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A genetic model of methionine restriction extends *Drosophila* health- and lifespan

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This PDF file includes:

Supplemental Methods

Stocks: *OreR* (lab stock); *UAS-Methioninase* (III) (generated for this paper); control *(yv; attP2(y+)* line used to generate *UAS-Methioninase* flies); *tubulin-Gal4,tubulin-Gal80ts* (lab stock); *elav-Gal4,tubulin-Gal80ts* (lab stock); *elav-Gal4* (lab stock); *Actin-GeneSwitch-Gal4* (gift from Dr. John Tower); *Elav-GeneSwitch-Gal4* (gift from Dr. John Tower); *nSyb-GeneSwitch-Gal4* (gift from Dr. Scott Pletcher); *TIGS-2* (gift from Dr. Scott Pletcher); *Whole body fat body–GeneSwitch–Gal4* (gift from Dr. John Tower); *UAS-Tau (wt)* (gift from Dr. Mel Feany).
Lifespan analysis. For survival analysis, flies were collected within 24 hrs from eclosion, sorted by sex under light CO_2 anesthesia, and reared at standard density (20-25 flies per vial) on cornmeal/soy flour/yeast fly food at 25°C and 60% humidity with 12 hrs on/off light cycle. Flies were transferred to fresh vials every two days, and the dead flies were counted. RU486 dissolved in ethanol was administered in the media at a final concentration of 150 ug/mL. All lifespan counts are listed in the Supplementary Table 1.

Antibodies and Immunoblot Analyses. A rabbit anti-Methioninase antibody was obtained from BioVision, and the anti-tubulin antibody was obtained from Sigma-Aldrich. For immunoblot analyses, 10 flies were grinded in a bullet blender in RIPA (Cell Signaling) lysis buffer with phosphatase and protease inhibitors (Roche). Whole-cell lysates were resolved by electrophoresis, and proteins were transferred onto PVDF membranes (Immobilon P; Millipore), blocked in Tris-buffered saline Tween-20 buffer (Cell Signaling Technology) containing 2.5% dry milk, and probed with the indicated antibodies diluted in this buffer.
qRT-PCR. Total RNA was extracted with the TRIzol reagent (Life Technologies), followed by DNase digestion using RQ1 RNase-Free DNase (Promega). Total RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed with the iQ SYBR Green Supermix (Bio-Rad) and a CFX96 Real- Time PCR Detection System (Bio-Rad). *RpL32* and *alpha-Tubulin 84B* were used as a normalization reference. Relative quantitation of mRNA levels was determined with the comparative C_T method.

Metabolite profiling (Dr. J. Asara's lab). 10 flies or 35 heads per sample (4 biological replicates) were collected, and the intracellular metabolites were extracted using 80% (v/v) aqueous methanol. A 5500 QTRAP hybrid triple quadrupole mass spectrometer (AB/SCIEX) coupled to a Prominence UFLC HPLC system (Shimadzu) was used for steady-state analyses of the samples. Selected reaction monitoring (SRM) of 287 polar metabolites using positive/negative switching with HILIC chromatography was performed. Peak areas from the total ion current for each metabolite SRM transition were integrated using the MultiQuant v2.1 software (AB/SCIEX). The resulting raw data from the MultiQuant software were analyzed using MetaboAnalyst (http://www.metaboanalyst.ca/MetaboAnalyst/).

Targeted Mass Spectrometry and data analyses (extended) (Dr. J. Asara's lab). 10 flies or 35 heads per sample (4 biological replicates) were collected under light CO_2 anesthesia, weighed, and rapidly snap frozen in liquid nitrogen. The intracellular metabolites were extracted using 1.6 mL of cold (-80°C) 80% (v/v) aqueous methanol. Flies were homogenized using 0.5 mm zirconium beads, and insoluble material in the lysates was centrifuged at 5,000*g* for 5 min. The resulting supernatant was evaporated using a speed vac. Samples were re-suspended using 20 µL HPLC grade water for mass spectrometry. 10 µL were injected and analyzed using a 5500 QTRAP triple quadrupole mass spectrometer (AB/SCIEX) coupled to a Prominence UFLC HPLC system (Shimadzu) via selected reaction monitoring (SRM) of a total of 287 endogenous water-soluble metabolites for steady-state analyses of the samples. Some metabolites were targeted in both the positive and negative ion mode for a total of 287 SRM transitions using positive/negative polarity switching. ESI voltage was +4900 V in the positive ion mode and –4500 V in the negative ion mode. The dwell time was 3 ms per SRM transition, and the total cycle time was 1.55 seconds. Approximately 10-14 data points were acquired per detected metabolite. Samples were delivered to the mass spectrometer via normal phase chromatography using a 4.6 mm i.d x 10 cm Amide Xbridge HILIC column (Waters Corp.) at 350 µL/min. Gradients were run starting from 85% buffer B (HPLC grade acetonitrile) to 42% B from 0-5 minutes; 42% B to 0% B from 5-16

minutes; 0% B was held from 16-24 minutes; 0% B to 85% B from 24-25 minutes; 85% B was held for 7 minutes to re-equilibrate the column. Buffer A was comprised of 20 mM ammonium hydroxide/20 mM ammonium acetate (pH=9.0) in 95:5 water:acetonitrile. Peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v2.0 software (AB/SCIEX).

The resulting raw data from the MultiQuant software were uploaded in metaboanalyst (http://www.metaboanalyst.ca/MetaboAnalyst/), and subsequent data processing and analyses were performed using this tool. Metabolites that were not detected in 40% of the samples were excluded from the analysis. Data were normalized to the median (per sample) and processed through log transformation. A heat map with hierarchical clustering was generated using Pearson correlations and Ward's method. Metabolite Set Enrichment Analysis (MSEA) was performed using Metaboanalyst, which uses the KEGG pathway database (http://www.genome.jp/kegg/pathway.html). Metabolite sets containing at least 5 compounds were employed in the analysis. MSEA calculates hypergeometrics test scores based on cumulative binominal distribution.

Metabolite profiling. (Dr. J. Rabinowitz's lab). 10 flies or 35 heads per sample were collected under light CO_2 anesthesia, weighted, rapidly snap frozen in liquid nitrogen, and the frozen tissues were ground by a Cyromill (Retsch, Newtown, PA). Intracellular metabolites were extracted with -20℃ 40:40:20 (methanol: acetonitrile: water) with 0.5% formic acid. Samples were vortexed before neutralizing with 8 ul of 15% ammonium bicarbonate per 100 ul of extraction solvent. The extract was centrifuged twice at 16,000Xg for 20 min at 4 ℃. The supernatant was transferred to LC-MS tubes for analysis. LC separation was on a XBridge BEH Amide column (2.1 mmm×150mm×2.5um particle size, Water, Milford, MA) using a gradient of solvent A (20 mM ammonium acetate, 20 mM ammonium hydroxide in 95:5 water:acetonitrile, pH 9.45) and solvent B (acetonitrile). LC gradient was: 0 min, 85% B; 2 min, 85% B; 3 min, 80% B; 5 min, 80% B; 6 min, 75%B; 8 min, 70% B; 9 min, 70% B: 10 min, 50% B; 12 min, 50% B; 13 min, 25% B; 16 min, 25% B; 18 min, 0%B; 23 min, 0%B; 24 min, 85% B (1). MS analysis was conducted on a quadrupole-orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific, San Jose, CA) in both positive and negative modes. Scans were performed from m/z 70 to 1000 at 1 Hz and a resolution of 140,000.

Food intake. DNA oligomers 1 and 2, designed by Park et al. (2), were mixed in ddH_2O to form a final concentration of 1.75 and 3.5 µg/µL, respectively. 90 uL of the oligomer solution were added to the center of each food vial to ensure a uniform spread. After the food was dry, flies separated by sex were transferred to the choice vials and kept overnight at 25°C. The flies were then collected under light CO_2 anesthesia, with 5 flies per sample. For the extraction procedure, squishing buffer was prepared using 10 mM Tris-buffered saline (Cell Signaling Technology), 25 mM NaCl, 1 mM EDTA, and 200 µg/mL Proteinase K (Thermo Fisher Scientific). 70 µL of squishing buffer was added to each sample, and the flies were homogenized using 0.5 mm zirconium beads. The samples were incubated for 40 min at 37°C for the digestion step, and were then incubated at 95°C for 5 min.

Climbing assay. Flies were tested for vertical climbing ability every week for 7 weeks. In this assay, vials with flies separated by sex and food condition were rapidly tapped three times and observed for 10 seconds. The percentage of flies that climbed the length of the vial during the observation interval was recorded. Consecutive trials were separated by 30 s of rest.

Smurf assay. The gut permeability of flies was measured using the Smurf assay described by (3). Fly food with RU486 or solvent was prepared with 2.5 % (wt/vol) of FD&C blue dye #1. Aged flies at 50 days were transferred to the vials and kept overnight at 25 °C. After 24 hours, female flies were sorted and counted under light CO_2 anesthesia according to Smurf status.

Egg laying. For egg laying behavior analysis, flies were collected within 24 hrs after eclosion, mated for 2 days, and transferred to fresh vials containing RU486 or solvent, with 2 females per

vial. Between 6 and 20 days after collection, the flies were transferred to fresh vials every 24 hours, and the number of eggs in each vial were counted and recorded.

Statistical analysis. Statistical analyses were performed in either JMP (SAS, Cary, NC, USA), metaboanalyst.ca, or Excel.

Supplemental Figures

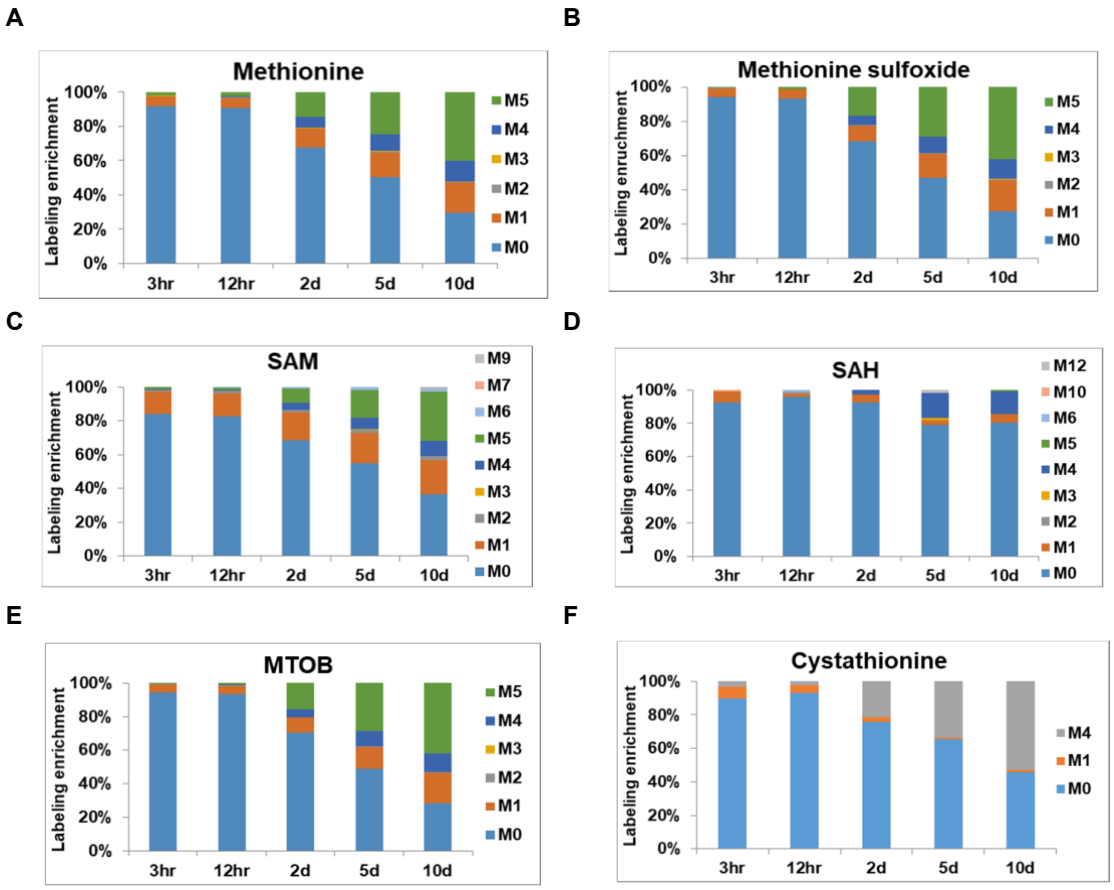

Fig. S1. **Gradual increase of labeled methionine and methionine metabolism metabolites over time.**
Relative labeling enrichment for methionine (A), methionine sulfoxide (B), SAM (C), SAH (D), MTOB (E), and cystathionine (F) in whole male flies fed with 1 mM of labeled ^{13}C5-methionine tracer in chemically-defined food (lacking endogenous methionine) for the indicated time. M0-M12 mark the number of labeled carbons. Labeling enrichment is the proportion of a particular labeled metabolite form relative to all measured isoforms. Note that although multiple isoforms of the same metabolite with different numbers of labeled carbons were measured, many of them are not present in *Drosophila* cells, and they are not displayed on the graph. 4 biological replicates / condition.

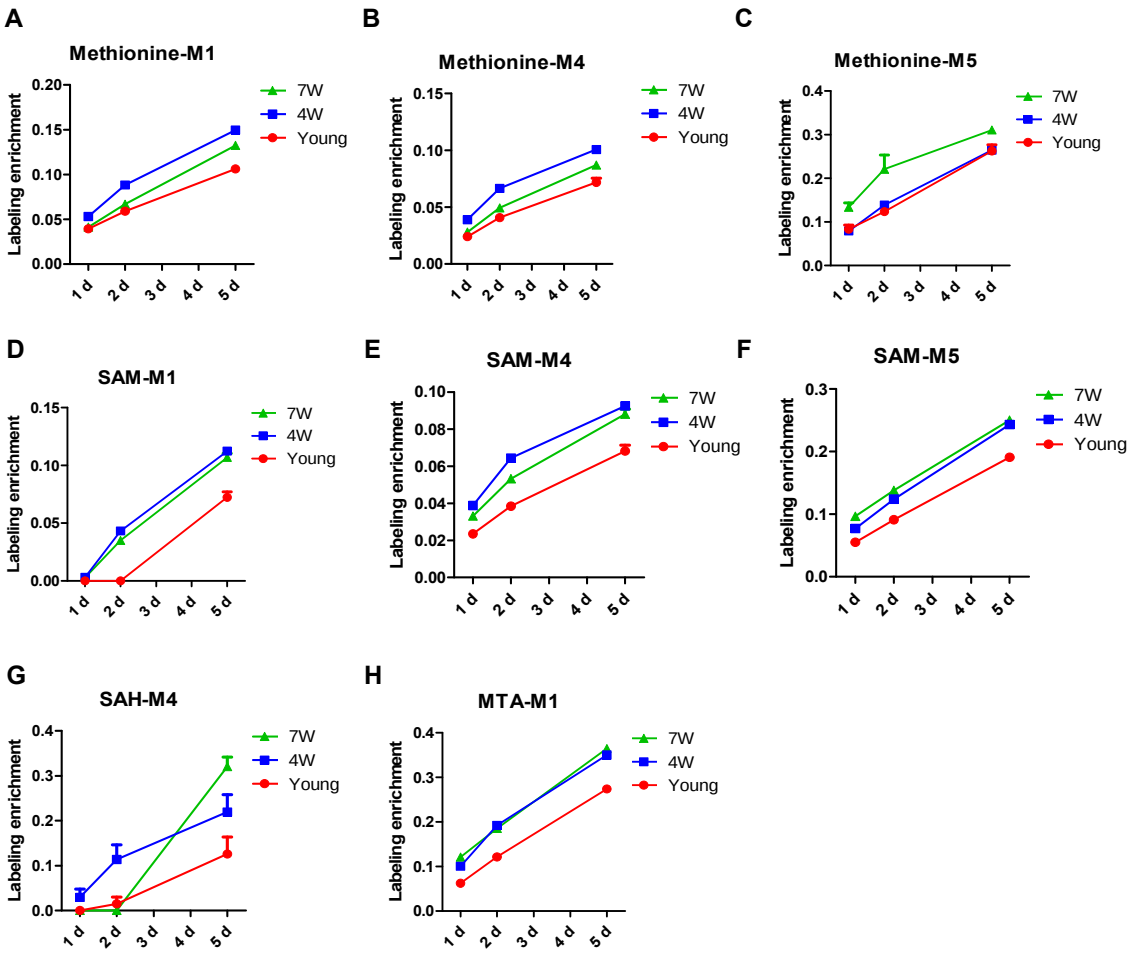

Fig. S2. **Age-dependent changes of the fate of labeled methionine.**
Time-dependent labeling enrichment for methionine-M1 (A), methionine-M4 (B), methionine-M5 (C), SAM-M1 (D), SAM-M4 (E), SAM-M5 (F), SAH-M4 (G), and MTA-M1 (H).

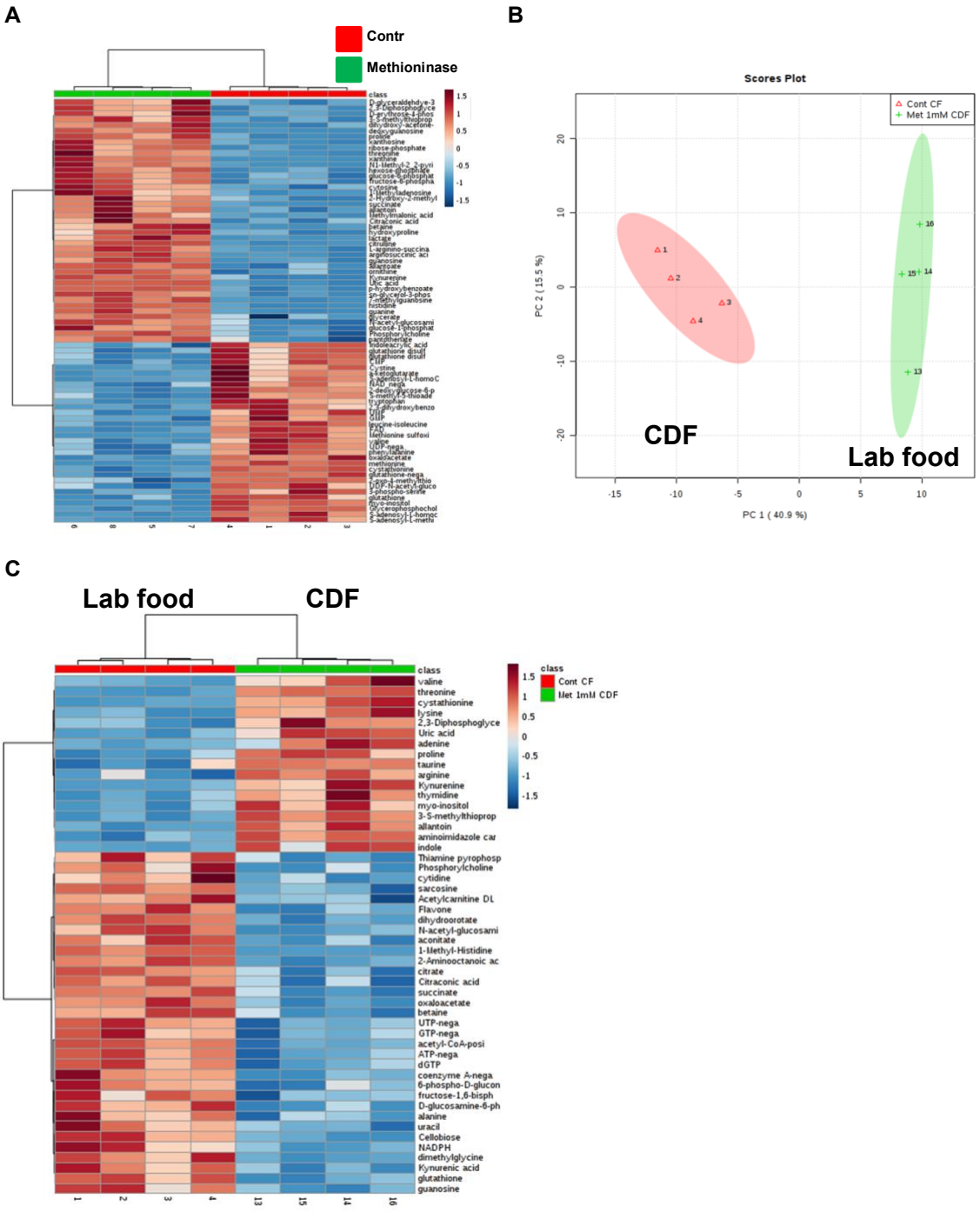

Fig. S3. **Dietary-dependent metabolic reprogramming**
(A) Heat map showing the significantly changed metabolites in *tubulin-Gal80ts, tubulin-Gal4>* control flies or flies expressing *Methioninase*. 4 biological replicates / condition.

(B) Principal component analysis and (C) heat maps of control flies maintained on standard lab food and chemically-defined food.

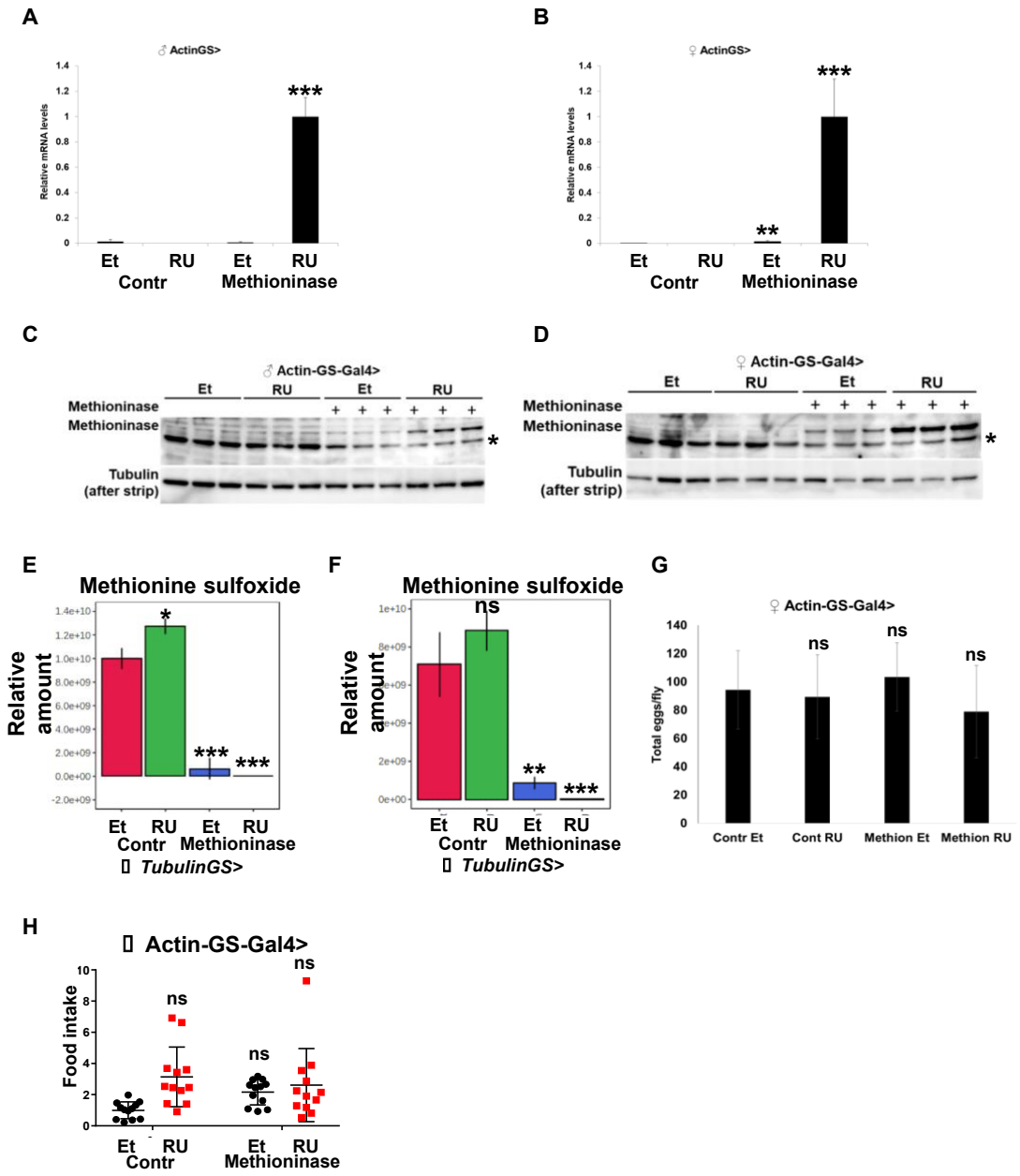

Fig. S4. **Low levels of Methioninase decrease levels of methionine and extend lifespan**
Relative mRNA levels of *Methioninase* in male (A) and female (B) *ActinGS>control* or *Methioninase* flies fed with either EtOH or RU486. Means ± SD. **p<0.01, ***p<0.001.

Immunoblot analysis of Methioninase and tubulin in male (C) and female (D) *ActinGS>control* or *Methioninase* flies fed with either EtOH or RU486. (in triplicates). * - marks nonspecific band.

Relative levels of methionine sulfoxide in male (E) and female (F) *TubulinGS>control* or *Methioninase* flies fed with either EtOH or RU486.

(G) Cumulative number of eggs in female *ActinGS>control* or *Methioninase* flies fed with either EtOH or RU486.

(H) Food uptake in female *ActinGS>control* or *Methioninase* flies fed with either EtOH or RU486.

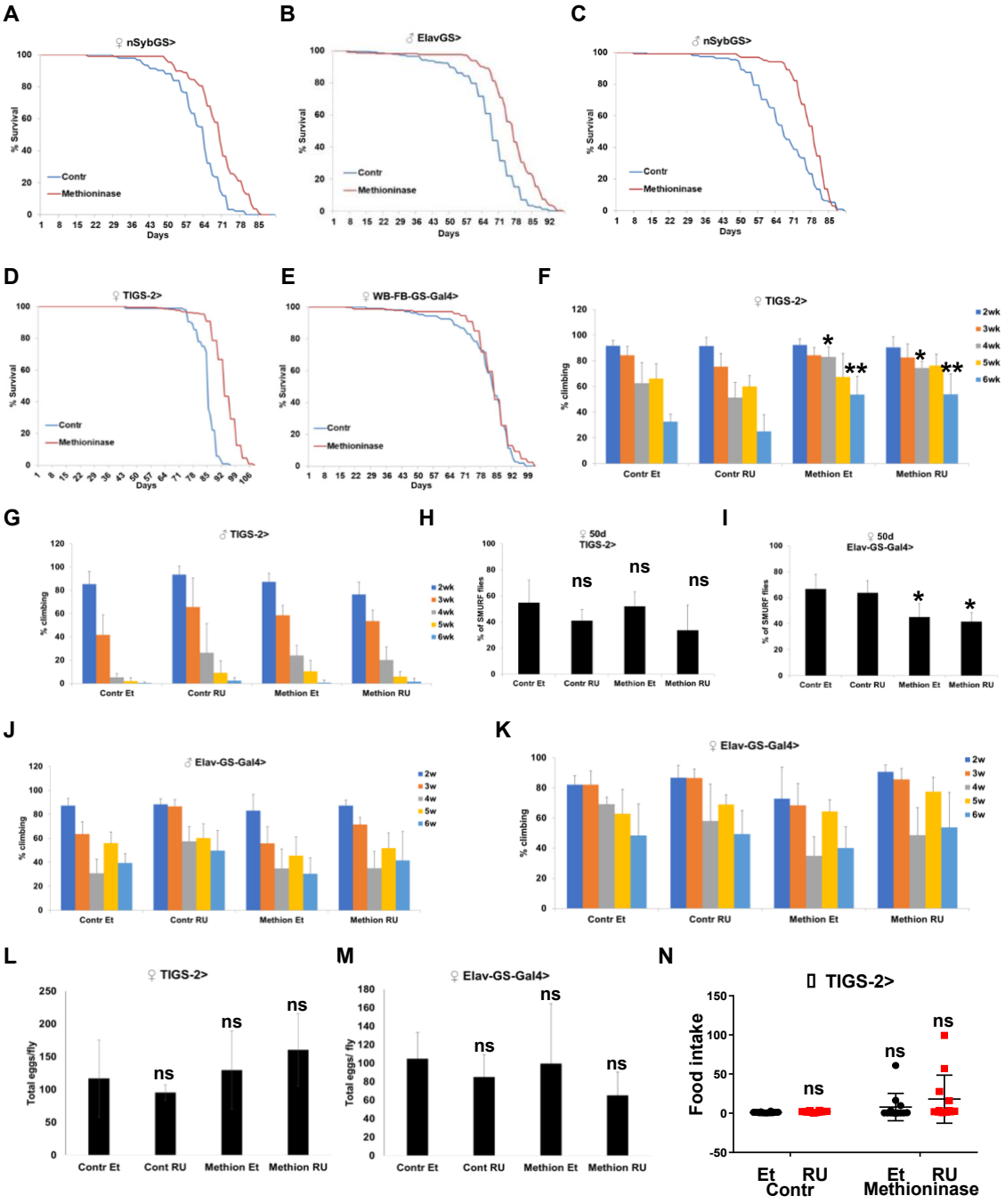

Fig. S5. **Tissue-specific *Methioninase* expression extends lifespan independently of the amino acids status in the food.**
(A) Neuronal-specific leaky expression of *Methioninase* in *nSybGS> Methioninase* female flies extends lifespan.

(B) Neuronal-specific leaky expression of *Methioninase* in *ElavGS> Methioninase* male flies extends lifespan.

(C) Neuronal-specific leaky expression of *Methioninase* in *nSybGS> Methioninase* male flies extends lifespan.

(D) Intestine-specific leaky expression of *Methioninase* in *TIGS-2> Methioninase f*emale flies extends lifespan.

(E) Fat body-specific leaky expression of *Methioninase* in *WB-FB-GS> Methioninase* female flies does not affect lifespan.

Climbing of female (F) and male (G) *TIGS-2>control* or *Methioninase* flies fed with either EtOH or RU486.

(H) Proportion of “smurf” females at 50 days of age in female *TIGS-2> control* or *Methioninase* flies fed with either EtOH or RU486.

(I) Proportion of “smurf” females at 50 days of age in female *Elav-GS> control* or *Methioninase* flies fed with either EtOH or RU486.

Climbing of male (J) and female (K) *ElavGS>control* or *Methioninase* flies fed with either EtOH or RU486.

(L) Cumulative number of eggs in female *TIGS-2>control* or *Methioninase* flies fed with either EtOH or RU486.

(M) Cumulative number of eggs in female *Elav-GS>control* or *Methioninase* flies fed with either EtOH or RU486.

(N) Food uptake Food consumption in male *TIGS-2>control* or *Methioninase* flies measured by qPCR

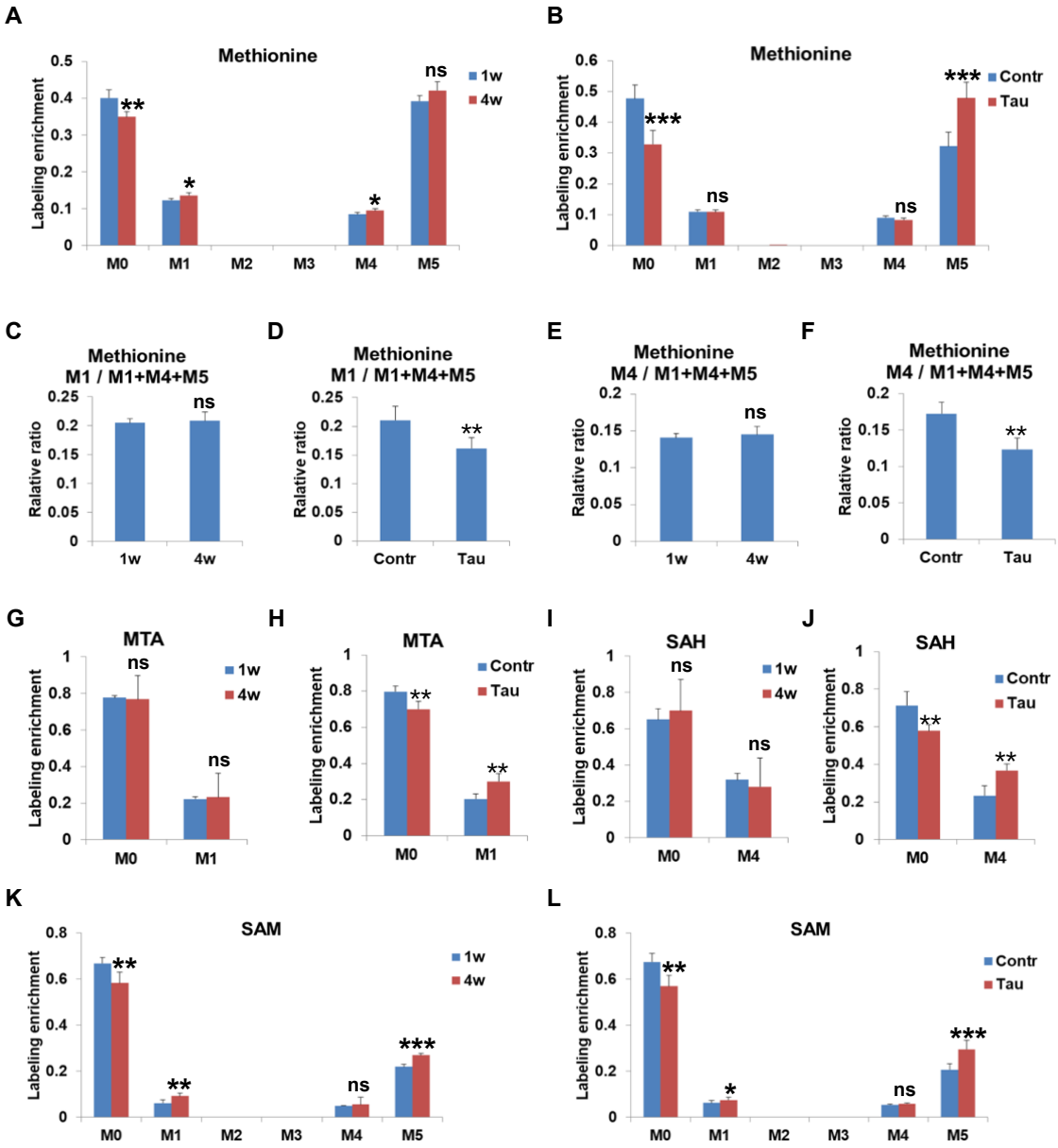

Fig. S6. Age-dependent and AD-relevant changes of the fate of labeled methionine in *Drosophila* heads.

M0-M12 marks the number of labeled carbons. Labeling enrichment is the proportion of a particular labeled metabolite form relative to all measured isoforms. Note that although multiple isoforms of the same metabolite with different numbers of labeled carbons were measured, many of them are not present in *Drosophila* cells, and they are not displayed on the graph. 5 biological replicates / condition.

Relative labeling enrichment for methionine in aged (A) and Tau-expressing (B) heads of male flies fed with 1 mM of labeled ^{13}C5-methionine tracer in chemically-defined food (lacking endogenous methionine) for 5 days. Means ± SD. *p<0.05, **p<0.01, ***p<0.001
The enrichment ratio (M1/M4+M5+M1) in aged (C) and Tau-expressing (D) heads of male flies between methionine (M1) produced from the salvage pathway and total labeled methionine

(M4+M5+M1) reflects the activity of the salvage (5'-methylthioadenosine (MTA) cycle) pathway. Means ± SD. **p<0.01

The enrichment ratio (M4/M4+M5+M1) in aged (E) and Tau-expressing (F) heads of male flies between methionine (M4) produced from the methionine cycle and total labeled methionine (M4+M5+M1) reflects the activity of the methionine cycle. Means ± SD. **p<0.01

Relative labeling enrichment for MTA (G, H), SAH (I, J), and SAM (K, L) in aged (G,I, K) and Tau-expressing (H, J, L) heads of male flies fed with 1 mM of labeled ^{13}C5-methionine tracer in chemically-defined food (lacking endogenous methionine) for 5 days. Means ± SD. *p<0.05, **p<0.01, ***p<0.001

Dataset S1 (Separate file). Lifespan counts
Lifespan counts for lifespan experiments

References:

1. L. Wang *et al.*, Peak Annotation and Verification Engine for Untargeted LC-MS Metabolomics. *Anal Chem* **91**, 1838-1846 (2019).
2. A. Park, T. Tran, N. S. Atkinson, Monitoring food preference in Drosophila by oligonucleotide tagging. *Proceedings of the National Academy of Sciences of the United States of America* **115**, 9020-9025 (2018).
3. M. Rera *et al.*, Modulation of longevity and tissue homeostasis by the Drosophila PGC-1 homolog. *Cell metabolism* **14**, 623-634 (2011).