**Overview**

Several collaborators including Vera Gorbunova’s group at U. of Rochester (see [The Gorbunova & Seluanov Laboratory – University of Rochester](http://www.sas.rochester.edu/bio/labs/Gorbunova/) ), Rich Miller’s group at U. of Michigan (see [Richard Miller Laboratory Home Page (richmillerlab.com)](https://www.richmillerlab.com/)), Vadim Gladyshev’s group at Harvard (see [Gladyshev Lab (harvard.edu)](https://gladyshevlab.bwh.harvard.edu/)), and Nik Schork’s group at TGen (see [Nicholas Schork (tgen.org)](https://www.tgen.org/faculty-profiles/nik-schork/)) are employing diverse “omics” approaches to identify/quantify molecular features from ~40 species of mammals and ~20 species of birds that correlate with health and longevity (maximum lifespan). These studies are also part of a larger effort to compare cross-species results with data from human centenarians led by Tom Perls group at Boston U. ([Thomas Perls | Chobanian & Avedisian School of Medicine (bu.edu)](https://www.bumc.bu.edu/camed/profile/thomas-perls/)) and Paola Sebastiani’s group at Tufts U. ([Paola Sebastiani, PhD | Tufts Medicine](https://www.tuftsmedicine.org/doctor/paola-sebastiani)) and others within the Integrative Longevity Omics Consortium (see <https://longevityomics.org/>).

**Methods**

***Mammalian species sample collection and tissue processing.*** All experiments were performed according to procedures approved by the University of Rochester Committee on Animal Resources (UCAR)***. Tissue processing.*** Tissues obtained from wild-caught animals were assumed to be of younger/middle age since predation normally precedes aging in the wild.Postmortem interval was minimized, and, in all cases, samples were kept on ice and frozen in less than 24h. At the earliest opportunity acter dissection, tissues from representative animals from each species were flash frozen in liquid nitrogen and stored at -80°C. Tissues were pulverized to a fine powder within a Biosafety cabinet under liquid nitrogen using a stainless-steel pulverizer Cell Crusher <https://www.fishersci.com/shop/products/cellcrusher-kit-with-tissue-pu/NC1824866>) chilled in liquid nitrogen and delivered to storage tubes with a scoop that had also been pre-chilled in liquid nitrogen and kept on dry ice. Similarly, when sampled for various “omics” processing, pulverized tissues were removed with a stainless-steel spatula that was pre-chilled in liquid nitrogen. Samples were never thawed after initial freezing until extractions were performed.

***Cross-species Transcriptomics.*** Two papers examining cross-species transcriptomics were recently published by ILO group members(Lu et al., 2022; Tyshkovskiy et al., 2023). Detailed methods related to cross-species transcriptomics have been published and are available at the publisher as STAR methods (<https://www.sciencedirect.com/science/article/pii/S1550413122001383#sec4>) and (https://www.cell.com/cell/fulltext/S0092-8674(23)00476-2?\_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS0092867423004762%3Fshowall%3Dtrue#secsectitle0095).

***Cross-species Proteomics.*** ***Tissue proteomics-*** We employed a “shotgun” style untargeted data-dependent acquisition (DDA) label-free quantitative (LFQ) approach. Approximately 5mg of tissue was mixed with 250µl of 50mM TEAB ph7.6; 5% SDS, mixed by pipetting, and briefly vortexed. Samples were sonicated in a chilled cup-horn Q800R3 Sonicator System (Qsonica; Newtown, CT) for a total of 15min at 30% output and duration of 30 x 30 sec pulses (with 30 sec in between pulses) at 6°C using a chilled circulating water bath. Samples were heated to 90°C for 2min and allowed to cool to room temperature (RT). Next, samples were centrifuged at 14,000xg for 10min to pellet insoluble debris and the supernatants were transferred to clean tubes. Total protein was quantified by the BCA assay and 100µg was reduced with 5mM dithiothreitol (DTT) for 30min at 60°C. Samples were cooled to RT and then alkylated with 10mM iodoacetamide (from a freshly prepared stock) for 30min at RT in the dark. Samples were processed using the standard S-trap mini column method (Protifi; Farmingdale, NY). Samples were digested with 4μg trypsin overnight at 37°C. Elution fractions were pooled and dried using a Speedvac (Labconco). Peptides were resuspended in 100μl MS-grade water (resistance ≥18MΩ) and quantified using the Pierce Quantitative Fluorometric Peptide Assay (Thermo). Common internal Retention Time standards (CiRT) peptide mix was added (50fmol mix/2µg tryptic peptides) and 2µg (in 4µL) of tryptic peptides were injected/analyzed by mass spectrometry (MS) on a Orbitrap Tribrid Fusion Lumos instrument (Thermo) equipped with an EASY-Spray HPLC Column (500mm x 75um 2um 100A P/N ES803A, Nano-Trap Pep Map C18 100A; Thermo). Buffer A was 0.1% formic acid and buffer B was 100% acetonitrile (ACN) with 0.1% FA. Flow rate was 300nl/min and runs were 150 min: 0-120 min, 5% B to 35% B; then from 120-120.5 min, 35-80% B; followed by a 9-minute 80% B wash until 130min. From 130-130.5min B was decreased to 5% and the column was re-equilibrated for the remaining 20-min at 5% B. the instrument was run in data dependent analysis (DDA) mode. MS2 fragmentation was with HCD (30% energy fixed) and dynamic exclusion was operative after a single time and lasted for 30sec. Additional instrument parameters may be found in the Thermo RAW files. ***Plasma proteomics*-** 10µl of plasma was added to 90µl of 5% SDS; 50mM TEAB pH 7.5 and then reduced with 5mM DTT for 30min at 60°C. Samples were cooled to RT and then alkylated with 10mM iodoacetamide (from a freshly prepared stock) for 30min in the dark. Samples were processed using the standard S-trap mini column method (Protifi; Farmingdale, NY) and MS like the processing of tissues.

***Computational-*** Raw files were analyzed directly with the MSFragger/Philosopher pipeline (da Veiga Leprevost et al., 2020; Kong et al., 2017) and included Peptide and Protein Prophet modules (Ma et al., 2012) for additional quality control. Quantitation at the level of MS1 was performed with the “LFQ-MBR; label-free quant match-between-runs” workflow using default parameters. This allows for alignment of chromatographic peaks between separate runs. Methionine oxidation and N-terminal acetylation were set as variable modifications. MaxLFQ with a minimum of two ions was implemented and normalization of intensity across runs was selected (Yu et al., 2021). Proteome databases are available upon request. Databases were translations of de novo assembled pan-tissue RNA libraries constructed with the Trinity pipeline (Grabherr et al., 2011; Haas et al., 2013). Most of the Trinity-derived libraries used were published in Yu et al. (Yu et al., 2020) and newer ones will be made available. When possible, publicly available proteome databases were also used for comparison. Search and refinement iterations converged in their stringency to keep the FDR below 1% for all peptide assignments. CiRT peptides (Escher et al., 2012) were also included since they were spiked into all samples and to enable an assessment of consistency of retention times (RTs) and peak alignments between runs. Species-specific proteins were converted to their nearest human homologs determined by BLAST as previously described (Lu *et al.*, 2022) ) and added to the protein-level quant output file (from MSFragger) using Excel. Subsequently, human annotations were employed for cross-species comparisons. Microsoft Excel, R/R studio (RCoreTeam, 2021), and Perseus (Tyanova et al., 2016) were used for downstream processing and statistical analyses of the output results files/matrices from MSFragger. Proteins detected in fewer than 15 samples were excluded. Hierarchical clustering (based on Euclidean distance or Spearman Correlation) and Principal Component Analysis (PCA) are used to indicate agreement between biological replicates and distance between species. Reproducibility is also assessed by the coefficient of variation (standard deviation divided by the mean) of each protein of each biological replicate. Given that body mass (BM) correlates closely with MLS (de Magalhaes et al., 2007), we built a linear model using BM to predict the MLS and used the residuals as BM-corrected MLS to identify proteins associated with MLS independent of BM. A log file from a LFQ-MBR workflow is available.

***Enrichment and proteomic analysis of Extracellular Vesicles (EVs) from plasma/serum***. EVs were enriched from plasma or serum with MagReSyn Strong Anion Exchange (SAX) beads (<https://allumiqs.com/shop/magresyn-sax/>) using a protocol from the MacCoss lab (Heil et al., 2023) with only minor modifications. A detailed protocol is also available at ReSyn Biosciences (https://resynbio.com/wp-content/uploads/2023/09/Mag-Net-EV-Manual-Enrichment-Ver4.pdf). Plasma samples were from our in-house animal colonies or purchased from Innovative Research, Inc. (Novi, MI; https://www.innov-research.com/) or Lake Immunogenics (Ontario, NY; https://www.lakeimmunogenics.com/). For EV enrichment, 100µl of each plasma sample (or serum in the case of bowhead whale samples) was mixed with 100µl of 2x EV binding buffer (100mM Bis Tris Propane pH6.3, 150mM NaCl with cOmpleteTM EDTA free protease inhibiter (Roche/Millipore-Sigma) added at 1 tablet /10ml). Next 50µl of pre-equilibrated SAX beads were added and mixed by gentle inversion for 30min at 4°C to facilitate binding of the EVs to the beads. Next, samples were pulled down using a magnetic separator and processed according to the published protocol. After reduction and alkylation, proteins were precipitated with 1ml of 95% ACN and gently washed without mixing three additional times with 95% ACN. Next, samples were washed three times consecutively with 70% EtOH. Peptides were digested with 6µg of MS-grade trypsin (Thermo) in 200µl of 50mM TEAB overnight at 37°C. Digestions were quenched by adding formic acid (FA) to 0.5% final. After separation of free peptides in solution from the beads using the magnet, the beads were washed with an additional 250µl of 50mM TEAB. Next, the peptide supernatants were pooled, frozen by placing in dry ice and solvent was evaporated using a using a Speedvac (LabConco). Peptides were resuspended in 100µl of MS-grade water and quantified using the Pierce Quantitative Fluorometric Peptide Assay (Thermo). Prior to injection, samples were adjusted to 5% ACN; 0.2% FA. Common internal Retention Time standards (CiRT) peptide mix (Escher *et al.*, 2012) was added (50fmol/2ug) and 2µg of tryptic peptides (in 4µL) were injected/analyzed by mass spectrometry (MS) on a Orbitrap Tribrid Fusion Lumos instrument or Astral (Thermo) equipped with an EASY-Spray HPLC Column (500mm x 75um 2um 100A P/N ES803A, Nano-Trap Pep Map C18 100A; Thermo). Samples were run in DIA mode.

***Computational-*** Raw data files were converted to mzML files using ProteoWizard with peak picking set to 1-n, and demultiplexing selected (Adusumilli and Mallick, 2017; Chambers et al., 2012). The mzML files were searched using DIANN (Demichev et al., 2020) with along with FASTA file databases described above for tissue extracts using library-free search/library generation with deep learning-based spectra retention time (RT) and IMs prediction criteria selected. Mass accuracy was set to 20 and MS1 accuracy set to 5.0, and oxidized methionine (Ox(M)) was also selected. Nearest human homologs for each species’ protein (determined by BLAST as previously described (Lu *et al.*, 2022)) were added to the protein group matrix (DIANN output). As before, the human annotation was used to facilitate cross-species comparisons. A large matrix containing all human homologs and relative protein-level quantitation is then exported to R and/or Perseus (Tyanova *et al.*, 2016) for downstream statistical analyses as described above. A log file from DIANN analysis of DIA data is available.

***Analysis of Extracellular Vesicles (EVs) from plasma/serum by Nanoparticle Tracking Analysis (NTA).*** We measured the size and concentration of extracellular vesicles in plasma (or serum in a few cases) by nanoparticle tracking analysis (NTA) with the Nanosight NS300 instrument (Malvern Analytical). Plasma was thawed on ice and diluted 10-3 (2µL into 2µL) into phosphate buffer saline pH 7.0 (tissue culture grade) and directly measured by light scattering. Each sample was recorded for five thirty second increments and averaged (and the sample was advanced for each increment). Mean (and mode) diameters as well as the number of vesicles per mL were recorded.

***Cross-species Metabolomics/Lipidomics***. ***For tissues-*** Untargeted semi-quantitative metabolomics/Lipidomics analyses were performed. We followed a single methanol/ tert-Butyl methyl ether (MeOH/MTBE) extraction protocol that is generally accepted as a robust, non-toxic method (compared to methods that employ chloroform) to extract both water-soluble and hydrophobic/lipid-like metabolites from tissues (Cajka and Fiehn, 2014; Zukunft et al., 2018) and was recently employed by the Fiehn group to analyze the aging mouse brain (Ding et al., 2021). Approximately 10-20mg of pulverized tissues that were stored at -80°C were added to 2.0ml Reinforced Bead Mill Tubes (VWR cat# 10158-556) containing zirconium ceramic oxide 1.4mm beads (Fisher Scientific cat# 15-340-159) on dry ice to avoid thawing. Samples were weighed (after wiping to remove condensation) and subtracted from the weight of the tubes/beads that had been previously recorded. Ice-cold, oxygen-depleted (with nitrogen gas) LC-MS grade MeOH was added (225ul per 5mg equivalent) to each tube, vortexed for 10sec and immediately placed on ice. Next, extraction was facilitated by homogenization with a Bertin Evolution PreCellys instrument equipped with a CryoLys (<https://www.bertin-technologies.com/product/sample-preparation-homogenizers/cryolys-evolution/>) by four 15sec pulses at 5800 intensity separated by 30sec intervals without pulsing. Following bead beating, samples were kept on ice for at least 15min. Then, samples were briefly vortexed to promote a homogeneous extract and 225µl was placed in a new 1.5ml low-bind tube (<https://www.fishersci.com/shop/products/thermo-scientific-low-protein-binding-collection-tubes-1-5-ml/p-4937563>). Next, 750ul of MTBE containing 5ul of UltimateSPLASHTM One deuterated internal standard (Avanti Polar Lipids; <https://avantilipids.com/product/330820>) was added to each tube and they were vortexed for 5min. These standards contain a mixture of deuterated lipids from each class permitting normalization of the samples in MSDIAL later. Next, 188ul of MS-grade water was added and samples were vortexed again for 5min and then centrifuged at 14,000rpm for 2min. For the analysis of hydrophobic molecules/lipids, 350µl of the upper organic (MTBE) layer was evaporated to dryness in a 96-Deepwell Protein LoBind plate (Eppendorf cat # 951033308) under a steady stream of nitrogen gas in a microplate evaporator (Organomation; <https://www.organomation.com/products/nitrogen-evaporators/microvap/microplate-evaporator>) to avoid oxidation as much as possible. Plates containing dried samples were covered with parafilm and stored at -20°C until just prior to available times for MS analysis (often within a day and no more than a week). Just prior to injection, MTBE/upper samples were resuspended in 60µl of MeOH/toluene (9:1, v/v) with additional [12-[(cyclohexylamine) carbonyl]amino]-dodecanoic acid (CUDA) (SIGMA) as an additional spiked in standard permitting normalization. Samples were transferred to amber glass autosampler vials with inserts (<https://www.ibisscientific.com/ibis-scientific-9mm-300-l-amber-screw-top-fused-insert-vial-100-pk/>). Next, 3µl was injected and LC/MS was performed with a Waters ACQUITY UPLC CSH C18 Column (130Å, 1.7 µm, 2.1 mm X 100 mm) on a Exploris 240 instrument (Thermo) and data were collected in data-dependent fashion in centroid mode. For the analysis of aqueous/polar molecules by Hydrophilic Interaction Liquid Chromatography (HILIC), 125µl of the bottom portion of the MeOH/MTBE extract was evaporated to dryness and stored similarly. Next, samples were resuspended in 100µl of acetonitrile/water (4:1, v/v), containing additional metabolite standards (d3-DL-Aspartic acid, d5-L-Glutamine, d3-Creatinine, Val-Tyr-Val tripeptide, and CUDA allowing normalization of the runs; stable isotope standards were purchased from Cambridge Isotopes) and added to brown glass autosampler vials indicated above. Next, 3µl was injected and LC/MS was performed with a Waters ACQUITY UPLC BEH Amide Column (130Å, 1.7 µm, 2.1 mm X 150mm) on a Exploris 240 instrument (Thermo) and data were collected in data-dependent fashion in centroid mode. ***For Plasma-***We performed separate extractions for hydrophilic molecules and lipids according to Barupal et al. (Barupal et al., 2019). For the analysis of aqueous/polar molecules by HILIC, 10µL of plasma was mixed with 1mL of ACN:IPA:H2O (3:3:2, v/v/v). Samples were vortexed for 5min, kept on ice for 15min, and then centrifuged for 2min at 14000 rcf. Next, 450µL was dried in the microplate nitrogen evaporator and subsequently resuspended in 100µl of ACN:H2O (80:20, v:v) containing metabolite standards (d3-DL-Aspartic acid, d5-L-Glutamine, d3-Creatinine, Val-Tyr-Val tripeptide, and CUDA- purchased from Cambridge Isotopes or Sigma) and added to brown glass autosampler vials indicated above. Next, 3µl was injected and LC/MS was performed with a Waters ACQUITY UPLC BEH Amide Column (130Å, 1.7 µm, 2.1 mm X 150mm) on a Exploris 240 instrument (Thermo) and data were collected in data-dependent fashion in centroid mode. For Lipidomics, 10µL of plasma was mixed with ice cold methanol that had been saturated with nitrogen gas and contained 10µl of SPLASH® LIPIDOMIX® Mass Spec Standard for analysis of human plasma from Avanti Polar Lipids (see: [SPLASH® LIPIDOMIX® Mass Spec Standard (avantilipids.com)](https://avantilipids.com/product/330707)). After vortexing briefly, 750uL of MTBE was added and samples were vortexed briefly again. Next, 188 µL MS-grade water was added and samples were vortexed for 5min and centrifuged for 2 min at 14000 rcf. Next 350ul of the upper MTBE layer was dried in the microplate nitrogen evaporator and subsequently resuspended in 60µl of MeOH/toluene (9:1, v/v) containing CUDA. Vials were also prepared that lack samples but contained internal standards and these were run after every ten samples to control for chromatographic drift and normalization of retention times. Blank vials were also prepared that lack samples and standards to control for contaminants in solvents and plastic/vials. The ion intensities of Blanks were subtracted from all samples in MSDIAL. At least three (except in a few rare cases) and as many as six independent biological replicates were assayed. HILIC-LC/MS and CSH-C18-LC/MS was performed as described above for tissues.

***Computational-*** Thermo RAW files from the Exploris 240 were converted to mzML files with ProteoWizard and subsequently to abf files using the ABF converter (<https://www.reifycs.com/abfconverter/>). We employed MSDIAL (Tsugawa et al., 2015) for data deconvolution, peak assignments (including adducts) and quantitation by peak height in conjunction with its internal Lipidome Atlas (Tsugawa et al., 2020) and LipidBlast (Shen et al., 2023) and also external spectral libraries such as ESI(-/+)-MS/MS from standards+bio+in silico (and other library/msp files) available at Riken (<http://prime.psc.riken.jp/compms/msdial/main.html#MSP>). Samples were normalized by internal standards including CUDA. The exported peak height quantitation matrix file from MSDIAL was processed further with the Mass Spectral Feature List Optimizer (MS-FLO) tool available at the Fiehn Lab (see <https://msflo.fiehnlab.ucdavis.edu/#/)using> default parameters by simply submitting the exported peak height files from MSDIAL. MS-FLO reduces the number of features by revealing redundancies, thereby improving downstream statistical analyses (DeFelice et al., 2017). The MS-FLO cleaned matrix was imported into Perseus (Tyanova *et al.*, 2016) and/or R for downstream statistical analyses. After adding categorical (sample groups, i.e. where all replicates from each species represents a class) and numerical (maximal lifespan, adult weight/BM, female sexual maturity for each respective species/class) annotations, the total ion intensities of features were log2(x) transformed and the matrix was filtered for valid values. Traits for categorical annotations such as maximal lifespan, adult weight/BM, and time to female sexual maturity were obtained from the AnAge Database (de Magalhaes et al., 2023; de Magalhaes and Costa, 2009; Stuart et al., 2013) and also used as log2(x) transformed values. A matrix with at least 80% total valid values was used for downstream analyses. Missing values were replaced by artificial values 1.8 below the normal distribution (the default for imputation by Perseus). Hierarchical clustering (based on Euclidean distance or Spearman Correlation) and Principal Component Analysis (PCA) were used to indicate agreement between biological replicates and distance between species. Reproducibility was also assessed by the coefficient of variation (standard deviation divided by the mean) of each feature of each biological replicate. In cases where the total score for a library match by MSDIAL was >0.9, the annotation was accepted. In these cases, Total Scores (in MSDIAL) indicated a high level of confidence and these features segregated from other metabolites/lipids that remained unknown. We did not discard any feature since unknowns can still be quantified accurately and correlated with MLS. We kept track of unknown features of interest by their alignment ID from a particular MSDIAL run as well as their ID assigned by MS-FLO that contains their relative retention time and accurate mass. In addition, we also kept track of any MS2 data collected that can aid in identification of a feature using other methods. In cases of unknowns that show a high correlation with MLS, we exported msp and mgf files for the MS2 spectra to search with other software including Sirius (Duhrkop et al., 2019; Ludwig et al., 2020), MS-FINDER (Fraisier-Vannier et al., 2020; Tsugawa et al., 2017; Tsugawa et al., 2016), and NIST MS-search software with the most current NIST database (see: [chemdata:nistlibs []](https://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:nistlibs)). When possible, we have begun implementing the five levels of confidence routinely employed by the Compound Identification working group of the Metabolomics Society and discussed by the Metabolomics Standards Initiative (Blazenovic et al., 2018; Blazenovic et al., 2017) which consider parameters such as accurate mass, retention time and MS2 profiles (both matching peaks and their relative intensities). Identification of unknowns is expected to extend beyond the current funding period and is currently a lower priority. Yet, if identified, they have the potential to reveal novel aspects of cross-species biology that may impact MLS. In order to facilitate additional downstream informatics with the metabolites/lipids of interest that show a high degree of confidence in their annotation, we employed the Chemical Translation Service available through the Fiehn Lab (http://cts.fiehnlab.ucdavis.edu/ ) to convert compound identifiers, including CAS, CHEBI, compound formulas, Human Metabolome Database HMDB, InChI, InChIKey, IUPAC name, KEGG compound ID, LipidMaps, PubChem CID+SID, SMILES and chemical synonym names to facilitate pathway/other analyses. For lipids, we employed the Lipid Ontology Enrichment Analysis tool (see http://lipidontology.com/) for lipidomic data (Molenaar et al., 2023; Molenaar et al., 2019) to reveal properties of lipids that were correlated with MLS. We also employed ChemRich (Barupal and Fiehn, 2017) to reveal chemical similarities between molecules of interest and MetaboAnalyst tools for additional analyses of molecules of interest (Howell and Yaros, 2023; Pang et al., 2021; Pang et al., 2022). While log files are not available from MSDIAL, we used default parameters except for a few things that are shown in the screen captures below. For example, a typical analysis starts by defining POS/NEG ion mode, data collected in centroid mode, and CID/HCD for collision type for MS2. Next samples are uploaded and class settings such as species or BLANK or standards alone are specified. Then default values for mass tolerances. The minimum peak height is set to 100, 000 routinely since this is where the signal of the Exploris240 generally becomes less reliable and the default linear weighted moving average is employed for peak detection. Next, we generally accept all adducts that add or remove hydrogen, water, sodium, or potassium and any combination thereof as possibilities. Databases may be inserted as I indicated above, or in the case of lipids, we begin with the internal database (shown in the screen capture below). Lastly, for alignment parameters, I use one of the internal standards 9alone) runs that is approximately mid-way between all for the runs as a reference to align all the runs since the standards are present in all of the samples. I also subtract the intensities of features in blanks, require a minimum of 5-fold above background (samples/blank), and keep features without MS2 also. If any features without MS2 collected show high correlation with MLS, then we can include those on an inclusion list to be sampled in duplicate runs in a targeted fashion. After the alignment is generated, we can normalize based on features such as CUDA or the Splash lipidomix in the case of lipids (shown in the screen capture below). MSDIAL allows us to export the peak height and area quantitative alignment matrices with and without normalization in case we wish to normalize later in R, Perseus, or MetaboAnalyst based on mean or quantile.

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