3.58	GO0008234 CYSTEINE TYPE PEPTIDASE ACTIVITY	-1.95
GO0008289 LIPID BINDING	3.53	GO0004674 PROTEIN SERINE OR THREONINE KINASE ACTIV	-1.95
GO0007165 SIGNAL TRANSDUCTION	3.52	GO0007625 GROOMING BEHAVIOR	-1.96
GO0019452 L CYSTEINE CATABOLIC PROCESS TO TAURINE	3.48	GO0004812 AMINOACYL TRNA LIGASE ACTIVITY	-2.04
GO0019530 TAURINE METABOLIC PROCESS	3.48	GO0031529 RUFFLE ORGANIZATION AND BIOGENESIS	-2.05
GO0008035 HIGH DENSITY LIPOPROTEIN BINDING	3.47	GO0004221 UBIQUITIN THIOLESTERASE ACTIVITY	-2.07
GO0004867 SERINE TYPE ENDOPEPTIDASE INHIBITOR ACTI	3.46	GO0016019 PEPTIDOGLYCAN RECEPTOR ACTIVITY	-2.07
GO0004866 ENDOPEPTIDASE INHIBITOR ACTIVITY	3.44	GO0016559 PEROXISOME FISSION	-2.08
GO0006629 LIPID METABOLIC PROCESS	3.40	GO0004558 ALPHA GLUCOSIDASE ACTIVITY	-2.08
GO0004984 OLFACTORY RECEPTOR ACTIVITY	3.39	GO0000151 UBIQUITIN LIGASE COMPLEX	-2.10
GO0007608 SENSORY PERCEPTION OF SMELL	3.31	GO0001841 NEURAL TUBE FORMATION	-2.11
GO0005179 HORMONE ACTIVITY	3.26	GO0051301 CELL DIVISION	-2.12
GO0006569 TRYPTOPHAN CATABOLIC PROCESS	3.15	GO0005921 GAP JUNCTION	-2.14
GO0016829 LYASE ACTIVITY	3.04	GO0030145 MANGANESE ION BINDING	-2.18
GO0006954 INFLAMMATORY RESPONSE	2.99	GO0003924 GTPASE ACTIVITY	-2.21
GO0007275 MULTICELLULAR ORGANISMAL DEVELOPMENT	2.98	GO0007049 CELL CYCLE	-2.22
GO0005198 STRUCTURAL MOLECULE ACTIVITY	2.97	GO0046853 INOSITOL AND DERIVATIVE PHOSPHORYLATION	-2.23
GO0030216 KERATINOCYTE DIFFERENTIATION	2.95	GO0004842 UBIQUITIN PROTEIN LIGASE ACTIVITY	-2.26
GO0019843 RRNA BINDING	2.94	GO0004021 ALANINE TRANSAMINASE	-2.29

		ACTIVITY	
GO0005529 SUGAR BINDING	2.93	GO0006418 TRNA AMINOACYLATION FOR PROTEIN TRANSLAT	-2.30
GO0016491 OXIDOREDUCTASE ACTIVITY	2.85	GO0048286 ALVEOLUS DEVELOPMENT	-2.31
GO0006006 GLUCOSE METABOLIC PROCESS	2.73	GO0016192 VESICLE MEDIATED TRANSPORT	-2.31
GO0019886 ANTIGEN PROCESSING AND PRESENTATION OF E	2.72	GO0016798 HYDROLASE ACTIVITY ACTING ON GLYCOSYL B	-2.34
GO0006097 GLYOXYLATE CYCLE	2.70	GO0006888 ER TO GOLGI VESICLE MEDIATED TRANSPORT	-2.37
GO0004450 ISOCITRATE DEHYDROGENASE (NADP+) ACTIVIT	2.60	GO0019787 SMALL CONJUGATING PROTEIN LIGASE ACTIVIT	-2.41
GO0007166 CELL SURFACE RECEPTOR LINKED SIGNAL TRAN	2.60	GO0006424 GLUTAMYL TRNA AMINOACYLATION	-2.41
GO0008137 NADH DEHYDROGENASE (UBIQUINONE) ACTIVITY	2.60	GO0016757 TRANSFERASE ACTIVITY TRANSFERRING GLYCO	-2.42
GO0007155 CELL ADHESION	2.59	GO0030534 ADULT BEHAVIOR	-2.45
GO0019841 RETINOL BINDING	2.57	GO0017176 PHOSPHATIDYLINOSITOL N ACETYLGLUCOSAMINY	-2.47
GO0042593 GLUCOSE HOMEOSTASIS	2.55	GO0006886 INTRACELLULAR PROTEIN TRANSPORT	-2.56
GO0042802 IDENTICAL PROTEIN BINDING	2.50	GO0008535 CYTOCHROME C OXIDASE COMPLEX ASSEMBLY	-2.56
GO0007160 CELL MATRIX ADHESION	2.47	GO0004879 LIGAND DEPENDENT NUCLEAR RECEPTOR ACTIVI	-2.56
GO0006874 CELLULAR CALCIUM ION HOMEOSTASIS	2.42	GO0003756 PROTEIN DISULFIDE ISOMERASE ACTIVITY	-2.59
GO0006412 TRANSLATION	2.42	GO0006915 APOPTOSIS	-2.62
GO0004601 PEROXIDASE ACTIVITY	2.35	GO0008150 BIOLOGICAL PROCESS	-2.63
GO0004942 ANAPHYLATOXIN RECEPTOR ACTIVITY	2.34	GO0006464 PROTEIN MODIFICATION PROCESS	-2.67
GO0005080 PROTEIN KINASE C BINDING	2.33	GO0000910 CYTOKINESIS	-2.71
GO0005496 STEROID BINDING	2.26	GO0016776 PHOSPHOTRANSFERASE ACTIVITY PHOSPHATE G	-2.73
GO0030528 TRANSCRIPTION REGULATOR ACTIVITY	2.24	GO0006446 REGULATION OF TRANSLATIONAL INITIATION	-2.74
GO0001584 RHODOPSIN LIKE RECEPTOR ACTIVITY	2.23	GO0005528 FK506 BINDING	-2.75
GO0019835 CYTOLYSIS	2.20	GO0045598 REGULATION OF FAT CELL DIFFERENTIATION	-2.77
GO0005125 CYTOKINE ACTIVITY	2.19	GO0000139 GOLGI MEMBRANE	-2.86
GO0051258 PROTEIN POLYMERIZATION	2.19	GO0001892 EMBRYONIC PLACENTA DEVELOPMENT	-2.93
GO0030595 LEUKOCYTE CHEMOTAXIS	2.19	GO0006071 GLYCEROL METABOLIC PROCESS	-2.93
GO0005578 PROTEINACEOUS EXTRACELLULAR MATRIX	2.16	GO0005794 GOLGI APPARATUS	-2.95
GO0005887 INTEGRAL TO PLASMA MEMBRANE	2.12	GO0006914 AUTOPHAGY	-3.08
GO0002504 ANTIGEN PROCESSING AND PRESENTATION OF P	2.12	GO0007040 LYSOSOME ORGANIZATION AND BIOGENESIS	-3.10
GO0009887 ORGAN MORPHOGENESIS	2.11	GO0045444 FAT CELL DIFFERENTIATION	-3.13
GO0005172 VASCULAR ENDOTHELIAL GROWTH FACTOR RECEP	2.11	GO0003707 STEROID HORMONE RECEPTOR ACTIVITY	-3.17
GO0006122 MITOCHONDRIAL ELECTRON TRANSPORT UBIQUI	2.08	GO0007005 MITOCHONDRION ORGANIZATION AND BIOGENESI	-3.29
GO0003954 NADH DEHYDROGENASE ACTIVITY	2.03	GO0005525 GTP BINDING	-3.40
GO0004944 C5A ANAPHYLATOXIN RECEPTOR ACTIVITY	2.01	GO0006512 UBIQUITIN CYCLE	-3.51
GO0042613 MHC CLASS II PROTEIN COMPLEX	2.00	GO0032020 ISG15 PROTEIN CONJUGATION	-3.57
GO0007342 FUSION OF SPERM TO EGG PLASMA MEMBRANE	1.98	GO0008889 GLYCEROPHOSPHODIESTER PHOSPHODIESTERASE	-3.77

GO0045664 REGULATION OF NEURON DIFFERENTIATION	1.97	GO0019001 GUANYL NUCLEOTIDE BINDING	-4.07
GO0043254 REGULATION OF PROTEIN COMPLEX ASSEMBLY	1.97	GO0015031 PROTEIN TRANSPORT	-4.30
GO0001955 BLOOD VESSEL MATURATION	1.94	GO0005764 LYSOSOME	-4.72
GO0004602 GLUTATHIONE PEROXIDASE ACTIVITY	1.93	GO0005694 CHROMOSOME	-4.74
GO0001942 HAIR FOLLICLE DEVELOPMENT	1.92	GO0000786 NUCLEOSOME	-6.33
GO0043025 CELL SOMA	1.84	GO0007001 CHROMOSOME ORGANIZATION AND BIOGENESIS (	-6.67
GO0004522 PANCREATIC RIBONUCLEASE ACTIVITY	1.83	GO0006334 NUCLEOSOME ASSEMBLY	-6.87

Complete list of GO terms significantly altered by 0.1% metformin treatment. Metformin: Met.

Supplementary Table S7. Densitometry analysis of the western blots of HepG2 cells shown in the manuscript.

Protein	Untreated	1mM Metformin
AMPK	100 ±4	123 ±16
pAMPK	100 ±52	330±48*
ACC	100 ±14	98 ±29
pACC	100 ±45	196 ±22
PGC1α/β	100 ±30	97 ±51
Complex I	100 ±5	129 ±7*
Complex III	100 ±7	96 ±2
Complex V	100 ±11	96 ±7
LDH	100 ±7	130 ±5*
β-actin	100 ±7	98 ±5

n = 3 per group**.** Data are represented as the mean ± SEM. $P < 0.05$ versus untreated (*t*-test two tailed).

Supplemenary Table S8. Densitometry analysis of the western blots of the livers shown in the manuscript.

Protein	SD	0.1% Metformin	CR
AMPK	100 ±12	118 ±25	113 ±13
pAMPK	100 ±7	127 ±7*	129 ±24
ACC	100 ±6	84 ±15	73 ±12
pACC	100 ±27	269 ±31*	289 ±30*
PGC1α/β	100 ±4	74 ±12	122 ±4*
Complex I	100 ±7	129 ±4*	163 ±6*
Complex III	100 ±11	120 ±6	112 ±6
Complex V	100 ±1	101 ±5	119 ±6*
SOD1	100 ±9	145 ±2*	119 ±2
TRXR1	100 ±13	199 ±6*	144 ±11
NQO2	100 ±19	238 ±37*	139 ±35
NQO1	100 ±21	184 ±13*	163 ±24
NFκB	100 ±7	112 ±7	65 ±7*
pNFκB	100 ±5	36 ±4*	35 ±4*
JNK	100 ±7	87 ±12	60 ±5*
pJNK	100 ±11	21 ±8*	17 ±13*
LDH	100 ±40	446 ±41*	45 ±27
β-actin	100 ±6	88 ±5	71 ±6*

n = 4-6 per group**.** Data are represented as the mean ± SEM. * $p < 0.05$ versus standard diet (SD) (*t*-test two tailed); CR, calorie restricted

Supplementary Table S9. List of primers used in this study.

Gene	Forward primer sequence	Reverse primer sequence
TNFα	5'– CCCTCACACTCAGATCATCTTCT –3'	5'– GCTACGACGTGGGCTACAG –3'
CISH	5'– GCATAGCCAAGACGTTCTCC –3'	5'– AATGTACCCTCCGGCATCTT –3'
NFκB	5'– GGAGGGATCTTCGGTAGTGG –3'	5'– CCCTGCGTTGGATTTCGTG –3'
IL6	5'– TAGTCCTTCCTACCCCAATTTCC –3'	5'– TTGGTCCTTAGCCACTCCTTC –3'
SOCS2	5'– TGTGCAAGGATAAACGGACA –3'	5'– GGTAAAGGGAGTCCCCAGA –3'
IL10	5'– GCAACTGTTCCTGAACTCAACT –3'	5'– ATCTTTTGGGGTCCGTCAACT –3'
Rsp18	5'– TGTGTTAGGGGACTGGTGGACA –3'	5'– CATCACCCACTTACCCCCAAAA –3'
COX2	5'– ATAACCGAGTCGTTCTGCCAAT –3'	5'– TTTCAGAGCATTGGCCATAGAA –3'
PGC1-α	5'– CACCAAACCCACAGAAAACAG –3'	5'– GGGTCAGAGGAAGAGATAAAGTTG –3'
PGC-1β	5'– GGTGTTCGGTGAGATTGTAGAG –3'	5'– GTGATAAAACCGTGCTTCTGG –3'
NRF1	5'– AATGTCCGCAGTGATGTCC –3'	5'– GCCTGAGTTTGTGTTTGCTG –3'
TFAM	5'– CACCCAGATGCCAAACTTTCAG –3'	5'– CTGCTCTTTATACTTGCTCACAG –3'
NDUFAB1	5'– GGACCGAGTTCTGTATGTCTTG –3'	5'– AAACCCAAATTCGTCTTCCATG –3'
Uqcrc1	5'– ATCAAGGCACTGTCCAAGG –3'	5'– TCATTTTCCTGCATCTCCCG –3'
COX5b	5'– ACCCTAATCTAGTCCCGTCC –3'	5'– CAGCCAAAACCAGATGACAG –3'
ATP5a1	5'– CATTGGTGATGGTATTGCGC –3'	5'– TCCCAAACACGACAACTCC –3'
MCAD	5'– TGTTAATCGGTGAAGGAGCAG –3'	5'– CTATCCAGGGCATACTTCGTG –3'
LCAD	5'– GGTGGAAAACGGAATGAAAGG –3'	5'– GGCAATCGGACATCTTCAAAG –3'
CPT1α	5'– AGACAAGAACCCCAACATCC –3'	5'– CAAAGGTGTCAAATGGGAAGG –3'
PEPCK	5'– CCATCCCAACTCGAGATTCTG –3'	5'– CTGAGGGCTTCATAGACAAGG –3'
G6Pase	5'– TCTTGTGGTTGGGATTCTGG –3'	5'– CGGATGTGGCTGAAAGTTTC –3'
LPK	5'– AGTCTTCCCCTTGCTCTACC –3'	5'– AATCACCAGATCACCAACTCG –3'
GK	5'– CTTCACCTTCTCCTTCCCTG –3'	5'– ATCTCAAAGTCCCCTCTCCT –3'
ND1	5'– TGCACCTACCCTATCACTCA –3'	5'– GGCTCATCCTGACATAGAATGG –3'
Cytb	5'– CCCACCGCATATTAAACCCG –3'	5'– GAGGTATGAAGGAAAGGTATAAGGG –3'
COX1	5'– CCCAGATATAGCATTCCCACG –3'	5'– ACTGTTCATCCTGTTCCTGC –3'
ATP6	5'– TCCCAATCGTTGTAGCCATC –3'	5'– TGTTGGAAAGAAGGAGTCGG –3'
GAPDH	5'– CACCAACTGCTTAGCCCC –3'	5'– TCTTCTGGGTGGCAGTGATG –3'
β-actin	5'– ACCTTCTACAATGAGCTGCG –3'	5'– CTGGATGGCTACGTACATGG –3'

Supplementary Methods

Glyceride determination. Livers (20mg) were homogenized in 1ml of methanol and protein concentration was determined by Bradford assay. Lipid extraction from samples was performed as according to the Folch method[52]. Briefly, 2 ml of chloroform were added followed by vortex. Then, 1 ml of ddH2O was added to separate the lipid-soluble phases. After centrifugation at 2000 x g for 5 min, the organic phase was harvested. The organic phase was dried under nitrogen stream and re-suspended in 1 ml of methanol. Free glycerol content was first determined spectrophotometrically with a Free Glycerol Reagent (Sigma-aldrich). Total Glycerol was determined after the addition of 40 µl of Reconstituted Triglyceride Reagent (Sigma-aldrich). Glyceride content was determined by the subtraction of the free glycerol from the total glycerol. n = 6-8 per group; age = 84 weeks; diet = 30 weeks.

Cell Culture. Primary MEFs were immortalized in culture using a standard 3T3 protocol[53]. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (high glucose) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C under humidified 5% CO_2. The medium was replenished every 2-3 d, and cells were subcultured after reaching confluence. ARE-*bla* HepG2 cells were purchased from Invitrogen Corporation (Carlsbad, CA) and maintained in 90% DMEM supplemented with Glutamax (Invitrogen), 10% dialyzed FBS, nonessential amino acids, penicillin/streptomycin, HEPES (25 mM, pH 7.3), and 5 µg/mL blasticidin (Invitrogen) under standard cell culture conditions (5% CO_2, 95% air, 37 °C). Primary rat hepatocytes were cultured according to the manufacturer's instructions (Lonza, Walkersville, MD). Where indicated, 1 mM metformin was added to the media and cells were harvested 16 h later. n = 3 per group.

Palmitate Oxidation. Palmitate oxidation was determined according to previously published methods with minor modifications[54]. In brief, primary rat hepatocytes (Lonza, Walkersville, MD) or MEFs were cultured in 12-well plates and cells were incubated overnight with 1mM metformin. After a 30-min preincubation with SAB (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.16 mM MgSO4, 2.5 mM CaCl2, 20 mM HEPES, and 1% fatty acid-free bovine serum albumin, pH 7.4) containing 3 mM glucose, [1-^{14}C]palmitate (Perkin Elmer, Wellesley, MA), glucose, and l-carnitine were added to final concentrations of 3 or 20 and 0.8 mM, respectively. A paper filter with 150µl of 10N KOH was stuck to each well by using a 0 mm

bushing (Home depot, Apin Hill, MD) in a optical adhesive film (Applied biosystems, Rockville, MD) and plates were sealed. After a 2-h incubation at 37 °C, 100 µl of 7% perchloric acid (Sigma-Aldrich) was injected into each well. Following 2 h at 37 °C, the KOH filters with captured $^{14}CO_2$. were collected and put in a scintillation vial. Scintillation mixture was dispensed into the vials, and the palmitate derived $^{14}CO_2$ was counted. n = 3-10 per group.

Glucose Incorporation into Lipids. Palmitate oxidation was determined according to previously published methods with minor modifications[54]. In brief, primary hepatocytes or MEFs were cultured in 12-well plates and cells were incubated overnight with 1mM metformin. After a 60-min preincubation with SAB, 0.5 Ci/mol [3-^{3}H]glucose (Perkin Elmer) and 5 Ci/mol [1-^{14}C]palmitate (Perkin Elmer) were included and plates were sealed with an optical adhesive film (Applied biosystems). [1-^{14}C]palmitate was included as a tracer. After 2h 1 ml of methanol:PBS (2:3) was added to the cells. Cells were collected with gentle pipeting, centrifuged at 700 × g, and washed twice with PBS. 200 µl of 0.2 m NaCl was added to the cell pellet and the mixture was immediately frozen in liquid N_2. The lipid and aqueous soluble products were separated by the following procedure. To the thawed cell suspension, 750 µl of $CHCl_3$:methanol (2:1) and 50 µl of 0.1 n KOH was added and, after vigorous vortexing, the phases were separated by centrifugation at 2000 × g for 20 min. The top aqueous layer was removed, and the bottom lipid-soluble layer was washed with 200 µl of methanol:water:$CHCl_3$ (48:47:3). 200 µl of the lipid soluble phase was added to scintillation mixture and incorporation of radiolabel into lipids was quantified. n = 3-10 per group.

Cataracts Assessment. Age-related lens opacity was scored by an experienced pathologist using a slit lamp in all living mice following a previously described method Assessment was 0 to 4, with 0 representing lens with no opacity, 4 representing complete opacity and mature cataract[55]. (*n* = 93-124 per group; age = 105 weeks; diet = 51 weeks).

Insulin, OGTT and ITT. To determine glucose level, blood samples were taken by venipuncture as previously described[56]. To quantify insulin level, whole blood was obtained after a 20-h fast and spun at 14,000 rpm for 5 min to pellet blood cells. Serum was transferred to a fresh tube and placed on dry ice and stored at -80ºC. Insulin levels were measured in plasma by using ELISA kits (Crystal Chem Inc., Downers Grove, IL). For OGTT, mice were fasted for 6 h and received an oral dose of 2 g.kg^{-1} of glucose (Sigma-Aldrich, St. Louis, MO) by gavage. At

baseline and 15, 30, 60 and 120 min after glucose administration, blood glucose levels were determined by tail venipuncture using an Ascensia Elite glucose meter (Bayer, Mishawaka, IN) (*n* = 8 per group; age = 93 weeks; diet = 39 weeks). For ITT, mice were fasted for 3 h and received an intraperitoneal dose of 1.5 IU.kg^{-1} of insulin. At baseline and 15, 30, 60, 90 and 120 min after glucose administration, blood glucose levels were measured, as previously described for OGTT (*n* = 9 per group; age = 81 weeks; diet = 27 weeks). Areas under the curves (AUC) were determined using the "Area Below Curves" function in Sigma Plot 10.0 (Systat, Point Richmond, CA).

Homeostasis Model Assessment (HOMA). Insulin resistance was estimated using the HOMA2 Calculator software available from the Oxford Centre for Diabetes, Endocrinology and Metabolism Diabetes Trials Unit website as described by Levy *et al.* [57].

Microarray Analysis. Principal components were calculated using DIANE 6.0. For the calculation of pairwise distances between samples, each microarray was considered as a point in a high-dimensional space since we treated each probe as a variable. For parametric analysis of gene set enrichment (PAGE), our expression data was tested using the PAGE method as previously described[58]. Briefly, for each pathway under each pair of conditions, an aggregated Z score was computed as:

$$zscore\ \ pathway = \frac{\overline{n_{pathway}}\ zratio\ \ \ genes\ in\ the\ pathway\ \ - zratio\ \ \ genes\ on\ the\ array}{\sigma_{sample}}$$

where $n_{pathway}$ is the number of genes in the specific pathway and σ_{sample} is the standard derivation of z-ratio on the comparison sample arrays. For each Z (pathway) a *P* value was computed (JMP 6.0 software) to the total z-ratio in comparison by z-test. Ingenuity Pathways Analysis© was performed by using the tools supplied by Ingenuity Inc. (Ingenuity Systems; Redwood City, CA) (*n* = 4-5 per group; age = 84 weeks; diet = 30 weeks).

Western Blot Analysis. Tissues were lysed in radioimmunoprecipitation buffer supplemented with ethylenediaminetetraacetic acid and ethylene glycol tetraacetic acid (Boston BioProducts, Ashland, MA). Insoluble material was removed by centrifugation (11,000 rpm, 20 min at 4ºC), and the Bradford assay method (Bio-Rad, Hercules, CA) used to determine protein concentration. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel

electrophoresis under reducing conditions and then transferred to nitrocellulose membranes. Western blots were performed according to standard methods, which involved blocking in 5% bovine serum albumin and incubations with the antibody of interest, followed by incubations with a secondary antibody conjugated with the enzyme horseradish peroxidase. The visualization of immunoreactive bands was performed using the ECL Plus Western blotting detection system (GE Healthcare, Pascataway, NJ). The quantification was done by volume densitometry using ImageJ software (National Institutes of Health, Bethesda, MD) and normalization to β-actin. In this study, the primary antibodies were directed against PGC1α (1:200 dilution) (Cat#:sc-13067), UCP2 (1:200 dilution) (Cat#:sc-6526), SOD2 (1:200 dilution) (Cat#:sc-30080), LDH (1:200 dilution) (Cat#:sc-27230), NQO1 (1:200 dilution) (Cat#:sc-16464), NQO2 (1:200 dilution) (Cat#:sc-32942) and TrxR1 (1:200 dilution) (Cat#:sc-20147) (Santa Cruz Biotechnology, Santa Cruz, CA); AMPK (1:1000 dilution) (Cat#:2532), p-AMPK (1:1000 dilution) (Cat#:2531), pNF-κβ (1:1000 dilution) (Cat#:3033), JNK (1:1000 dilution) (Cat#:9258) and pJNK (1:1000 dilution) (Cat#:9251) (Cell Signaling Technology, Beverly, Ma); ACC (1:500 dilution) (Cat#:04-322) and p-ACC (1:1000 dilution) (Cat#:05-373) (Millipore, Bedford, MA); Complex III (1:2000 dilution) (Cat#:MS304) and Complex V (1:2000 dilution) (Cat#:MS507) (MitoSciences, Eugene, OR); β-actin (1:1000 dilution) (Cat#:AB6276) and Complex I (1:2000 dilution) (Cat#:AB14713) (Abcam Inc., Cambridge, MA); NF-κβ (1:500 dilution) (Cat#:1546-1) (Epitomics, Burlingame, CA); 4-HNE (1:1000 dilution) (Cat#:393206) (EMD-Calbiochem, La Jolla, CA). In animal experiments, *n* = 4-6 per group; age = 84 weeks; diet = 30 weeks.

ATP determination. Tissues were homogenized and treated with ATP-releasing buffer (100 mM potassium phosphate buffer at pH 7.8, 1% Triton X-100, 2 mm EDTA, and 1 mM dithiothreitol) for the determination of ATP levels using a luciferin-based ATP determination kit (Invitrogen). n= 4-5 per group.

Oxygen Consumption. Oxygen consumption in wild-type MEFs was measured using the Seahorse 24XF instrument, according to the manufacturer's protocol (Seahorse Biosciences, North Billerica, MA). MEFs were seeded into a Seahorse tissue culture plate at a density of 50,000 MEFs per well in 100 μl DMEM. Two h later, another 100 μl of medium containing 2 mM metformin was added (final concentration of 1 mM metformin), and the cells were incubated for an additional 24 h. The medium was removed and replaced with unbuffered XF assay media (Seahorse Biosciences), pH 7.4, supplemented with 25 mM glucose, 1 mM sodium

pyruvate and 1 mM GlutaMAX. One h later, the oxygen consumption rate and extracellular acidification were measured in the Seahorse XF24 analyzer in 4 blocks of three 3-min periods. The first block measured the basal respiration rate. Next, 1 μM oligomycin (EMD Chemicals, Gibbstown, NJ) was added to inhibit complex V, and the second block was measured. Then, 0.3 μM carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP) (Sigma-Aldrich) was added to uncouple respiration, and the third block was measured. Finally, 2 μM antimycin A (Sigma-Aldrich) was added to inhibit complex III, and the last measurements were performed.

mtDNA Analysis. Total DNA was extracted with DNeasy blood and tissue kit (QIAGEN, Valencia, CA). mtDNA was amplified using primers specific for the mitochondrial cytochrome c oxidase subunit 2 (*Cox2*) gene and normalized to genomic DNA by amplification of the ribosomal protein s18 (*Rps18*) nuclear gene. Primers were designed using the IDT software (IDT, Dallas, TX) and the primer sequences can be found in the Supplementary section (Supplementary Table S9) (n = 5-8 per group; age = 84 weeks; diet = 30 weeks).

Nrf2/ARE Pathway Activity Assay. ARE-*bla* HepG2 cells were seeded at a density of 35,000 cells/well in white-walled 96-well tissue culture plates and allowed to expand until 80% confluence. Cells were treated with various concentrations of metformin or 25 μM of *tert*-butylhydroquinone (tBHQ, Fisher, Pittsburgh, PA) as a positive control, and incubated at 37 °C for 16 h. After treatment, the medium was removed, and the cells were washed with 1 X PBS followed by incubation with a β-lactamase substrate (Lytic Blazer, Invitrogen) for 4 h at 37 °C. Relative fluorescence values were determined at an excitation/emission wavelength of 485/530nm in a 1420 multilabel counter (Perkin Elmer, Wellesley, MA). Values for wells without treatment were subtracted from all readings. $n \geq 3$ per group.

MTT Assay. Cells were treated with metformin or tBHQ as described above. Medium was removed and replaced with a 0.5% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (Sigma-Aldrich). Plates were incubated for an additional 3 h, after which the MTT solution was removed and blue formazan crystals were solubilized with 100 μl of dimethylsulfoxide (Sigma-Aldrich). Optical density was determined at 590 nm using a 96-well plate-reader 1420 multilabel counter. $n \geq 3$ per group.

Calcein AM Assay. Cells were treated with metformin or tBHQ as described above. Medium was removed, and cells were washed with 100 μL of Calcein AM DW Buffer (Sigma-Aldrich). Then, 50 μL of fresh 1X Calcein AM DW Buffer was added to cells followed by 50 μL

of freshly prepared 2X Calcein AM Solution (Sigma-Aldrich). Plates were incubated for 30 min at 37 °C, and fluorescence was determined using a 490/520 nm excitation/emission filter. n ≥ 3 per group.

Measurement of Mitochondrial Activities. Mitochondrial activities were determined in approximately 50 µg of protein lysates following the method described by Bernier et al.[50]. Activities of NADH:coenzyme Q oxidoreductase (complex I), succinate dehydrogenase (complex II), ubiquinol:cytochrome *c* oxidoreductase (complex III), cytochrome *c* oxidase (complex IV) , NADH:cytochrome *c* reductase (complex I to III), succinate:cytochrome *c* reductase (complex II to complex III), and citrate synthase were determined by spectrophotometric methods. Results were expressed in nmol.mg protein^{-1}.min^{-1} (*n* = 5-6 per group; age = 84 weeks; diet = 30 weeks).

Quantitative RT–PCR. Total RNA was extracted from frozen tissue samples or cells using the RNeasy kit (Qiagen). Complementary DNA was synthesized from total RNA with the SuperScript First-Strand Synthesis System (Invitrogen) and random hexamer primers. The real-time polymerase chain reaction measurement was performed on individual cDNAs by using SYBR green dye to measure duplex DNA formation with the Roche Lightcycler system. The calculation of mRNA expression was performed by the $2^{-\Delta\Delta CT}$ method normalized to the expression of β-actin and/or GAPDH[50]. The primers and probes used in real time RT–PCR are listed in Supplementary Table S6 (*n* = 5 per group; age = 84 weeks; diet = 30 weeks).

Histology analysis of tissues. Following euthanasia, liver was excised and fixed in formaldehyde. Slides were stained with hematoxylin and eosin (FD NeuroTechnologies, Columbia, MD). After rinsing in distilled water, liver sections were mounted with Super Mount Aqueous mounting medium (Biogenex, Fremont, CA) and photographed by using light microscopy at 20 x magnification using standard protocols. For quantifications liver infiltrated macrophages, at least five microscope fields at ×20 magnification were scored for positive cells. The values represented are relative to untreated controls. (*n* = 4 per group; age = 115 weeks; diet = 61 weeks).

Supplementary References

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