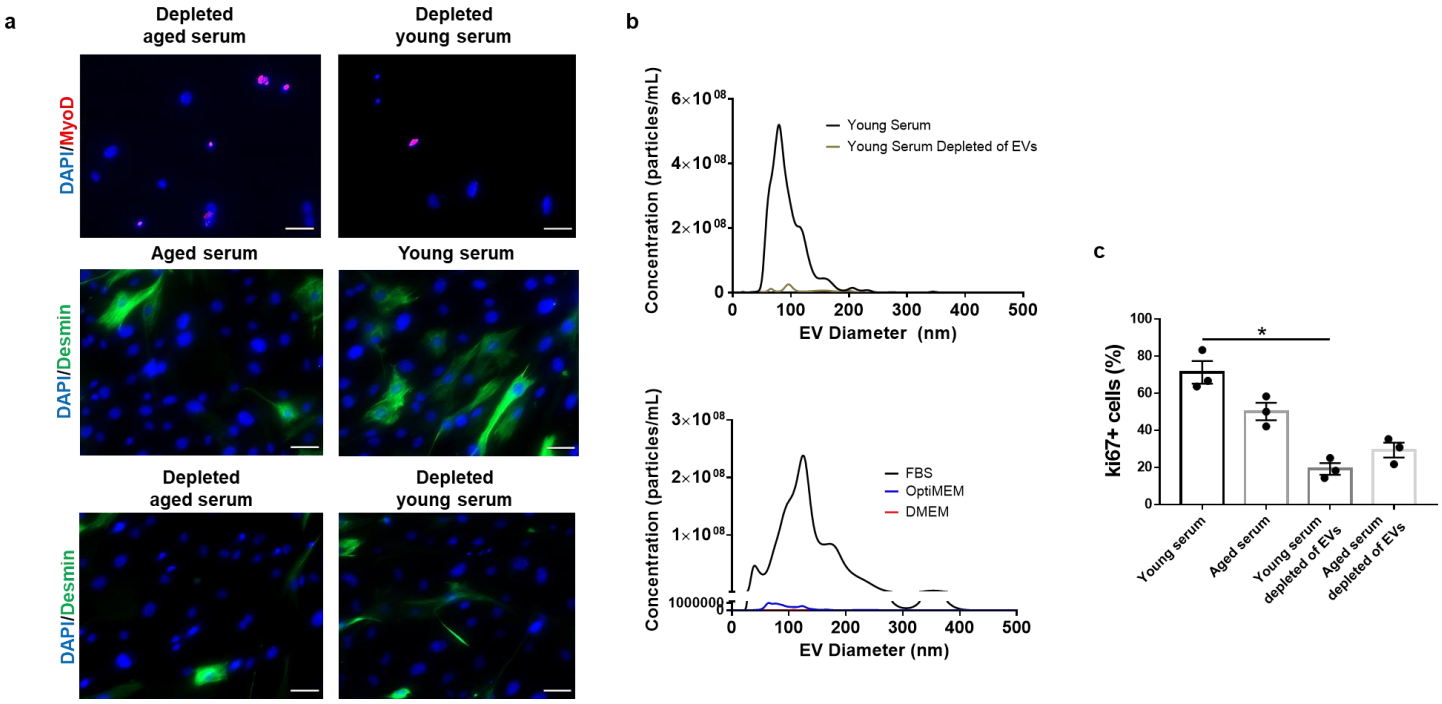
Regulation of aged skeletal muscle regeneration by circulating extracellular vesicles

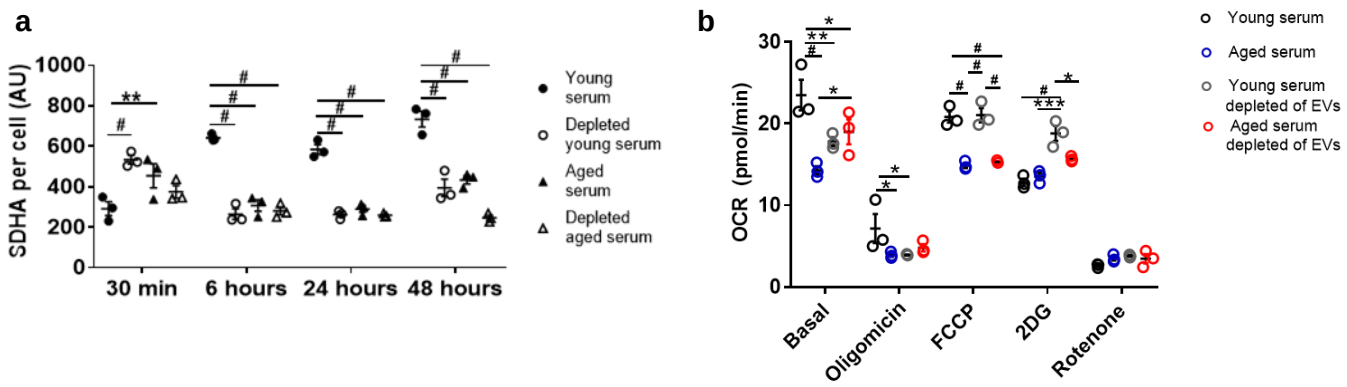
Amrita Sahu[1,2], Zachary J. Clemens[2], Sunita N. Shinde[1], Sruthi Sivakumar[1,3], Abish Pius[1,4], Ankit Bhatia[5], Silvia Picciolini[6], Cristiano Carlomagno[6], Alice Gualerzi[6], Marzia Bedoni[6], Bennett Van Houten[7], Mita Lovalekar[8], Nicholas F. Fitz[2], Iliya Lefterov[2], Aaron Barchowsky[2, 7], Radosveta Koldamova[2], *Fabrisia Ambrosio[1, 2, 3, 9]

**Corresponding author*

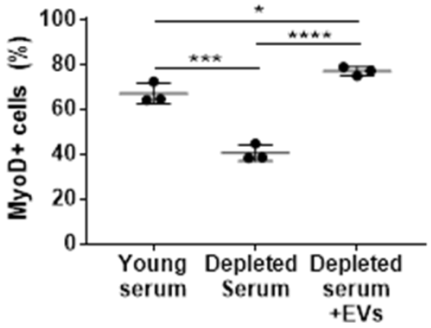
SUPPLEMENTARY FIGURES



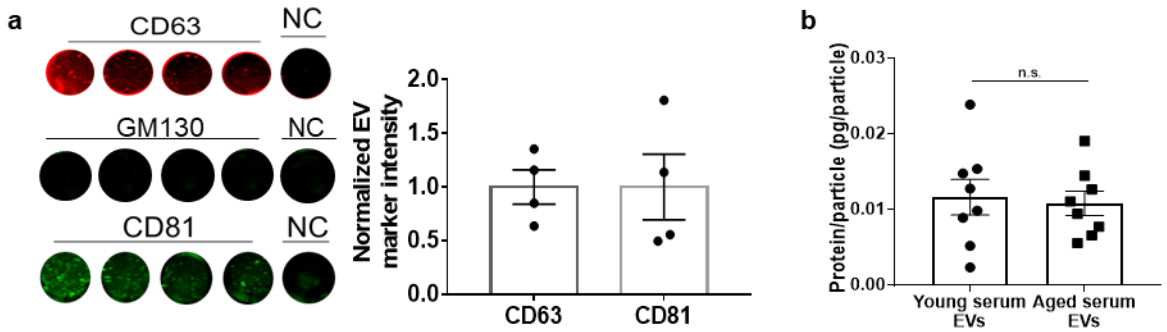
<u>Supplementary Figure 1.</u> Effect of EV depletion on myogenicity and cellular proliferation. a, Representative images of MyoD and Desmin expression in aged MPCs when treated with aged or young serum depleted of EVs. Scale: 50 µm. **b,** NTA plot of young serum vs. EV-depleted young serum (left) and fetal bovine serum (FBS), and FBS-free media (OptiMEM) and DMEM (right). **c,** Quantification of ki67+(%) aged MPCs in response to young serum, aged serum, or EV-depleted young or aged serum (*p<0.05, one-way ANOVA with Tukey's comparison, n=3 wells/group). Data presented as mean + SEM.



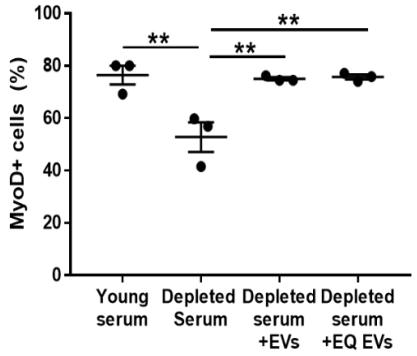
Supplemental Figure 2. Serum treatment increased target cell mitochondrial health in an age-and EV-dependent manner a, Quantification of SDHA in aged MPCs receiving serum treatments with or without EVs at timepoints: 30 minutes, 6 hours, 24 hours, and 48 hours. (**p<0.01, #p<0.0001, two-way ANOVA with Dunn's multiple comparisons to young serum as control). **b,** Fibro-adipogenic progenitor cells (FAPs) were exposed to young or aged serum with/without EVs for 48 hours, and bioenergetics was assessed using Seahorse XFe96 analyzer. Eight wells were evaluated for bioenergetics per cell type and treatment group. Oxygen Consumption Rate (OCR) is represented as the average of the three time points for Basal, Oligomicin treatment, FCCP treatment, 2DG treatment and Rotenone treatment ±SEM (8 wells/group, data points represent n=3 time points, *p≤0.05, **p<0.01, ***p<0.001, #p<0.0001; two-way ANOVA with repeated measures and Tukey's multiple comparisons). Data presented as mean + SEM.



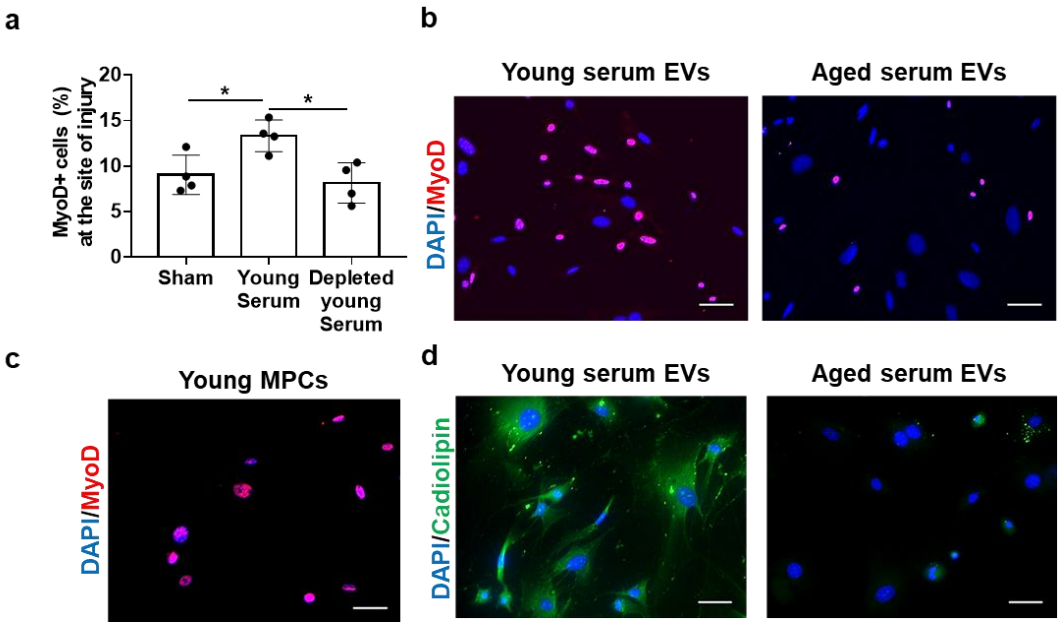
Supplemental Figure 3. Cells treated with young serum depleted of EVs display decreased MyoD expression, but the effect is reversed when serum is repleted with isolated EVs. Removal of EVs from young serum significantly reduced the MyoD expression of aged myogenic progenitor cells. However, supplementation of the EV-depleted serum with purified column-derived EVs enhanced MyoD expression of target muscle cells. (*p<0.05, ***p<0.001, ****p<0.0001, n=3 wells/group, one-way ANOVA with Tukey's multiple comparison). Data presented as mean + SEM.



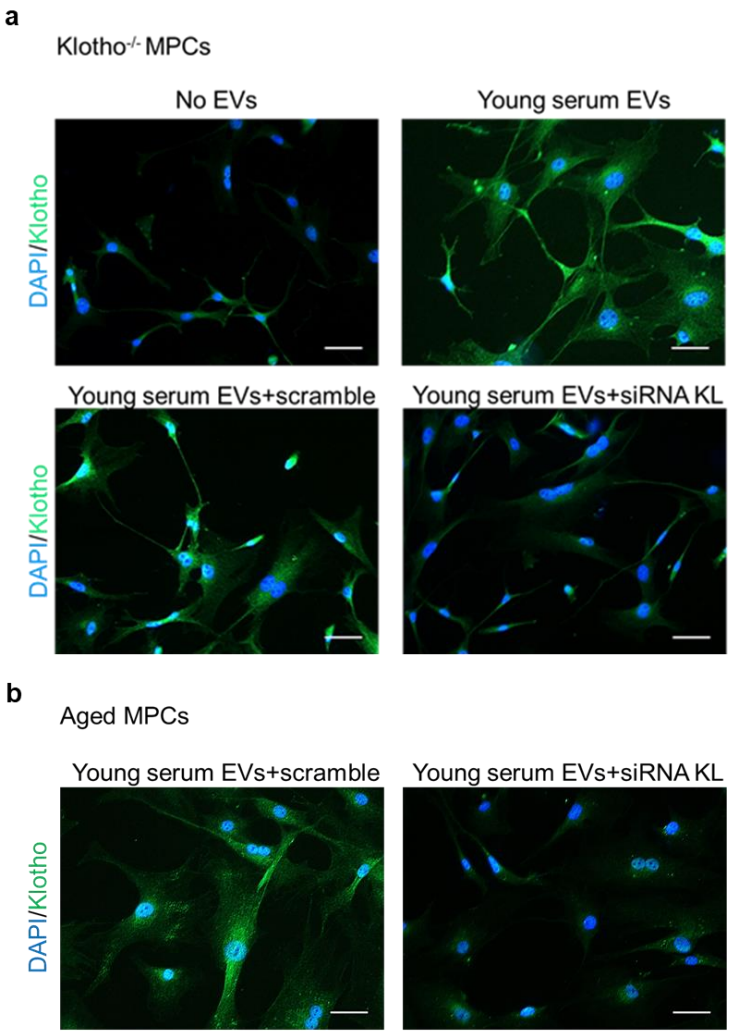
Supplemental Figure 4. Characterization and protein quantification of circulating EVs in young and aged serum. a, In-well immunofluorescence western analysis of young serum EVs reveals the presence of EV surface markers, CD63 and CD81, and absence of cytosolic contaminant protein, GM130. CD63 and CD81 intensity scores were divided by final EV concentrations in the wells and normalized to average intensity of surface markers of young serum EVs. (NC=negative control). **b,** Bicinchoninic acid assay was used to quantify the protein content per EV ($p>0.05$, two-tailed Student's t test with Welch's correction). Data presented as mean + SEM.



Supplemental Figure 5. The decreased MyoD expression in cells treated with young EV-depleted serum is restored when serum is repleted with either isolated EVs (Depleted serum+ EVs) or Exoquick elute (Depleted serum+EQ EVs). Removal of EVs from young serum significantly reduced the MyoD expression of aged myogenic progenitor cells. However, supplementation of the EV-depleted serum with purified column-derived EVs or Exo-quick precipitated EVs equally enhanced MyoD expression of target muscle cells. (**$p<0.01$, n=3 wells/group, one-way ANOVA with Tukey's multiple comparison). Data presented as mean + SEM.

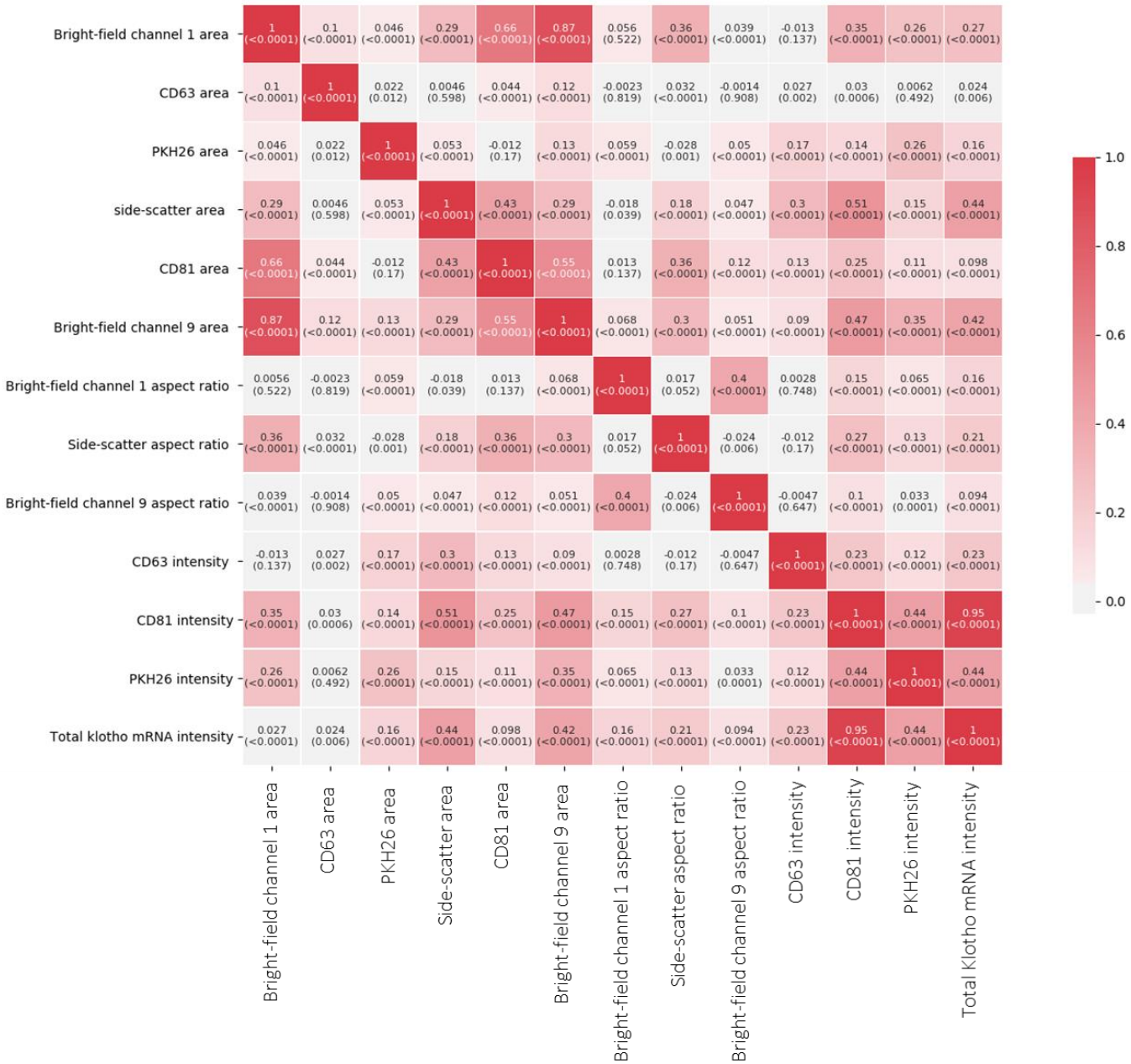


<u>Supplemental Figure 6.</u> MyoD expression and cardiolipin content in aged muscle cells is increased in the presence of young serum in an EV-dependent manner. a, Quantification of MyoD *in vivo* three days post injury in aged animals receiving sham, young serum, or EV-depleted young serum treatment. (*p<0.05, one-way ANOVA with Tukey's multiple comparisons, n=4/group). **b,** Representative images of MyoD in aged MPCs treated with young or aged serum EVs. Scale: 50 µm.**c,** Representative image of MyoD in Young MPCs. Scale: 50 µm. **d,** Representative images of cardiolipin content in aged MPCs treated with young or aged serum EVs. Scale: 50 µm. Data presented as mean + SEM.

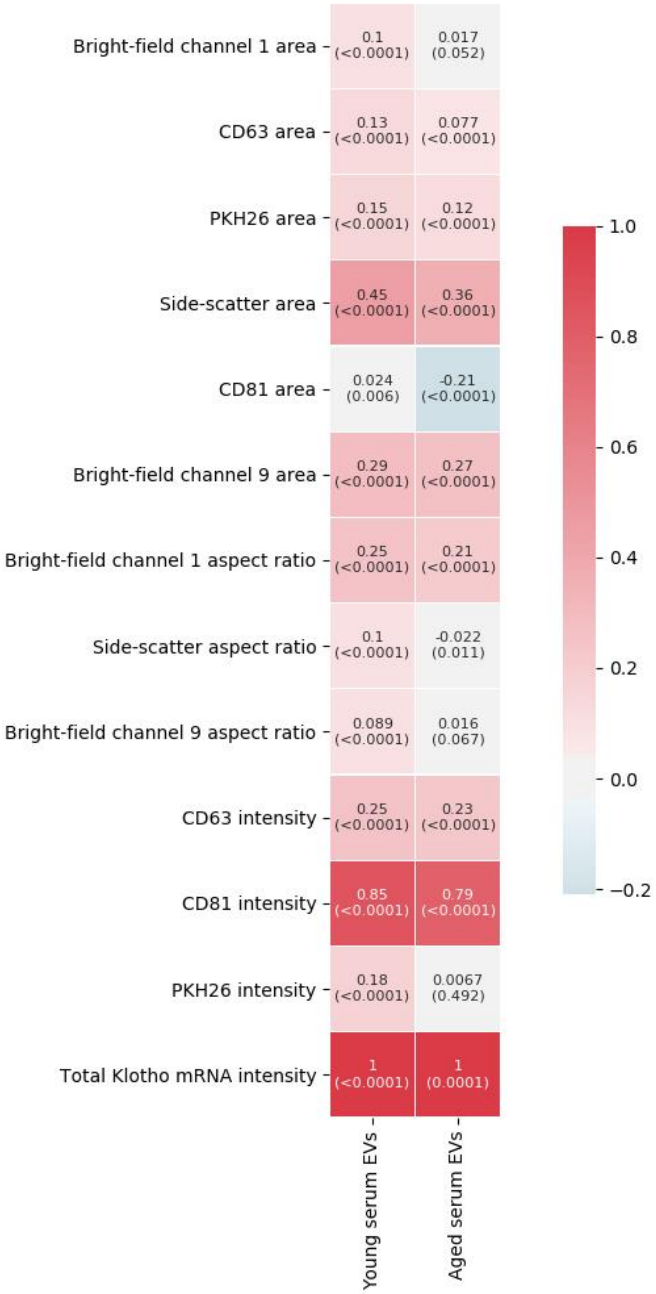


Supplemental Figure 7. Representative images of Klotho protein in *Klotho*$^{-/-}$ MPCs or aged MPCs administered with young serum EVs treated with a non-targeting RNA (scramble) or silencing RNA (siRNA) to *Klotho.* Scale: 50 μm.

a

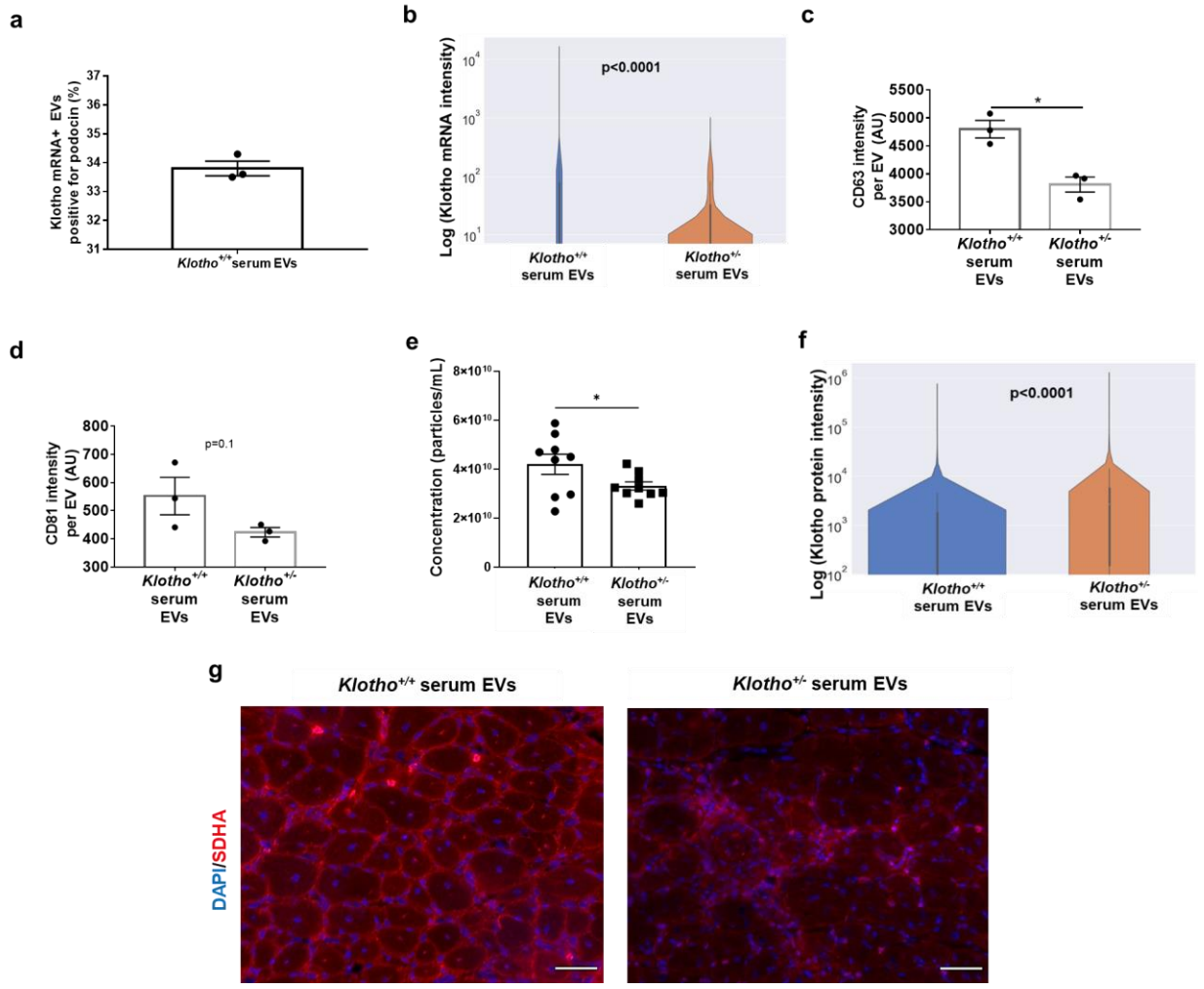

Bright-field channel 1 area
CD63 area
PKH26 area
side-scatter area
CD81 area
Bright-field channel 9 area
Bright-field channel 1 aspect ratio
Side-scatter aspect ratio
Bright-field channel 9 aspect ratio
CD63 intensity
CD81 intensity
PKH26 intensity
Total klotho mRNA intensity
1.0
0.8
0.6
0.4
0.2
0.0

b

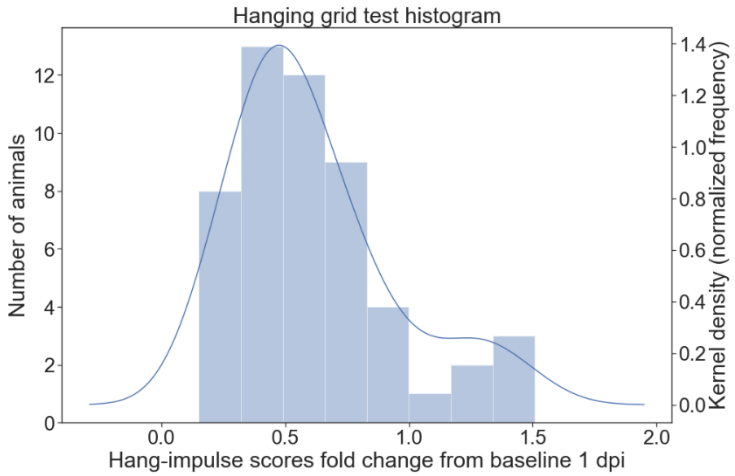


<u>Supplemental Figure 8.</u> Correlation matrices of EV structural and compositional features. (A) Pearson r coefficients were evaluated for correlation between features extracted from the dataset. Size (area and aspect ratio) and texture (modulation) features were extracted from the ImageStream image-files using IDEAS software. Modulation in the context of fluorescent images refers to dispersion or accumulation of the tagged protein of interest within the region of interest. The intensity feature was evaluated using classical computer vision-based image processing. (B) Pearson r coefficients were evaluated for correlation of Klotho mRNA intensity with size and intensity features of bright-field, side-scatter, PKH26, CD63, and CD81 channels. Size (area and aspect ratio) features were extracted from the

ImageStream image-files using IDEAS software. The intensity feature was evaluated using classical computer vision-based image processing. From the filtered dataset of 13,206 total particles, 130 particles that satisfied the criteria of total klotho<15 and cd81>150, were eliminated for correlation matrix as they were observed to be in the noise range when plotted. The p-values for each correlation is indicated in brackets below the Pearson r coefficients.



<u>Supplemental Figure 9.</u> Administration of EVs from *Klotho*$^{+/-}$ animals ultimately regulates *in vivo* muscle SDHA expression, a small portion of which express kidney marker, podocin. a, Quantification of the number of Klotho mRNA positive EVs that are positive for kidney marker, podocin (%), as determined by imaging flow cytometry. **b,** Violin plots of Klotho mRNA intensity values per EV, extracted using imaging flow cytometry analysis of EVs ($p<0.0001$, n=19,992 (*Klotho*$^{+/+}$), 38,524 (*Klotho*$^{+/-)}$ EVs for this experimental run were pooled from 4 young or 4 aged serum samples; Mann Whitney t test, experiment repeated in triplicates). Violin plot minima, maxima, median, 25th percentile and 75th percentile are 0, 16530.5, 0, 0, 78.8 for Klotho$^{+/+}$ EVs and 0, 991.2, 0, 0, and 32.6 for *Klotho*$^{+/-}$ EVs. Quantification of **c,** CD63 and **d,** CD81 content per EV in the circulation of *Klotho*$^{+/+}$ or *Klotho*$^{+/-}$ animals ($p>0.05$, n=3/group, two-tailed Mann Whitney t test). **e,** Quantification of EV concentration in circulation of *Klotho*$^{+/+}$ or *Klotho*$^{+/-}$ animals ($p>0.05$, n=9/group, two-tailed Welch's t test). **f,** Violin plots of Klotho protein intensity values extracted using imaging flow cytometry analysis of EVs ($p<0.0001$, n=19,992 (*Klotho*$^{+/+}$), 38,577 (*Klotho*$^{+/-}$) EVs for this experimental run, pooled from 4 young or 4 aged serum samples, two-tailed Mann Whitney t test, experiment repeated in triplicates). Violin plot minima, maxima, median, 25th percentile and 75th percentile are 0, 765296.2, 95.4, 0, and 1797.4 for *Klotho*$^{+/+}$ EVs and 0,1310203.4, 2715.4, 152.1, and 5759.9 for Klotho$^{+/-}$ EVs. **g,** Representative images of SDHA content in injured aged muscles 14dpi, treated with EVs isolated from *Klotho*$^{+/+}$ or *Klotho*$^{+/-}$ serum. Scale: 50 μm. Data presented as mean + SEM.



Supplemental Figure 10. Histogram and kernel density plot for overall muscle endurance of aged animals one day post injury. Variability in the overall muscle endurance as determined by the hanging grid test as observed at one-day post injury (1 dpi) when normalized to baseline, pre-injury scores. Only animals performing within 25-75% percentile of the median (range:0.40-0.74) were included in the study.

Study accession	Number of samples	Sample type	Isolation method	RNA isolation	Age	Sex
GSE100206	32	plasma	Total exosome isolation kit from Invitrogen	exoRNeasy Serum/Plasma Maxi kit	Not collected	Not specified

Supplemental Table 1. Table outlining sample demographic information and methods summary of publicly archived RNAseq data used for quantification of Klotho transcripts within circulating EVs. The Klotho transcript values shown in Figure 3I were obtained from www.exorbase.org.

SUPPLEMENTARY METHODS

Animal experimentation

All animal experiments were performed with prior approval from the Institutional Animal Care and Use Committee of the University of Pittsburgh (IACUC protocol # 20087744, 20098045). Aged male C57BL/6 mice used in these studies were obtained from NIA (21-24 months). Young male C57/BL6 mice were obtained from Jackson Laboratories. The original breeders for Klotho strain mice were obtained from MMRCC, UC Davis. The mouse colony for the laboratory was maintained in house. *Klotho* homozygotes (3-6 months, Klotho$^{+/+}$, B6; 129S5-Kltm1-Lex), *Klotho* heterozygotes (3-6 months, Klotho$^{+/-}$; B6; 129S5-Kltm1-Lex), young, and aged male mice were used to obtain EVs from serum. *Klotho* knockout (4-6 weeks, Klotho$^{-/-}$; B6; 129S5-Kltm1-Lex), young, and aged male mice were used to isolate muscle progenitor cells (MPCs).

Muscle progenitor cell (MPC) isolation

Muscle progenitor cells were isolated as previously described[1]. Briefly, hindlimb muscles were harvested and minced, after which time the tissue homogenate was weighed and treated with successive digestive enzymes. The homogenate was digested in 2 mg/mL of collagenase XI for one hour, followed by 2.4 U/mL dispase for 45 minutes and 0.1% trypsin for 30 minutes. The final homogenate was then centrifuged at 500 g for 5 minutes at 8˚C, after which time the final pellet was re-suspended in high serum medium (Dulbecco's modified eagle's medium, 20% fetal bovine serum, 1% penicillin/streptomycin and 0.5% chick embryo extract). The suspension was then plated in collagen I-coated plates for 24 hours, after which time the supernatant was transferred to another collagen coated plate. For *in vitro* experiments, MPCs were freshly isolated and were not used beyond two passages. Expression levels of Pax7 and MyoD in isolated MPCs were confirmed to be 82% and 97%, respectively.

Fibro-adipogenic progenitor isolation

Hindlimb muscles were isolated from aged mice and were washed in HBSS to remove any hair, debris or blot clots. Muscles were chopped in wash media (HBSS+10%horse serum+1%pen/strep) until the suspension could pass through a 10 mL pipette. The muscle suspension was transferred into a 50 mL tube and centrifuged at 900g, 8ºC for five minutes. The

muscles went through a successive digestion process of 750U/mL collagenase II (1.5 hours-step 1), 11 U/mL dispase and 1000 U/mL collagenase II (both together for 45 minutes-step 2). A modified flow cytometry protocol was applied to isolate FAPs[2]. Cells were sorted as $CD31^-$ (Fisher Scientific, cat#50-951-7), $CD45^-$ (Invitrogen, cat# 11-0451-82), $Sca1^+$(Invitrogen, cat#25-5981-82) and α-7 $integrin^-$ (Invitrogen, cat# MA5-23555) population and grown in high serum media (Dulbecco's modified eagle's medium, 20% fetal bovine serum, 1% penicillin/streptomycin and 0.5% chick embryo extract).

Serum collection

Blood was extracted from animals placed in supine position using the cardiac puncture method. Briefly, animals were anesthetized using isofluorane. Then, the skin was cut from the abdomen to the neck. The diaphragm was cut open for easy access to the apex of the heart. A pair of mosquito scissors were used to pull open the chest cavity. Blood was then drawn from the apex of the heart using a 25 5/8 gauge 1 mL needle. To avoid hemolysis due to shear stress, blood was then placed into a 1.5mL Eppendorf tube after removing the needle from the syringe. Blood was centrifuged for 20 minutes at 16,100 g after incubation at room temperature for 60 minutes. The serum was then aliquoted into 1.5 mL centrifuge tubes (Eppendorf) using a 200 µL micro-pipette and were preserved in the -20°C freezer for subsequent use. Any samples displaying hemolysis (as evidenced by significant pink/red coloration) were not included in the experiment.

Administration of serum on cells

Aged MPCs were plated at a density of 8,000 or 10,000 cells per well in an 8-well chamber slide or at 60,000 cells per well in a 6-well plate. Fibro-adipogenic progenitors (FAPs) were plated at a density of 60,000 cells per well in a 6-well plate. Cells were cultured for 24 hours and then treated with 10% young or aged serum in FBS-free media (optiMEM) with or without EVs for 48 hours prior to performing any end-point analysis.

Depletion of EVs from serum

Bulk EVs were depleted from young and aged serum using ExoQuick (Product# EXOQ5A-1) following the manufacturer's recommended protocol. Briefly, 63 µL of Exoquick solution was

added to 250 µL serum and the mixture was incubated on ice for 30 minutes. Next, samples were centrifuged at 1500 g for 30 minutes at 4C. The EVs were pelleted at the bottom of the tube and the EV-free serum was used for *in vitro* applications. Exoquick removed >95% EVs from the blood serum (Supplemental Fig. 2).

Analysis of bioenergetics

Muscle progenitors or FAPs were first cultured for 24 hours on 6-well plates at a density of 60,000 cells per well, after which time they were treated with young serum, aged serum, or EV-depleted young or aged serum. The cells were trypsinized 48 hours after treatment and plated on a 96-well plate at a density of 30,000 cells per well using CellTak. The cells were then cultured in un-buffered DMEM for 1 hour at 37˚C without CO_2. Cells were exposed to stressors with successive injections of 1 µM Oligomycin, 300 nM FCCP (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone), 100 mM 2-DG (2-Deoxy-D-glucose) and 1 µM Rotenone. The average of the first three time points prior to oligomycin treatment were averaged to get the basal oxygen consumption rate (OCR). Each experiment was repeated at least in triplicates. The investigator performing the seahorse experiments was blinded to the hypotheses at the time of data analysis.

Serum injections

Wild-type aged male C57/BL6 (21-23 months, NIA) were randomized into one of three cohorts that received tail-vein injections of 100 µL of young serum, 100 µL of young serum depleted of EVs, or sham injections. For studies of muscle function, animals were injected every three days for a total of eight injections. Animals were injured with 10 µL of 1 mg/mL of cardiotoxin on Day 12 of the experimental paradigm and muscle function was evaluated on Day 23. For cognition studies, animals received a total of ten injections, and cognitive tests were administered at the end of the ninth and tenth injection.

We tested four serum samples randomly for platelet contamination using a hemoanalyzer (Hemvet 950FS, University of Pittsburgh Vascular Medicine Institute) and observed minimal-to-no presence of platelets in the samples (average of 5K/µL serum).

EV isolation using size exclusion chromatography (SEC)

EVs were isolated using iZon qEVsingle 35 nm or 70 nm size-exclusion chromatography columns (Product Code: SP6). Columns were first allowed to equilibrate to room temperature prior to use. Blood serum samples were thawed at room temperature, and centrifuged at 1,500 g for 10 minutes, to remove any remaining cellular debris. After equilibrating the column to room temperature, the cap and stopper were removed, and the column was washed with 1 mL of PBS (Sigma: P5368). After washing, 100 µL of serum was added onto the top filter of the column. The eluted volume was collected in small Eppendorf centrifuge tubes. The first five fractions (200 µL each) were collected in a 1.5 mL tube. These fractions contain minimal amount of EVs and are considered to be void fractions according to manufacturer's guidelines. Majority of the EVs were eluted in fractions 6-11 (200 µL each). These fractions, a total of 1.2 mL, were collected together in a 2 mL tube. After isolation, EVs were stored at 4˚C for up to one week. Any unused EVs were then frozen and stored at -20˚C for future use.

EVs were characterized for different surface markers (CD63 and CD81) using imaging flow cytometry (Fig. 3), surface plasmon resonance imaging (Fig. 5), and in-well immunofluorescence westerns (Supplemental Fig. 4).

Nanoparticle Tracking Analysis of EVs

Nanoparticle Tracking Analysis (NTA) was performed using an NS300 NanoSight device (Malvern Panalytical). Ten microliters from each EV sample was diluted 1:100 in type 1 EV-free water and infused through the flow-cell using a syringe pump (Harvard Apparatus 98-4730). Three 45-second videos were recorded for each sample, with the camera level set to 14. These videos were batch analyzed by the software (NTA 3.3) with the detection threshold set to 3. The flow-cell was washed with 1 mL of type 1 water between each sample.

Repletion of EVs in EV-depleted serum

EVs were depleted from 100 µL young serum as described above. From the same mouse, another aliquot of 100 µL young serum, EVs were isolated using size-exclusion chromatography, as described above. The eluted EVs that were collected in 1.2 mL solution were subjected to concentrating columns to concentrate the particles into a volume of 50 µL (Product number: UFC801008D, Millipore Sigma). The eluted samples were added to the 15 mL column and

centrifuged at 7,500 g for 10-20 minutes until the desired volume was attained. The concentrated EVs were then added to the EV-depleted serum for downstream *in vitro* and *in vivo* studies.

To deplete EVs from 100 µL serum, 25.2 µL of Exoquick solution was added and the mixture was placed on ice for 30 minutes in a 1.5 mL Eppendorf tube. Next, the mixture was centrifuged at 1500 g for 30 minutes at 4C. The EVs were precipitated at the bottom, and the supernatant was placed into another 1.5 mL Eppendorf tube. Another 100 µL aliquot from the same lot of serum was used to isolate EVs using size-exclusion chromatography. 100 µL serum yielded ~2 x 10^{10} EVs/mL in 1.2 mL eluted PBS solution. These EVs were then concentrated into 50 µL solution using the method described above. This volume was then added to the 100 µL EV-depleted serum. Cells were treated cultured in 10% EV-depleted serum or EV-restored serum conditions.

EV tracking in vivo

EVs were depleted from 100 µL young serum as described above. From the same batch and another aliquot of 100 µL young serum, EVs were isolated using size-exclusion chromatography, as described above. The eluted EVs were then stained with 1 µL PKH26 dye (P9691, Sigma) and 2 µL of near-IR dye (EXOGV900A-1, System Biosciences) for one hour. EVs were then concentrated to 50 µL using the concentrating columns, as described above. The dyed EVs were then added to the EV-depleted serum, which was injected via tail vein three days after administering a cardiotoxin injury to one of the TAs of aged mice (contralateral TA uninjured). The mice were euthanized 48 hours after the injection. TAs were isolated and imaged on the LiCOR Odyssey Clx system in the near-IR (800 nm) channel. TAs were subsequently harvested and frozen in nitrogen-cooled 2-methylbutane for histological analysis by a blinded investigator.

EV injections

To investigate whether young EVs have a beneficial effect on skeletal muscle regeneration of aged male mice, animals were injected with ~5e8 EVs intramuscularly to the TA muscle three days after injury. Next, aged mice were injected with EVs from young Klotho$^{+/+}$ and Klotho$^{+/-}$ mice. For this, the aged mice received two intramuscular injections of ~7.5e8 EVs at three- and

five-days post-injury. EVs were pooled from isolations of at least four different mouse serum samples.

Animal injury model

Mice were first anesthetized with 2% isoflurane and then received bilateral injuries to the tibialis anterior (TA) muscles via intramuscular injection of cardiotoxin (10 μL of 1mg/mL cardiotoxin per TA). Animals were provided with an ingestible medigel, Carprofen, for pain management. Fourteen days following injury, the animals were subjected to an *in situ* contractile testing protocol to evaluate the injured muscle's force producing capacity. Following the contractile testing, blood was isolated from the mice using a cardiac puncture. The animals were then euthanized, and TAs were harvested for histological analysis.

Animals displaying evidence of external injuries and/or tumor growths were not included in the study. We have found that some animals exhibit very severe injuries following injection, whereas other animals display almost no functional deficit, as determined by the hang-impulse testing. Therefore, with the goal of increasing the homogeneity across groups, animals were tested one day after cardiotoxin injection, and only those animals displaying a hang-impulse score within 25-75th percentile of the mean (range: 0.40-0.74 fold-change 14 dpi score when compared to 1 dpi score) were subsequently randomized to one of the experimental groups (a total of 16 animals fell outside the range). We chose the 25-75th percentile as a conventional range to exclude potential outliers that fall in the 1st or 4th quartiles of the data set**.**

In situ contractile testing

Contractile testing was performed 14 days after injury using an *in situ* testing apparatus (Model 809B, Aurora Scientific Inc, Canada), stimulator (Model 701C, Aurora Scientific Inc, Canada), and force transducer (Aurora Scientific Inc, Canada). Briefly, the peroneal nerve of anesthetized animals was isolated through a small incision lateral to the left knee. Mice were then placed supine on a 37^{o}C-heated platform and the foot being tested was positioned on the footplate. The left hindlimb used for testing was stabilized with cloth tape on the knee and foot. Muscles were stimulated through the peroneal nerve by an electrode inserted beneath the skin. Muscle peak twitch, time to peak twitch, and half-relaxation time with the ankle positioned at 20^{o} of plantarflexion (the position that we determined to result in the greatest force output) were

quantified. Stimulations at 10, 30, 50, 80, 100, 120, 150 Hz were elicited to obtain a force-frequency curve, with a 2-minute rest between each contraction. Bilateral TA muscles were subsequently harvested and frozen in nitrogen-cooled 2-methylbutane for histological analysis by a blinded investigator.

Immunofluorescence imaging

Cell-seeded chamber slides were fixed with warm 2% Paraformaldehyde for 15 minutes, then washed with phosphate-buffered-saline (PBS) three times. The samples were then permeabilized with 0.1% Triton-X for 15 minutes after which the samples were blocked with 3% BSA and 0.1% Triton-X for 45 minutes. A similar process was followed for muscle cryo-sections up to the point of the blocking step. After blocking, the samples were incubated overnight at 4ºC with the following primary antibodies in antibody solution (3% BSA+5% Goat Serum+0.1% Triton-X), at the dilutions mentioned below:

Antibody	**Host-species**	**Product number**	**Dilution**
MyoD	Rabbit	SCBT, sc-760	1:500
MyoD	Mouse	sc-377460	1:500
Desmin	Rabbit	Abcam, ab15200	1:500
Klotho	Rat	R&D systems, MAB1819, Lot# KGN0315101	1:400
Laminin	Rabbit	Abcam, ab11575	1:500
Collagen I	Rabbit	Abcam, ab21286	1:500
SDHA	Mouse	Abcam, ab14715	1:500
Pax-7	Rabbit	Abcam, ab187339	1:100
Pax-7	Mouse	DSHB	5 µg/mL

After incubating the samples with primary antibodies overnight, samples were washed with PBS three times, after which they were incubated with host-specific secondary antibodies in antibody solution for one hour at room temperature at the dilutions mentioned in the table below:

Antibody	Host-species	Product number	Dilution
AlexaFluor 594	Rabbit	Life Technologies, A11012	1:500
AlexaFluor 488	Rat	Life Technologies, A11006	1:500
AlexaFluor 594	Rat	Life Technologies, A11007	1:500
AlexaFluor 488	Mouse IgG1	Life Technologies, A21121	1:500
AlexaFluor 594	Mouse IgG2b	Life Technologies, A21145	1:500
Phalloidin 647	N/A	Life Technologies, A22287	1:500

After a triple wash with PBS, the cells were incubated with their respective secondary antibodies, goat-anti Rat Alexa Fluor 488 or 594, goat anti-Rabbit Alexa Fluor 546 and Phalloidin 647 in 3% BSA+5% Goat Serum+0.1% Triton-X was diluted 1:500 for 60 minutes. Following a triple wash with PBS, the chamber slides were stained with DAPI for 2 minutes and then washed with PBS again. The chamber sides were mounted with a glass coverslip using Gelvatol (Source: Center for Biologic Imaging (CBI), University of Pittsburgh) as a mounting media. These were dried in 4ºC for at least three hours before imaging. Imaging was performed using 20X magnification on Zeiss-Axiovision microscope.

For Nonyl Acridine Orange (NAO) staining (Thermofisher, A1372), the fixed and permeablized cells were stained with 5 µM NAO made in HBSS (-Ca^{2+}, -Mg^{2+}), for 15 minutes. The cells were then washed with PBS, following which the cells were stained with DAPI for two minutes and washed with PBS again. The cells were mounted with a glass coverslip using Gelvatol (Source: Center of Biologic Imaging, University of Pittsburgh). The slides were dried at 4ºC and imaged using 20X magnification on Zeiss-Axiovision microscope using the FITC channel to measure reduced cardiolipin.

Imaging analysis was done using ImageJ. For *in vitro* experiments, Klotho, NAO, and SDHA were quantified as protein per cell. Pax7, MyoD, and Desmin were quantified as percentage of cells positive for those markers divided by total number of cells within an image. For *in vivo* experiments, SDHA was quantified as a function of protein level in every centrally nucleated (regenerating) fiber. For this, the myofibers were manually traced and integrated density of SDHA was computed for each of the traced myofiber as the output. A total of SDHA expression in all regenerating myofibers were quantified as output for each image. Collagen I

was quantified as total protein per unit area of image taken. Area of regenerating myofibers were quantified by manually tracing the laminin in the myofibers.

RNA-seq analysis

TA muscles from the three groups of aged animals (young serum injections, depleted young serum, or saline injections) were collected for RNA sequencing on day 23 to Novogene Corporation, Inc. Quality control of all RNA samples was performed on an Agilent 2100 Bioanalyzer instrument and samples with RIN > 5.5 and total RNA yield > 400 ng were further used for library construction. Only those samples that passed the quality check requirements for library preparation with poly A enrichment were utilized in this study (3 animals from sham, 4 animals from young serum, and 4 animals from young serum depleted of EVs groups). Libraries were sequenced on NovaSeq 6000 with PE150 strategy. Poor quality reads were eliminated with the criteria for Phred score Q30 >80%. Reads with adaptor contamination and uncertain nucleotides (N) with > 10% content was removed. PartekFlow STAR - 2.7.3a was used to align reads to mm10 genome. Uniquely aligned reads were used for downstream analysis.

Principal component analysis (PCA) performed in R revealed the distinction between groups. Linear discriminant analysis (LDA) was done with first 6 PCs was used to reduce dimensionality and visualize clearly separate clusters. Further DeSeq2 was performed with FDR<0.1. The Venn diagram (Fig 3b) shows global differentially expressed (DE) genes with log fold change (LFC) magnitude > 0.1. Gene Ontology (GO) enrichment analysis was performed using Enrichr (Maa'yan lab) with cut-offs of FDR < 0.1, LFC > 1.5. First, genes with LFC magnitude greater than 1.5 was performed. The GO terms were ranked based on combined score ranking.

Human serum collection

Human serum was purchased from a commercially available vendor, Innovative Research. Blood was collected from young males (18-35 years) and aged males (65-80 years) and serum was collected off-the-clot. No collection was performed at the University of Pittsburgh.

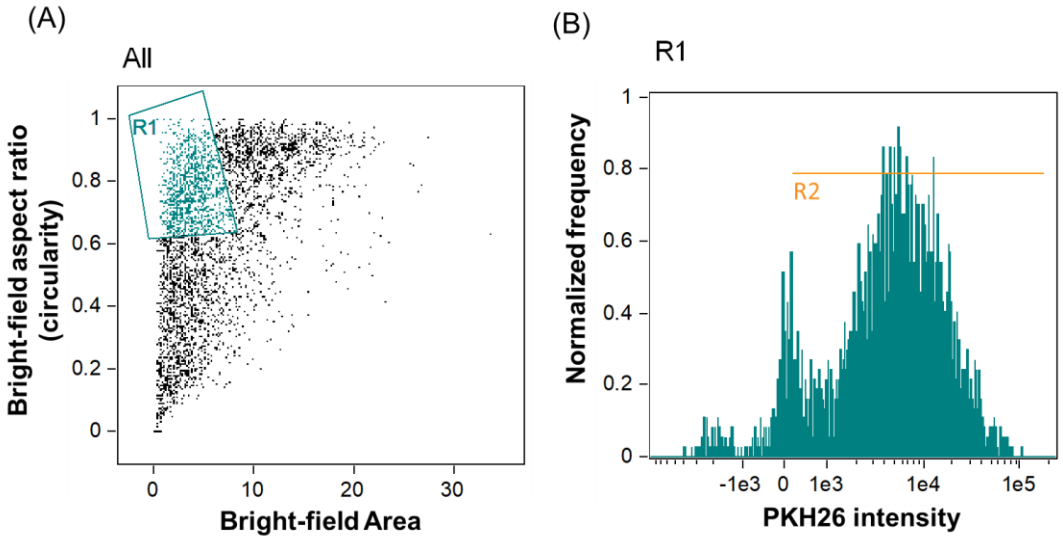
Probing CD63, CD81 and Klotho mRNA in circulating EVs for ImageStream analysis

PrimeFlow™ was performed according to the manufacturer's instructions. Two standard 20bDNAs Mouse Klotho oligos probe sets VB1-6001084 (Part No. 6003837) and VB10-6001085 (Part No. 6003838)) tagged with Type 1 Alexafluor AF647 and Type 10 Alexafluor AF568 dyes, respectively were utilized. Expression of total mRNA was reported based on the sum of fluorescence intensities of type 1 and type 10, at the single EV resolution. The Type 1 probe was designed to hybridize to region 2803-3753 of the full canonical Klotho sequence. The Type 10 probe was designed to hybridize to region 2585-3597 of the full Klotho_202 sequence. To probe for human Klotho mRNA, probe set VA1-3005823-PF tagged with Type 1 Alexafluor AF647 was utilized. Expression of total mRNA was reported based on total intensity of Klotho mRNA per EV. The sequences targeted by the probe are listed below.

Circulating EVs from four young animals and four aged animals were pooled together to form one sample each of the young and aged groups. The EVs were then fixed with equal parts of RNA Fixation Buffer 1A and 1B for 30 minutes at 4ºC. The EVs were then centrifuged at 16,100 g at 4ºC for 30 minutes and the supernatant was discarded. EVs were incubated with CD63 (Santa Cruz, sc-5275) and CD81 (Santa Cruz, sc-7637) in RNA permeabilization buffer with 1X RNase Inhibitors, at 4ºC in the dark for two hours. The target probes (VB1-6001084 (Part No. 6003837) and VB10-6001085 (Part No. 6003838)) were then thawed to room temperature. In the meantime, the EV suspensions were washed with RNA wash buffer and centrifuged at 16,100 g at 4ºC for 30 minutes. Target probes were diluted 1:20 in target probe diluent and 100 µL of the diluted probes were added to each EV suspension. These were incubated for two hours in a dry oven at 40ºC. After the incubation period, wash the samples with RNA wash buffer with 1X RNAse inhibitors and centrifuge at 16,100 g for 30 minutes at room temperature. The supernatant was removed till the 100 µL level, re-suspended in the residual volume and stored in the dark at 4ºC, overnight.

The next day, the EV samples were brought to room temperature and 100 µL of pre-amp mix was added to each sample. These samples were incubated at 40ºC for 1.5 hours. After washing with RNA wash buffer, the samples were centrifuged at 16,100 g for 30 minutes at room temperature. During this time, the probe labels were thawed on ice. Next, 100 µL of amp-mix was added to each sample and incubated at 40ºC for 1.5 hours. Next, the samples were mixed with wash buffer, centrifuged at 16,100 g for 30 minutes at room temperature. The

supernatant was removed until the 100 µL mark and the residual volume was re-suspended. The thawed probe labels were diluted 1:100 in label probe diluent and 100 µL of this was added to each of the EV sample. The samples were incubated at 40ºC for one hour. The samples were then mixed with wash buffer and centrifuged at 16,100 g for 30 minutes at room temperature. The supernatant was removed, and the residual volume was stored in IC fixation buffer provided in the kit, overnight at 4ºC. Prior to taking the samples to flow imaging, the samples received 1 µL of PKH26 for 10 minutes. The samples were washed in wash buffer and centrifuged at 16,100 g for 30 minutes at 4ºC. The supernatant was removed until the 100 µL mark and the residual volume was mixed gently. These samples were imaged on Imagestreamx MarkII system at the flow cytometry core of the Department of Immunology at the University of Pittsburgh. The EVs were gated for size and positive signal of PKH26 (see below for gating strategy). Three independent runs were done for staining and imaging of EVs.



Representative gating strategy for acquisition of EVs on ImageStream instrument. A gating strategy to include nanoparticles with (A) high circularity and small area (R1), and (B) positive PKH26 signal (R2) was used to image the young and aged serum EVs.

Information on the probes designed to target Klotho transcripts in circulating EVs are as follows:

1. **Type 1 probe (mouse Klotho):**

Accession: NM_013823.2

Region covered by Probe-set: 2803 – 3753 (951bp) (red region)

GATAATCATTGCTCGTGGGGCGGCGGGAGCGGGGGTGGGCACCGCGTAGGGAGGGCGGCGGGGCGCG
GGCATATAGGGGCGCGGCGCGGTGCCCTCCCGGCTCCCGCAGCATGCTAGCCCGCGCCCCTCCTCGCC
GCCCGCCGCGGCTGGTGCTGCTCCGTTTGCTGTTGCTGCATCTGCTGCTGCTCGCCCTGCGCGCCCGCT
GCCTGAGCGCTGAGCCGGGTCAGGGCGCGCAGACCTGGGCTCGCTTCGCGCGCGCTCCTGCCCCAGAG
GCCGCTGGCCTCCTCCACGACACCTTCCCCGACGGTTTCCTCTGGGCGGTAGGCAGCGCCGCCTATCAG
ACCGAGGGCGGCTGGCGACAGCACGGCAAAGGCGCGTCCATCTGGGACACTTTCACCCATCACTCTGG
GGCGGCCCCGTCCGACTCCCCGATCGTCGTGGCGCCGTCGGGTGCCCCGTCGCCTCCCCTGTCCTCCAC
TGGAGATGTGGCCAGCGATAGTTACAACAACGTCTACCGCGACACAGAGGGGCTGCGCGAACTGGGG
GTCACCCACTACCGCTTCTCCATATCGTGGGCGCGGGTGCTCCCCAATGGCACCGCGGGCACTCCCAA
CCGCGAGGGGCTGCGCTACTACCGGCGGCTGCTGGAGCGGCTGCGGGAGCTGGGCGTGCAGCCGGTG
GTTACCCTGTACCATTGGGACCTGCCACAGCGCCTGCAGGACACCTATGGCGGATGGGCCAATCGCGC
CCTGGCCGACCATTTCAGGGATTATGCCGAGCTCTGCTTCCGCCACTTCGGTGGTCAGGTCAAGTACTG
GATCACCATTGACAACCCCTACGTGGTGGCCTGGCACGGGTATGCCACCGGGCGCCTGGCCCCGGGCG
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CATCTCTACAACACCTCTTTCCGCCCCACACAGGGAGGCCGGGTGTCTATCGCCTTAAGCTCCCATTGG
ATCAATCCTCGAAGAATGACTGACTATAATATCAGAGAATGCCAGAAGTCTCTTGACTTTGTGCTAGG
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CGGACCAACCTTGAGCTTTCAGCTATTGGACCCTAACATGAAGTTCCGCCAATTGGAGTCTCCCAACCT
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CAAGGAGCTGTTGCCAAAGTCTTCGGCCTTGTTCTACCAAAAGCTGATAGAGGACAATGGCTTTCCTC
CTTTACCTGAAAACCAGCCCCTTGAAGGGACATTTCCCTGTGACTTTGCTTGGGGAGTTGTTGACAACT
ACGTTCAAGTGGACACTACTCTCTCTCAGTTTACTGACCCGAATGTCTATCTGTGGGATGTGCATCACA
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GCCATCCGGCCTCAGATAACCTTACTTCGAGAAATGCGGGTCACCCACTTTCGCTTCTCCCTGGACTGG
GCCCTGATCTTGCCTCTGGGTAACCAGACCCAAGTGAACCACACGGTTCTGCACTTCTACCGCTGCATG
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AACACACGGAACATGACCTATCGTGCCGGGCACCACCTCCTGAGAGCCCATGCCTTGGCTTGGCATCT
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CAAGAGCAAAACTTTAATAGTTATGGAGAATATTGCCAGAACAGCTCAAAACTGTTTTATTTGTTGGT
CCAATTTCCCATTAATTAGTTCAATAATAAATATCATTTAGAAATAAAAAAAA

2. **Type 10 probe (mouse Klotho):**

Accesion:ENSMUST00000202096.1 Kl-202

Region covered by probe-set: 2585 – 3597 (1013bp) (red region)

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cc

3. **Type 1 probe (human Klotho):**

Accession: NM_004795.3

cgcgcagcat gcccgccagc gccccgccgc gccgcccgcg gccgccgccg ccgtcgctgt
cgctgctgct ggtgctgctg ggcctgggcg gccgccgcct gcgtgcggag ccgggcgacg
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631 ggggcagccc gcggctcggg tacctggtgg cgcacaacct cctcctggct catgccaaag
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633 taagctctca ctggatcaat cctcgaagaa tgaccgacca cagcatcaaa gaatgtcaaa
634 aatctctgga ctttgtacta ggttggtttg ccaaacccgt atttattgat ggtgactatc
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636 agttcatcaa aggaactgct gacttttttg ctctttgctt tggacccacc ttgagttttc
637 aacttttgga ccctcacatg aagttccgcc aattggaatc tcccaacctg aggcaactgc
638 tttcctggat tgaccttgaa tttaaccatc ctcaaatatt tattgtggaa aatggctggt
639 ttgtctcagg gaccaccaag agagatgatg ccaaatatat gtattacctc aaaaagttca
640 tcatggaaac cttaaaagcc atcaagctgg atggggtgga tgtcatcggg tataccgcat
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643 aaaagctgat agagaaaaat ggcttccctc ctttacctga aaatcagccc ctagaaggga
644 catttccctg tgactttgct tggggagttg ttgacaacta cattcaagta gataccactc
645 tgtctcagtt taccgacctg aatgtttacc tgtgggatgt ccaccacagt aaaaggctta
646 ttaaagtgga tggggttgtg accaagaaga ggaaatccta ctgtgttgac tttgctgcca
647 tccagcccca gatcgcttta ctccaggaaa tgcacgttac acattttcgc ttctccctgg
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649 actatcgctg catggccagc gagcttgtcc gtgtcaacat caccccagtg gtggccctgt
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652 gccatcacgt caagctttgg ataacgatga atgagccgta tacaaggaat atgacataca
653 gtgctggcca caaccttctg aaggcccatg ccctggcttg gcatgtgtac aatgaaaagt
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655 cctgcccttt ctcccaaaag gacaaagagg tggctgagag agttttggaa tttgacattg
656 gctggctggc tgagcccatt ttcggctctg gagattatcc atgggtgatg agggactggc
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675 agtagtaatt gcaagagttc gaatagaaag ttatgtacca agtaaccatt tctcagctgc
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678 ttctatcaaa tactagtatt aatttatgta tctggttaat gacatacttg gagagcaaat
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683 gtgcaacatt atgattaatc tgattataca ccatttttga gcagatcttg gaatgaatga
684 catgaccttt ccctagagaa taaggatgaa ataatcactc attctatgaa cagtgacact
685 actttctatt ctttagctgt actgtaattt ctttgagttg atagttttac aaattcttaa
686 taggttcaaa agcaatctgg tctgaataac actggatttg tttctgtgat ctctgaggtc

tattttatgt ttttgctgct acttctgtgg aagtagcttt gaactagttt tactttgaac
tttcacgctg aaacatgcta gtgatatcta gaaagggcta attaggtctc atcctttaat
gccccttaaa taagtcttgc tgattttcag acagggaagt ctctctatta cactggagct
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ccaacctcac tgacacaggg tcatagtgta taataatata ctgtactata taatatatca
tctttagagg tatgattttt tcatgaaaga taagcttttg gtaatattca ttttaaagtg
gacttattaa aattggatgc tagagaatca agtttatttt atgtatatat ttttctgatt
ataagagtaa tatatgttca ttgtaaaaat ttttaaaaca cagaaactat atgcaaagaa
aaaataaaaa ttatctataa tctcagaacc cagaaatagc cactattaac atttcctacg
tattttattt tacatagatc atattgtata tagttagtat ctttattaat ttttattatg
aaactttcct ttgtcattat tagtcttcaa aagcatgatt tttaatagtt gttgagtatt
ccaccacagg aatgtatcac aacttaaccg ttcccgtttg ttagactagt ttcttattaa
tgttgatgaa tgttgtttaa aaataatttt gttgctacat ttactttaat ttccttgact
gtaaagagaa gtaattttgc tccttgataa agtattatat taataataaa tctgcctgca
acttttgcc ttctttcata atcataaaaa aa

The yellow highlighted sequences are identical between the two mouse Klotho mRNA sequences. This region is Glycosyl hydrolase-1 1 region+. Two sets of ViewRNA® Probes were used to target 2803-3753 region of canonical mouse Klotho (NM_013823) and 2585-3597 region of mouse Klotho 202 (ENSMUST00000202096.1), respectively. These regions were found to be dissimilar by BLAST, suggesting that each probe set does not bind to the other Klotho mRNA tested.

ImageStream imaging and machine learning-based computational analysis

The flow cytometry for EV imaging was conducted using a 60X objective at a resolution of 0.3 μm^2/pixel. The reduced width of the core stream using the 60X magnification reduces the positional space of EVs within the focal plane, thereby increasing the frequency of microscopically focused objects. Filtered sheath buffers were used to ensure the absence of particulates. Samples were acquired using INSPIRE® software with the highest resolution (sensitivity) and lowest speed. All used lasers for fluorochromes employed were used at the optimal power setting.

Machine learning algorithms supported by classical computer vision methods were employed on the particles to filter EVs and compute most age-discriminative feature. The computer vision pipeline for filtering operated on the bright-field and side-scatter channels from a database of at least 20,000 samples (~10,000 each of young and aged serum EVs). Successive steps of image morphological operations, histogram-based background noise suppression, iso-data thresholding and h-maxima detection were used to filter spherical and singular EVs that resulted in a final

sample set of 13,206 samples. The features used for machine learning algorithms were based on size (area, aspect ratio), signal strength (intensity) and texture (modulation) of the EVs. Size and texture features were extracted from the IDEAS software used to analyze the ImageStream based acquisition of samples. Area and aspect ratio of the EVs indicate how big and circular the EVs are. The texture feature indicates the smoothness or roughness of the EV. This feature explores the dispersion of the protein of interest in the EV. This set of features was augmented with signal strength feature of total intensity, calculated by summing the intensities of all pixels within a binary mask computed using iso-data thresholding after background noise suppression. A total of 24 features were used to employ the machine learning algorithms on the filtered database.

Cross-correlations were computed for exploratory analysis using Spearman's method as the features were not assumed to be normally distributed. Kernel density estimation was used to compute the density distributions of the intensity channels associated with CD63, CD81 and Klotho mRNA markers extracted from young or aged specimens. In this method, a Gaussian kernel was placed at each data point. The kernel size was computed by Scott's method. The resulting Gaussian mixture estimates the continuous density of the feature. Total Klotho intensity was computed as a sum of intensities from channel 4 and channel 11 images since the probes do not tag same regions of the sequence. Gradient-boosted Decision Trees were used for classification of age with the above-mentioned curated features as input[3]. Decision tree classifiers are desirable for their ability to measure the importance of the feature to the classification task. Accuracy statistics were reported with 20-fold cross-validation to prevent overfitting.

Surface Plasmon Resonance Imaging (SPRi)

Young and aged serum EVs were analyzed by SPRi to study the presence and relative amount of Klotho protein and CD63 on their membranes. First, gold SPRi chips (Horiba Scientific SAS,SPRi-Biochip, Palaiseau, France) were functionalized with antibodies against CD63 (CD63 Antibody, MAB5048, R&D Systems), Klotho (Mouse Klotho Antibody, AF1819, R&D Systems) and IgG (Purified anti-rat IgG1 Antibody, 407402, Biolegend, as negative control). The surface of the chip was coated with a self-assembled monolayer of thiolated PEG molecules for the immobilization of antibodies through EDC/NHS chemistry. Following this, a microspotter (SPRi Arrayer, Horiba) was used to create spots with diameters of 0.7 mm of the

selected antibodies in distinct areas of the same SPRi chip. Four spots per antibody were made at ~70% relative humidity at room temperature. The chip was then blocked with a solution of ethanolamine 1 M, pH 9, for 30 min, washed with water and used in SPRi instrument XelPleX (Horiba Scientific SAS).

HBS-ET was used as a running buffer (1.5 M NaCl, 100 mM HEPES, 30 mM EDTA, Tween 0.5%, pH 7.4). EVs were isolated from 100 µl of serum by size-exclusion chromatography using qEV column (IZON, qEVsingle) with PBS as running buffer, collecting fractions from 6th to 11th and adding protease inhibitors. Aliquots of 500 µL of young and aged isolated EVs, resuspended in HBS-ET, were injected in the SPRi flow chamber with a flow rate of 10 µL/min and the SPRi signals were collected and analyzed by using EzSuite software and OriginLab. Sensorgrams were corrected by subtracting the signal related to the anti-rat IgG antibody.

Raman Spectroscopy

Young and aged EVs that were isolated by size-exclusion chromatography were concentrated using an ultracentrifugation step (100,000 g x 70min) for 9th to 11th fractions. The concentrated EVs were analyzed by Raman spectroscopy to obtain an overall biochemical (LabRAM, Horiba Jobin Yvon S.A.S. Lille, France) following a previous published protocol (Gualerzi A et al, Sci. Rep, 2017; Gualerzi A et al, JEV, 2019). Briefly, 5µl of the concentrated EV suspension was applied on calcium fluoride disks and Raman acquisitions were performed using the following characteristics: (a) 532 nm laser, (b) 50x objective, (c) grating 1800, (d) 400 µm entrance slit and (e) in the spectral ranges 400-1800 cm^{-1} and 2600-3200 cm^{-1}. A reference sample (Si) at 570.7 cm^{-1} was used to calibrate the instrument prior to running the experimental samples. Ten spectra per sample were collected following a line-map from the border of the drop to the edge, with an acquisition time of 30 seconds, following which the acquired data were analyzed through LabSpec6 and OriginLab sofwares. First, a despike (poly 5), baseline correction (fifth order polynomial curve), normalization by unit vector was performed following which multivariate statistical analysis was performed on the spectra. Principal Component Analysis (PCA) was performed for the data reduction identifying the principal components (PCs) that represent the differences in the spectra. Linear discriminant analysis (LDA) was performed by using a small number of PCs (n=15) in order to evaluate the possibility to discriminate the spectra of two

groups in statistically significant way (Mann-Whitney Test). PCA was performed using OriginLab PlugIn called "Principal Component Analysis for Spectroscopy".

Administration of EVs to aged cells and silencing Klotho mRNA in EVs

Muscle progenitors from aged or Klotho$^{-/-}$ mice were plated at a density of 10,000 cells per well in an 8-well chamber slide for 24 hours. The cells were then exposed to one billion young or aged EVs for a duration of 48 hours.

To test whether Klotho mRNA may be a driver for Klotho modulation within the recipient aged cells and Klotho$^{-/-}$ cells, young EVs were treated with non-targeting control scramble or siRNA to Klotho for a total of 50 minutes prior to administering them to aged cells. Every billion EVs received 10 µl of transfection reagent (Dharmacon, Dharmafect T-2001-01) and 10 µL of a smart-pool of 5µM non-targeting control (Dharmacon) or siRNA to Klotho (Dharmacon). First, the transfection reagent and scramble/siRNA were mixed and incubated in the incubator at 37C for 10 minutes. Following incubation, the EVs were placed on ice for 40 minutes prior to use for an *in vitro* application. Cells receiving the young, aged or treated young EVs were analyzed for MyoD, cardiolipin content and/or Klotho expression 48 hours post-administration.

ELISA

Muscle progenitors were plated on an 8 well chamber slide or 12-well plate at a density of 8,000 cells or 20,000 cells per well, respectively, prior to treatment with one or two billion EVs. Conditioned media from the samples (untreated aged cells/Klotho$^{-/-}$ cells and aged cells/ Klotho$^{-/-}$ cells treated with young EVs) were collected 48 hours after treatment.

Klotho protein levels in media were measured by a colorimetric sandwich enzyme immunoassay (ELISA Kit SEH757Mu, Cloud-Clone Corp, Lot#L170622859) according to manufacturer's instructions. Briefly, 100 µL of standards and samples were added to a 96-well microtiter plate that was pre-coated with a biotin-conjugated antibody specific to Klotho. The plate was then incubated for one hour at 37°C following which the samples and standards were removed. A 100 µL of detection reagent A (Biotin-conjugated antibody) was added to each well and incubated at 37°C for one hour. The plates were washed three times with washing buffer provided by the manufacturer. Next, 100 µL of detection reagent B (Avidin conjugated Horseradish-Peroxidase (HRP-avidin)) was added to each well and incubated for 30 minutes at

37 °C. The plate was then washed with washing buffer five times. Next, 90µL of tetramethylbenzidine substrate was added to the plate and incubated at 37 °C for 20 minutes. A 50 µL sulfuric acid stop solution was then added to terminate the color development reaction. The optical density (OD) of each well was measured at 450 nm. The OD of samples was compared to OD standard curve with known antigen concentrations to determine the concentration of samples. The standard curve had a concentration range from 3.25 pg/mL to 200 pg/mL. The data were then normalized to the number of cells per well. Samples were never subjected to freeze-thaw.

EV engineering with synthetic mRNA

Aged or Klotho$^{+/-}$ EVs isolated from the serum were transfected with the synthetic Klotho mRNA sequences using Exo-Fect™ exosome transfection reagent from System Biosciences (Cat#EXFT-10A1). Briefly, one billion EVs were treated with 10 µL exofect solution and 1 µg of synthetic Klotho mRNA. The unloaded control EVs were treated with just 10 µL exofect solution. This solution was mixed well by flicking the tube 3 times. Samples were incubated at 37˚C for 10 minutes. A stopping solution (30 µL) provided in the kit was added to the samples and incubated on ice for 30 minutes to precipitate the EVs. Samples were then centrifuged at 16,100 g at 4ºC. The supernatants were removed, and samples were re-suspended in culture media (DMEM+20% exosome-free FBS+1%penicillin/streptomycin) and administered to 10,000 aged muscle progenitors for 48 hours. The synthetic mRNA used was the Klotho mRNA with RNA length of 3321 NT. The sequence was substituted 25% with Cyanine 5-U and capped (Cap1) using CleanCap^{TM} AG (Trilink Biotechnologies, Lot no. WOTL25007). The opening reading frame used in constructing the synthetic mRNA is:

ATGCTAGCCCGCGCCCCTCCTCGCCGCCCGCCGCGGCTGGTGCTGCTCCGTTTGCTGTTG
CTGCATCTGCTGCTGCTCGCCCTGCGCGCCCGCTGCCTGAGCGCTGAGCCGGGTCAGGGC
GCGCAGACCTGGGCTCGCTTCGCGCGCGCTCCTGCCCCAGAGGCCGCTGGCCTCCTCCAC
GACACCTTCCCCGACGGTTTCCTCTGGGCGGTAGGCAGCGCCGCCTATCAGACCGAGGGC
GGCTGGCGACAGCACGGCAAAGGCGCGTCCATCTGGGACACTTTCACCCATCACTCTGGG
GCGGCCCCGTCCGACTCCCCGATCGTCGTGGCGCCGTCGGGTGCCCCGTCGCCTCCCCTG
TCCTCCACTGGAGATGTGGCCAGCGATAGTTACAACAACGTCTACCGCGACACAGAGGGG
CTGCGCGAACTGGGGGTCACCCACTACCGCTTCTCCATATCGTGGGCGCGGGTGCTCCCC
AATGGCACCGCGGGCACTCCCAACCGCGAGGGGCTGCGCTACTACCGGCGGCTGCTGGAG
CGGCTGCGGGAGCTGGGCGTGCAGCCGGTGGTTACCCTGTACCATTGGGACCTGCCACAG
CGCCTGCAGGACACCTATGGCGGATGGGCCAATCGCGCCCTGGCCGACCATTTCAGGGAT
TATGCCGAGCTCTGCTTCCGCCACTTCGGTGGTCAGGTCAAGTACTGGATCACCATTGAC
AACCCCTACGTGGTGGCCTGGCACGGGTATGCCACCGGGCGCCTGGCCCCGGGCGTGAGG
GGCAGCTCCAGGCTCGGGTACCTGGTTGCCCACAACCTACTTTTGGCTCATGCCAAAGTC

TGGCATCTCTACAACACCTCTTTCCGCCCCACACAGGGAGGCCGGGTGTCTATCGCCTTA
AGCTCCCATTGGATCAATCCTCGAAGAATGACTGACTATAATATCAGAGAATGCCAGAAG
TCTCTTGACTTTGTGCTAGGCTGGTTTGCCAAACCCATATTTATTGATGGCGACTACCCA
GAGAGTATGAAGAACAACCTCTCGTCTCTTCTGCCTGATTTTACTGAATCTGAGAAGAGG
CTCATCAGAGGAACTGCTGACTTTTTTGCTCTCTCCTTCGGACCAACCTTGAGCTTTCAG
CTATTGGACCCTAACATGAAGTTCCGCCAATTGGAGTCTCCCAACCTGAGGCAGCTTCTG
TCTTGGATAGATCTGGAATATAACCACCCTCCAATATTTATTGTGGAAAATGGCTGGTTT
GTCTCGGGAACCACCAAAAGGGATGATGCCAAATATATGTATTATCTCAAGAAGTTCATA
ATGGAAACCTTAAAAGCAATCAGACTGGATGGGGTCGACGTCATTGGGTACACCGCGTGG
TCGCTCATGGACGGTTTCGAGTGGCATAGGGGCTACAGCATCCGGCGAGGACTCTTCTAC
GTTGACTTTCTGAGTCAGGACAAGGAGCTGTTGCCAAAGTCTTCGGCCTTGTTCTACCAA
AAGCTGATAGAGGACAATGGCTTTCCTCCTTTACCTGAAAACCAGCCCCTTGAAGGGACA
TTTCCCTGTGACTTTGCTTGGGGAGTTGTTGACAACTACGTTCAAGTGGACACTACTCTC
TCTCAGTTTACTGACCCGAATGTCTATCTGTGGGATGTGCATCACAGTAAGAGGCTTATT
AAAGTAGACGGGGTTGTAGCCAAGAAGAGAAAACCTTACTGTGTTGATTTCTCTGCCATC
CGGCCTCAGATAACCTTACTTCGAGAAATGCGGGTCACCCACTTTCGCTTCTCCCTGGAC
TGGGCCCTGATCTTGCCTCTGGGTAACCAGACCCAAGTGAACCACACGGTTCTGCACTTC
TACCGCTGCATGATCAGCGAGCTGGTGCACGCCAACATCACTCCAGTGGTGGCCCTGTGG
CAGCCAGCAGCCCCGCACCAAGGCCTGCCACATGCCCTTGCAAAACATGGGGCCTGGGAG
AACCCGCACACTGCTCTGGCGTTTGCAGACTACGCAAACCTGTGTTTTAAAGAGTTGGGT
CACTGGGTCAATCTCTGGATCACCATGAACGAGCCAAACACACGGAACATGACCTATCGT
GCCGGGCACCACCTCCTGAGAGCCCATGCCTTGGCTTGGCATCTGTACGATGACAAGTTT
AGGGCGGCTCAGAAAGGCAAAATATCCATCGCCTTGCAGGCTGACTGGATAGAACCGGCC
TGCCCTTTCTCTCAAAATGACAAAGAAGTGGCCGAGAGAGTTTTGGAATTTGATATAGGC
TGGCTGGCAGAGCCTATTTTTGGTTCCGGAGATTATCCACGTGTGATGAGGGACTGGCTG
AACCAAAAAAACAATTTTCTTTTGCCCTATTTCACCGAAGATGAAAAAAAGCTAGTCCGG
GGTTCCTTTGACTTCCTGGCGGTGAGTCATTACACCACCATTCTGGTAGACTGGGAAAAG
GAGGATCCGATGAAATACAACGATTACTTGGAGGTACAGGAGATGACTGACATCACATGG
CTCAACTCTCCCAGTCAGGTGGCAGTGGTGCCTTGGGGGCTGCGCAAAGTGCTCAACTGG
CTAAGGTTCAAGTACGGAGACCTCCCGATGTATGTGACAGCCAATGGAATCGATGATGAC
CCCCACGCCGAGCAAGACTCACTGAGGATCTATTATATTAAGAATTATGTGAATGAGGCT
CTGAAAGCCTACGTGTTGGACGACATCAACCTTTGTGGCTACTTTGCGTATTCACTTAGT
GATCGCTCAGCTCCCAAGTCTGGCTTTTATCGATATGCTGCGAATCAGTTTGAGCCCAAA
CCATCTATGAAACATTACAGGAAAATTATTGACAGCAATGGCTTCCTGGGTTCTGGAACA
CTGGGAAGGTTTTGTCCAGAAGAATACACTGTGTGCACCGAATGTGGATTTTTTCAAACC
CGGAAGTCTTTGCTGGTCTTCATCTCGTTTCTTGTTTTTACTTTTATTATTTCTCTTGCT
CTCATTTTTCACTACTCCAAGAAAGGCCAGAGAAGTTATAAGTAA

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