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Supplemental Information

Methionine Restriction Extends Lifespan in Progeroid Mice and Alters Lipid and Bile Acid Metabolism

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SUPPLEMENTAL FIGURES

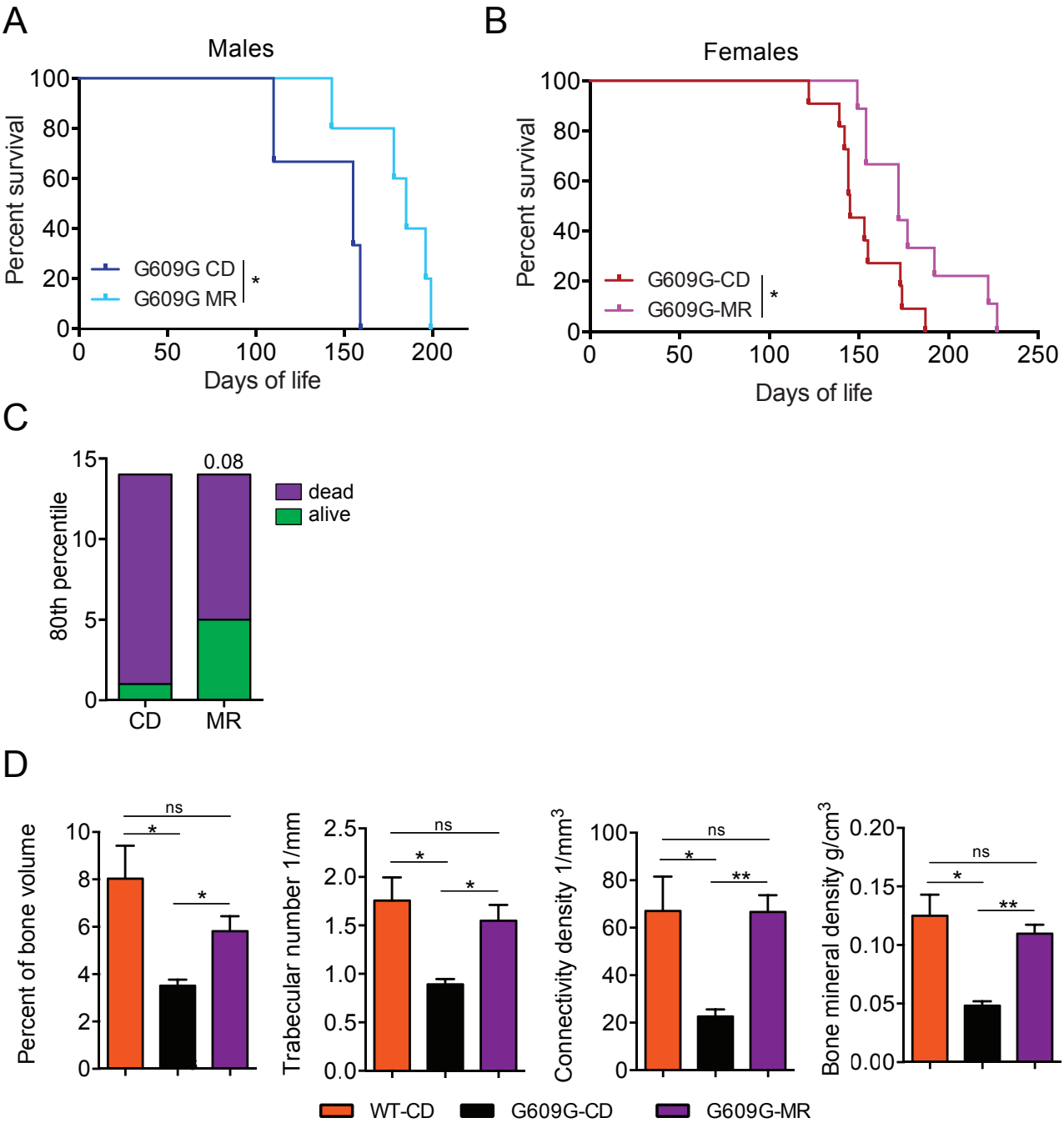

Figure S1. Related to Figure 1. Methionine restriction enhances healthspan and median and maximal lifespan of *Lmna*$^{G609G/G609G}$ mice. (A-B) Survival plot of *Lmna*$^{G609G/G609G}$ mice fed MR diet or control diet in (A) males (n=3-5) and (B) females (n=9-11). Median survival increment is 19.35% for males fed an MR diet ($p<0.05$) and 18.62% for females fed an MR diet ($p<0.05$). Survival curves were analyzed with Log-rank (Mantel-Cox) test (p=0.04 for males and 0.02 for females) and Gehan-Breslow-Wilcoxon test (p=0.07 for males and 0.02 for females). (C) Maximum survival analysis at 80th percentile using Fisher's exact test of *Lmna*$^{G609G/G609G}$ mice fed CD and MR diet. (D) Quantitative analysis of percent of bone volume (bone volume/tissue volume [BV/TV]), trabecular number per mm, connectivity density per mm^3 and bone mineral density (g/cm^3) (n=3). Mean values are represented and error bars indicate SEM. ns: not significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

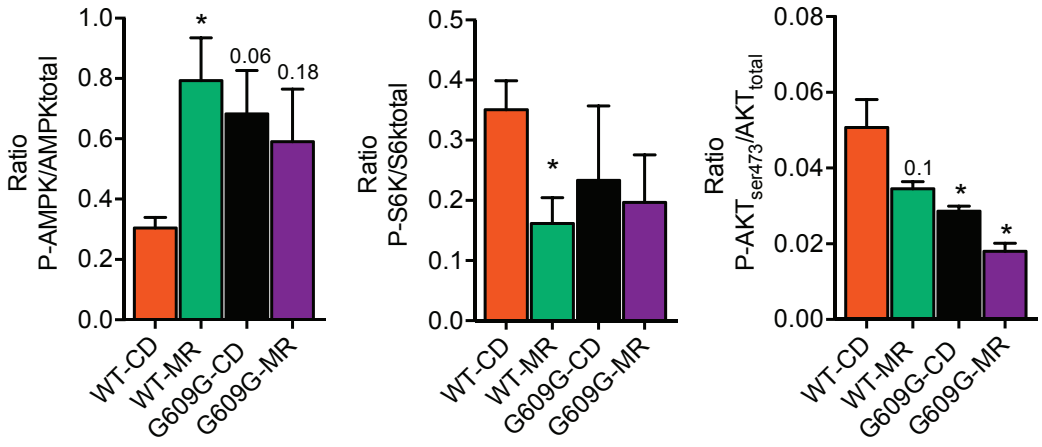

Figure S2. Related to Figure 2. Methionine restriction fails to induce the classical survival response in *Lmna*$^{G609G/G609G}$ mice. Quantification of the ratio of P-AMPK/total-AMPT, P-P70S6K/total-P70S6K and P-AKT(ser473)/total-AKT in liver protein extracts (n=3). *p<0.05.

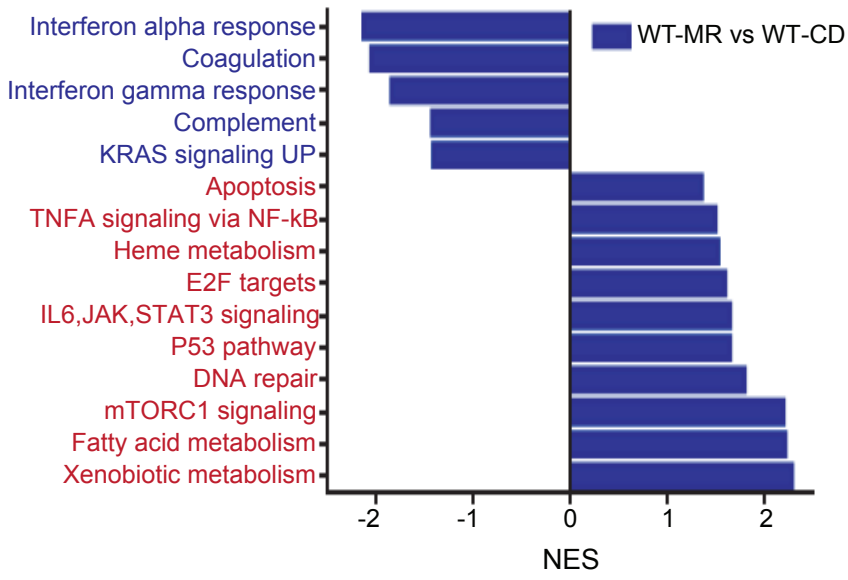

Figure S3. Related to Figure 3. Microarray analysis of liver samples show a shift under methionine restricted diet. GSEA normalized enrichment score (NES) analysis of WT-MR compared to WT-CD. Red color indicates upregulated pathways and blue color downregulated pathways.

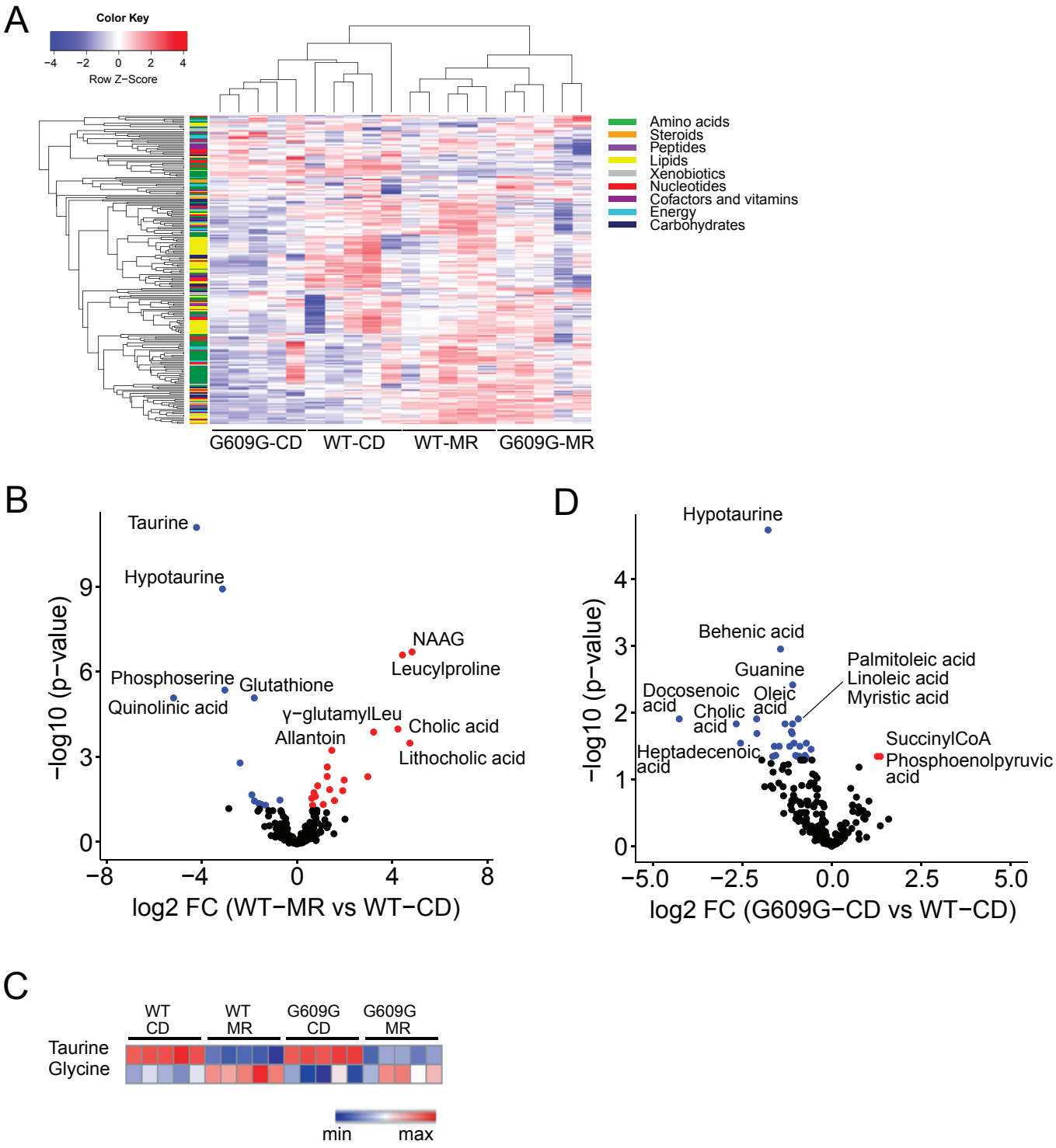

Figure S4. Related to Figure 4. Metabolomic analyses of liver samples show a metabolic shift under methionine restricted diet. (A) Metabolite heatmap based on the differential expression on the detected metabolites. Clusterization proves the similarity between MR samples. (B) Volcano plot showing the most relevant differences of individual metabolites in WT-MR compared to WT-CD mice. Upregulated metabolites are indicated in red and downregulated metabolites in blue. (C) Taurine and glycine relative levels in liver samples represented in a heatmap elaborated with Gene-E software. (D) Volcano plot showing the most relevant differences of individual metabolites in *Lmna*$^{G609G/G609G}$-CD compared to WT-CD mice. Upregulated metabolites are indicated in red and downregulated metabolites in blue.

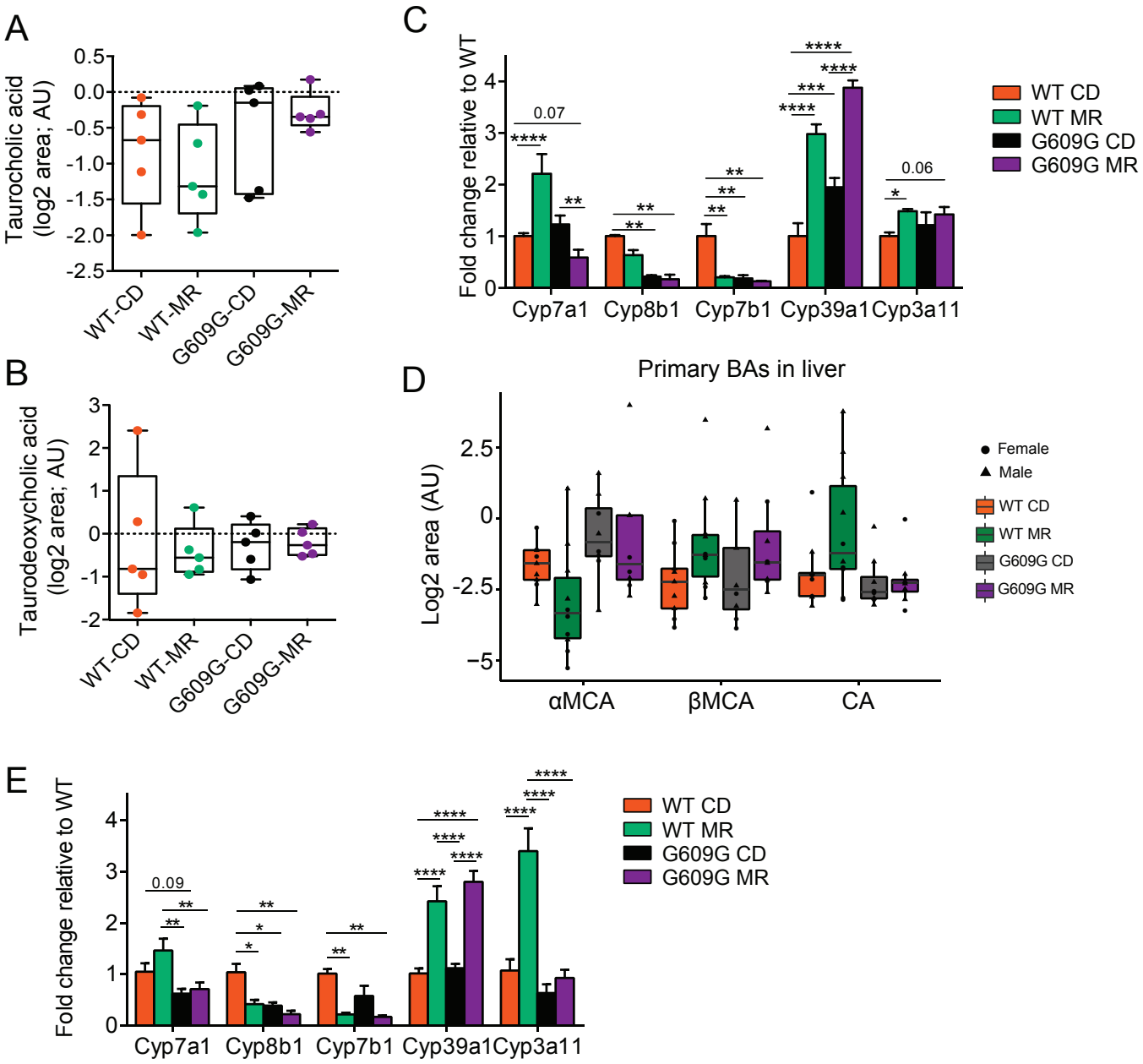

Figure S5. Related to Figure 5. Bile acids profile under methionine restriction in fasting and re-feeding conditions. (A-B) Boxplots showing the levels of (A) taurocholic acid and (B) taurodeoxycholic acid under fasting conditions in liver samples from WT-CD, WT-MR, *Lmna*$^{G609G/G609G}$-CD and *Lmna*$^{G609G/G609G}$-MR (n=5). Levels are indicated as the log2 of the normalized area in arbitrary units (AU). (C) mRNA expression levels of genes involved in bile acid synthesis in liver under fasting conditions. Values are shown as relative to WT-CD mice (n=5). (D) Primary bile acids levels in liver samples from WT-CD, WT-MR, *Lmna*$^{G609G/G609G}$-CD and *Lmna*$^{G609G/G609G}$-MR mice. Levels are indicated as the log2 of the normalized area in AU. Sex is indicated with different symbols (n=8). αMCA: α-muricholic acid; β MCA: β-muricholic acid; CA: cholic acid. (E) mRNA expression levels of genes involved in bile acid synthesis in liver under refeeding conditions. Values are shown as relative to WT-CD mice (n=5). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. See also Table S3.

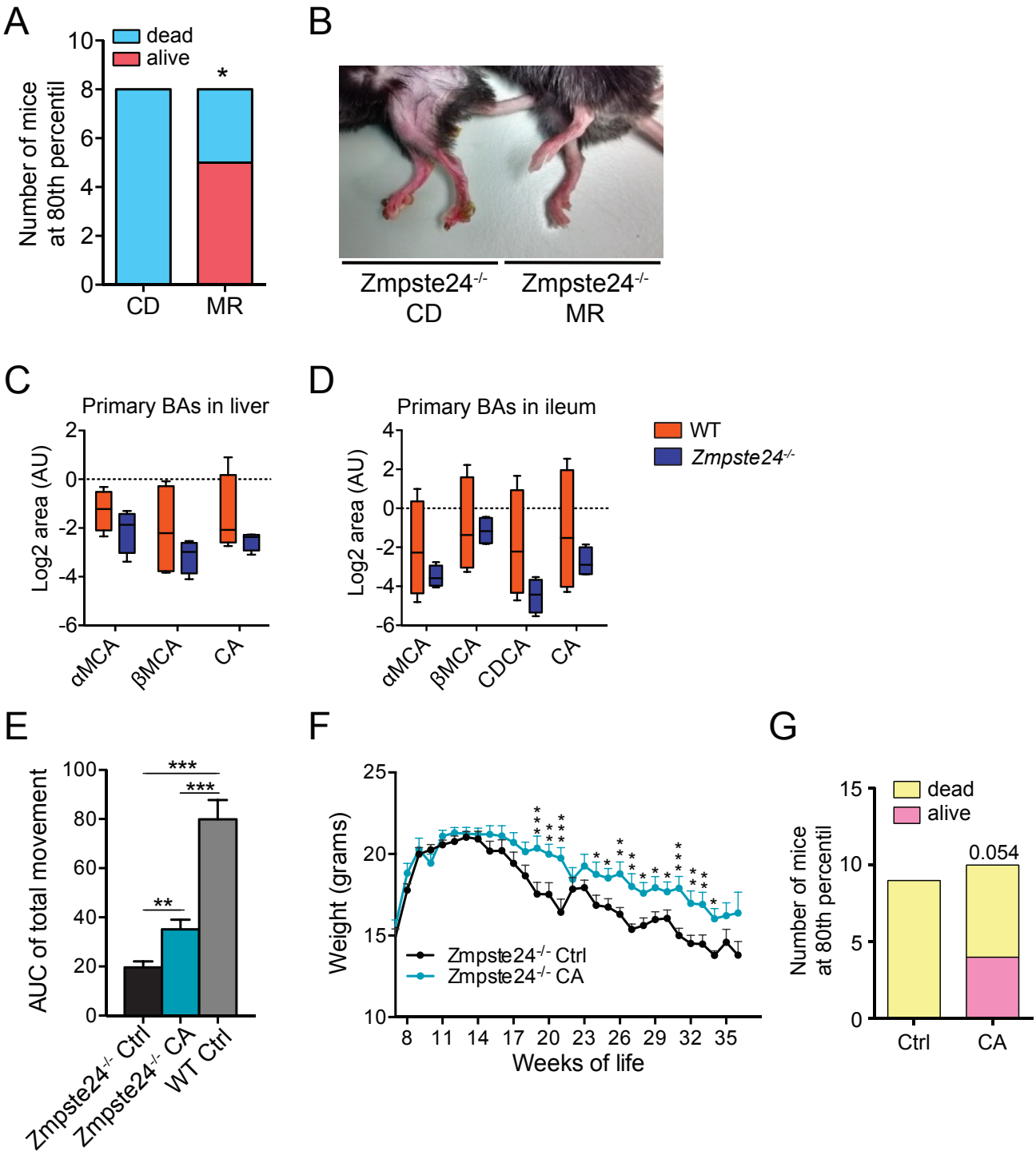

Figure S6. Relative to Figure 6. Methionine restriction and cholic acid supplementation enhance healthspan and lifespan in *Zmpste24*$^{-/-}$ mouse model of HGPS. (A) Maximum survival analysis at 80th percentile using Fisher's exact test of *Zmpste24*$^{-/-}$ mice fed CD and MR diet. (B) *Zmpste24*$^{-/-}$ MR mice (right) have an improved hind limb atrophy than *Zmpste24*$^{-/-}$ CD mice (left). (C-D) Primary bile acids levels in (C) liver and (D) ileum samples from WT and *Zmpste24*$^{-/-}$ mice. Levels are indicated as the log2 of the normalized area in arbitrary units (AU). αMCA: α-muricholic acid; β MCA: β-muricholic acid; CDCA: chenodeoxycholic acid; CA: cholic acid. (E) Area under the curve of the daily movement of WT, *Zmpste24*$^{-/-}$ CA and *Zmpste24*$^{-/-}$ control mice. (F) *Zmpste24*$^{-/-}$ CA show a milder phenotype-associated loss of weight than *Zmpste24*$^{-/-}$ control. (G) Maximum survival analysis at 80th percentile using Fisher's exact test of *Zmpste24*$^{-/-}$ control mice and supplemented with cholic acid. *p<0.05, **p<0.01, ***p<0.001.

Table S3. Related to Figure S5. List of primer sets used for qPCR analyses.

Gene	Forward (5'-3')	Reverse (5'-3')
Cyp3a11	GGTTTATGGAAATTCGACATGGA	GGGTCTGTGACAGCAAGG
Cyp7a1	CTCAGCTCTGGAGGGAATGC	CGCAGAGCCTCCTTGATGA
Cyp7b1	GCCACCTTACTGCTCTCGG	GCCAAGATAAGGAAGCCAACCT
Cyp8b1	CCATAAGACGCCATCCCTCC	AATGTGGGTGAGCCATCAG
Cyp39a1	GGCAACATAAAAGCTCACGGA	CCGTCTCCACCACTTCCAAT
Actb	CTGAGGAGCACCCTGTGCT	GTTGAAGGTCTCAAACATGATCTG
Gapdh	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGAT

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Analysis of bone structure

All tibia samples were scanned by high-resolution micro-computed tomography (SkyScan 1174, SkyScan, Kontich, Belgium). The small sample-holder device for μCT was used to fit the specimen with the long axis perpendicular to the floor of the specimen holder and the x-ray source. Images were obtained by 50 kV X ray tube voltage and 800 μA. All specimens were scanned using 0.5 mm aluminum filter and at 9.6 μm pixel size resolution. For each specimen, a series of 613 projection images were obtained with a rotation step of 0.3° and frame averaging 2 for a total 180° rotation. The scanning time for each sample was approximately 2 hours using an exposure time of 5500 ms. Flat field correction was performed at the beginning of each scan. The images obtained during scanning were reconstructed using the software NRecon (SkyScan). The correction values of attenuation coefficient, beam hardering, smoothing and ring-artifact reduction were the same in all samples. For morphometric analysis in 2D and 3D it was used the software provided by the manufacturer (CTAn). Region of interest (ROI) was manually delimited in each of the samples. For the analysis of the diaphyseal cortical region 100 slices were chosen. Global grayscale threshold levels for this area were between 88 and 250. For the trabecular region a total of 150 slices were selected and adaptative grayscale threshold levels between 63 and 250 were used. The morphometric parameters examined were bone mineral density (BMD), ratio of bone volume/ tissue volume (BV/TV), and trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp) for the trabecular area and connectivity density among trabeculae. The parameters were measured according to the ASBMR histomorphometry nomenclature (Parfitt et al., 1987).

Western blot analysis

Tissues were collected and immediately frozen in dry ice. About 50 mg of each sample of frozen tissue was homogenized in 300 μL of 100 mM Tris-HCl (pH 7.4), 2% SDS, and 50 mM EDTA with a Polytron homogenizer. After evaluation of the protein concentration with the bicinchoninic acid technique (Pierce BCA protein assay kit), equal amounts of proteins were loaded onto SDS-polyacrylamide gels. After electrophoresis, gels were electrotransferred onto Immobilon-FL polyvinylidene fluoride membranes (Millipore). Membranes were stained with Ponceau to confirm the proper electrotransfer and the equal protein loading. Then, membranes were washed with TBS-T buffer (20 mM Tris at pH 7.4, 150 mM NaCl, 0.05% Tween 20), blocked with 5% nonfat dry milk in TBS-T and incubated overnight at 4 °C with the different primary antibodies. Finally, blots were incubated with 1:10,000 secondary antibody conjugated with horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories) in 1.5% nonfat milk in TBS-T for 1 h at room temperature. After washing off the secondary antibody with TBST-T buffer, we developed the immunoreactive bands with Immobilon Western chemiluminescent HRP substrate (Millipore) in a LAS-3000 Imaging System (Fujifilm). For the generation of Figure 2D, three blots with the same samples were ran in parallel. Each total form was analyzed in the same blot as the phosphorylated form after membrane stripping following standard protocols. α -Tubulin was used as sample processing control for protein lysates.

RNA preparation and quantitative PCR

Total RNA from about 30 mg of frozen tissues was extracted using Trizol (Life Technologies) and resuspended in nuclease-free water (Life Technologies). 1–2 μg of total RNA was used for reverse transcription using the QuantiTect Reverse Transcription kit (QIAGEN). 10× diluted cDNA was used for quantitative PCR (qPCR) reactions using Power SYBR Green PCR Master Mix (Life Technologies) in an Applied Biosystems 7300 HT Real-Time PCR System. Results are represented as relative quantification using RQ value ($RQ=2^{-\Delta\Delta Ct}$). Primer sets for qPCR analyses are shown in Table S3.

Serum fatty acid and liver triglycerides

Free fatty acids in serum were assayed using the luminometric Free Fatty Acid Assay Kit from Abnova (KA1667). Triglycerides in liver were assayed using the EnzyChrom Triglyceride Assay Kit from BioAssay Systems (ETGA-200). From liver preparation, 50 mg of tissue was homogenized in 500 μL of 5% Triton X-100 (T9284, Sigma) in phosphate buffered saline. Once homogenized, samples were introduced in a water bath at 80 °C and left inside for 5 min allowing them to reach 100 °C in the water bath. We repeated this two times, allowing the samples to settle at room temperature between cycles. After that, samples were centrifuged at 13,000 rpm for 5 min. The supernatant was recovered and diluted 1:8 in Milli-Q water. 10 μL of this dilution was used for the analysis.

Untargeted metabolomics analysis

30 mg of liver for each condition were first weighted and solubilized into 1.5 mL polypropylene Precellys lysis tubes, with 500 µL of cold (-20 °C) lysate buffer (MeOH/ Water/Chloroform, 9/1/1, -20 °C). They were then homogenized three times for 20 s at 5,000 rpm using Precellys 24 tissue homogenator (Bertin Technologies), followed by a centrifugation (10 min at 15,000 x g, 4 °C). 600 µL of the upper phase of the supernatant were collected. The supernatant was then evaporated in microcentrifuge tubes at 40 °C in a pneumatically-assisted concentrator (Techne DB3). 300 µL of methanol were added to the dried extract and split in two aliquots of 150 µL: the first one was used for the GC-MS analysis and the second one for the LC-MS experiment. Analytical methods and data processing were performed as previously described (Enot et al., 2015). Standard reagents used (acetonitrile, methanol, chloroform, acetic acid and dibutylamine acetate concentrate –DBAA) were all acquired from Sigma Aldrich.

Targeted metabolomics analysis

30 mg of tissues (liver/ileon) for each condition were first weighted and solubilized into 1.5 mL polypropylene microcentrifuge tubes with ceramic beads with 1 mL of cold lysate buffer (MeOH/Water/Chloroform, 9/1/1, -20 °C). They were then homogenized three times for 20 s at 5500 rpm using Precellys 24 tissue homogenator (Bertin Technologies, Montigny-le-Bretonneux, France), followed by a centrifugation (10 min at 15000 g, 4 °C). Targeted metabolomics was perform using Ultra High-Pressure Liquid Chromatography coupled by Mass Spectrometry (UHPLC/MS). Collected supernatant were evaporated in microcentrifuge tubes at 40 °C in a pneumatically-assisted concentrator (Techne DB3, Staffordshire, UK). The LC-MS dried extracts were solubilized with 450 µL of MilliQ water and aliquoted in 4 microcentrifuge tubes (100 µL). For bile acids measurements, one aliquot was transferred to LC vials and injected into LC/MS or kept at -80 °C until injection. Targeted analysis was performed on a RRLC 1260 system (Agilent Technologies, Waldbronn, Germany) coupled to a Triple Quadrupole 6410 (Agilent Technologies) equipped with an electrospray source operating in positive mode. The gas temperature was set to 325 °C with a gas flow of 12 L/min. The capillary voltage was set to 4.5 kV. 10 µL of sample were injected on a Column Poroshell 120 EC-C8 (100 mm x 2.1 mm particle size 2.7 µm) from Agilent technologies, protected by a guard column XDB-C18 (5 mm × 2.1 mm particle size 1.8 µm) and heated at 40 °C by a pelletier oven. The gradient mobile phase consisted of water with 0.2% of formic acid (A) and acetonitrile/isopropanol (1/1; v/v) (B) freshly made. The flow rate was set to 0.3 mL/min, and gradient as follow: initial condition was 70% phase A and 30% phase B, maintained during 1.5 min. Molecules were then eluted using a gradient from 30% to 60% phase B over 9 min. The column was washed using 98% mobile phase B for 2 min and equilibrated using 30% mobile phase B for 2 min. After each injection, the needle was washed twice with isopropanol and thrice with water. The autosampler was kept at 4°C. At the end of the batch of analysis, column was rinsed with 0.3 mL/min of MilliQ water (phase A) and acetonitrile (phase B) as follow: 10% phase B during 20 min, to 90% phase B in 20 min, and maintained during 20 min before shutdown. The collision gas was nitrogen. The scan mode used was the MRM for biological samples. Peak detection and integration of the analytes was performed using the Agilent Mass Hunter quantitative software (B.07.01).

Metabolomics quality control policy

A daily qualification of the instrumentation was set up with automatic tune and calibration processes. These qualifications were completed with double injections of standards mixes, at the beginning and at the end of the run, as for a blank extracted sample to control the background impurities. Mixtures were adapted for each chromatographic method. After the extraction, fractions of each biological sample were pooled to create a Quality Control (QC) sample, use to passivate the column before the analysis with the proper biological matrix and re-injected during the batch to monitor and correct analytical bias occurring during the batch (m/z, retention time and sensitivity drifts) during post acquisition treatment signal.

SUPPLEMENTAL REFERENCES

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Parfitt, A.M., Drezner, M.K., Glorieux, F.H., Kanis, J.A., Malluche, H., Meunier, P.J., Ott, S.M., and Recker, R.R. (1987). Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Min. Res *2*, 595–610.