844-663-8749
support@invivobiosystems.com
invivobiosystems.com

InVivo Biosystems

InVivo Longevity Platform

Example Service Report

FIRST OF ALL

THANK YOU

FOR CONSIDERING US!

At InVivo Biosystems, our mission is to help our clients develop and deliver solutions that improve the quality of human health.

Our technology and expertise benefit early-stage investigations the most by focusing on proof-of-principle experiments that provide the preliminary data needed to make a go/no-go decision.

We provide the infrastructure and experienced personnel that give your project a strong start to help you accelerate the development of compounds and move them down the pipeline.

Our scientists work one-on-one with our clients to provide the consultation and research needed to detect early risks and move new projects forward quickly.

Starting with a thorough understanding of your needs, we will work with you closely to bring your project to successful closure. We will always ensure timely communication and the most efficient solutions to your problems.

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REPORT SUMMARY

Customer	**ABC Aging**
Project Contact	**John Doe**
Project Code	**ABCA-001**
Project Scope	1. **Understand whether compound Lu0128 has an effect on lifespan and healthspan.** 2. **Identify all the genes whose expression is affected by compound Lu0128.** 3. **Identify pathways that are targeted by compound Lu0128.** 4. **Determine the extent to which known aging-related pathways are targeted by compound Lu0128.**

Project Scope and Aims

Use the nematode *C. elegans* as a model to gain insight into the impact of compounds Lu0128 on lifespan, healthspan, gene expression and aging-specific pathways.

Using a 3-pronged approach, we:

1. Measured toxicity and determined dosage of Lu0128 for lifespan/healthspan studies.
2. Measured the effect of compounds Lu0128 on lifespan.
3. Identified Lu0128's mode of action.

Step 1: We determined a physiologically effective dosage and delivery method for Lu0128, and assessed the toxicity of Lu0128 to *C. elegans.*

Step 2: We determined whether Lu0128 extends lifespan and healthspan using automated survival measurement and in-depth healthspan analysis.

Step 3: We Identified genes and pathways related to Lu0128-mediated pro-longevity effects using whole transcription analysis.

Executive Summary

Oxidative stress has been shown to play a role in aging and age-related illness. ABC Aging's product Lu0128 is a derivative of Glutathione that has been modified for enhanced potency and biostability. The nematode worm C. elegans is an established and validated model system for testing products that modulate lifespan or treat age-related illness. Lu0128 significantly increased the average lifespan of C. elegans, similar to the well-established life-extending compound that we used as a positive control. Furthermore, deep phenotyping revealed that Lu0128 improved healthspan relative to both vehicle (no treatment) and positive controls. Whole transcription analysis analysis indicates that the effect of Lu0128 on lifespan and healthspan likely results from modulation of oxidative stress, consistent with this class of compounds.

Project Conclussions

Step I. Dosage, Toxicity, and Feasibility tests

- Compound Lu0128 is water-soluble and can be delivered to worms by permeating solid agar media with an aqueous solution.
- Based on the dose response curves of two stress biosensors, a working EC50 of 21µM (rounded to 20µM) was selected as a benchmark for further testing.
- Growth, viability, and toxicity assays indicated a maximum non-toxic dosage of 10µM Lu0128.
- Recommended proceeding with a dosage of 10µM Lu0128 for lifespan assay.

Step II. Lifespan assay and survival analysis.

- Worms treated with Lu0128 showed an increased median and maximum lifespan.
- Worms treated with 10µM Lu0128 showed significantly increased lifespan relative to vehicle control (Log-rank test, P-value = <0.0001).
- Worms treated with Lu0128 remained in a mobile state longer than those treated with vehicle control.
- Principal components analysis revealed that the movement features of worms treated with Lu0128 clustered exclusively from those treated with vehicle control and similarly to those treated with the positive control.

Step III. Identify aging pathways

- A total of 74 genes showed significantly ($p<0.00001$) different expression at day 10 of adulthood in Lu0128 treated vs. untreated animals.
- Lu0128 showed a significant change in the activity of pathways linked to oxidative phosphorylation and oxidative stress.

Summary conclusions for this project

- Lu0128 significantly increased lifespan and healthspan.
- Lu0128 possibly influences lifespan through modulation of oxidative phosphorylation and oxidative stress.

RESULTS AND DATA

Step I. Dosage, Toxicity, and Feasibility.

The goal of this set of experiments is to determine experimental conditions for steps II and III.

1.1 Stress response assay and dose selection

The EC**50** is the concentration of a drug that gives half-maximal response. We determine a working EC**50** for Lu0128 by measuring physiological stress in response to varying concentrations using our fluorescent stress biosensor C. elegans strains.

Transgenic biosensor worms express red fluorescent protein (RFP) in response to stress and constitutively express green fluorescent protein (GFP).

- Day 1 adult biosensor worms were treated with a 1 µM-10 mM range of Lu0128 concentrations.
- Fluorescent images were captured after 24 hours of treatment.
- Stress response was quantified by normalizing the RFP signal to the GFP signal.

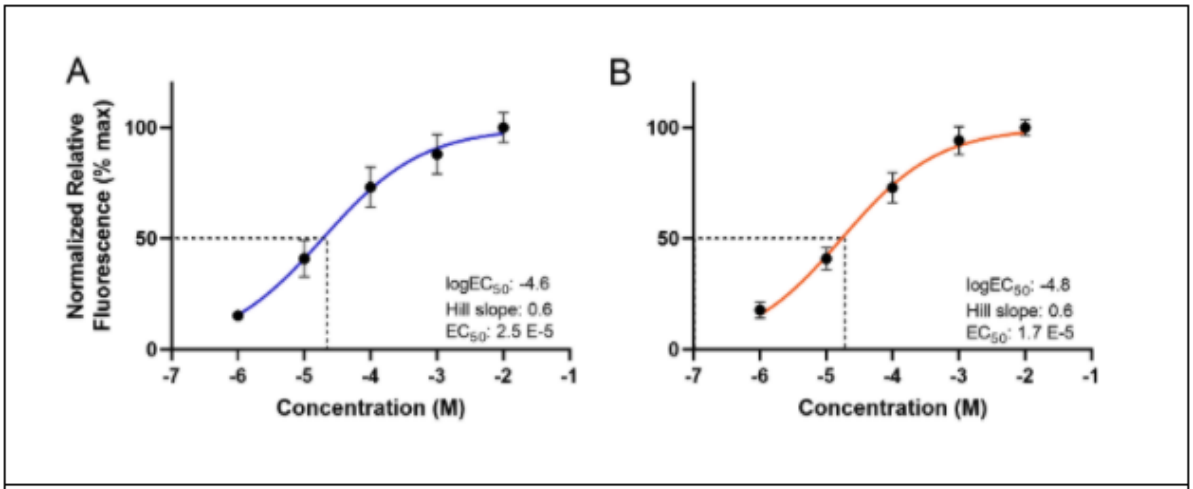

Figure 1.1. Dose-response curve for Lu0128 using two fluorescent stress biosensor worm strains. Day 1 adult worms were treated with Lu0128 at 1µM, 10µM, 100µM, 1mM, and 10mM for 12 hours. RFP signal was quantified by normalizing to constitutive GFP signal. (A) Xenobiotic stress biosensor response assay. (B) Oxidative stress response assay.
Calculated working EC_{50} = 21µM.

Compound Lu0128 is water-soluble and can be delivered to worms by permeating solid agar media with an aqueous solution. Based on the dose response curves of two stress biosensors (Figure 1.1.), a working EC50 of 21µM (rounded to 20µM) was selected for further testing.

1.2 Viability, Growth, and Development assay to determine toxicity in *C. elegans.*

- Worms were exposed to three concentrations of compounds (EC25, EC50, EC100) from embryo to first day of adulthood.
- 50 treated embryos were plated on each of three replicate dishes.
- Quantity and size of worms was measured each day through day 1 of adulthood.

The goal of this set of experiments is to determine experimental conditions for steps II and III.

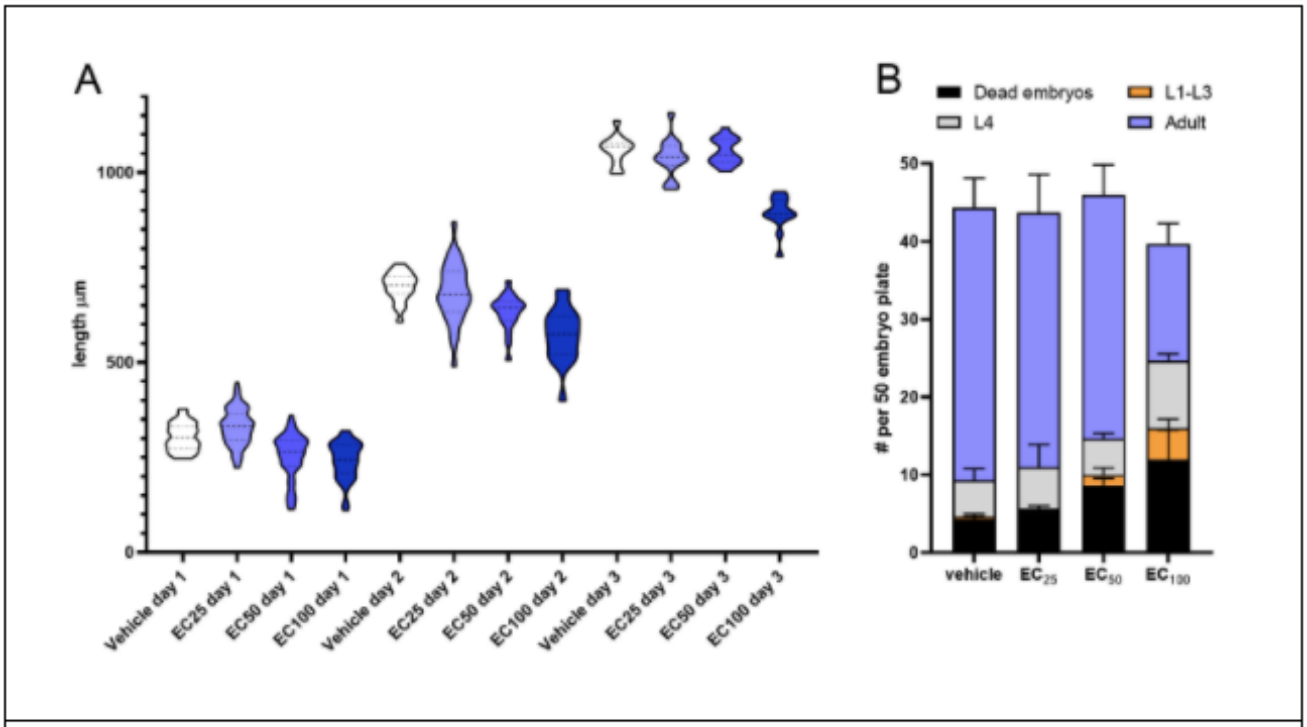

Figure 1.2. Viability, Growth, and Development assay to determine toxicity of Lu0128 in *C. elegans*. A) Violin plot showing distribution of worm lengths during development from larvae to adult when treated with varying concentrations of Lu0128. B) Day three snapshot of worm development including embryonic lethality (black). EC_{25}, EC_{50}, EC_{100} are based on dose-response curves in Experiment 1.1 and correspond to concentrations of 10µM, 20µM, and 40µM.

In viability assays, Lu0128 exposure at the EC100 caused a clear developmental delay (Figure 1.2.A.) and reduced viability (Figure 1.2.B.) . A slight developmental delay was observed at the EC50. Worms treated with 10µM Lu0128 (EC25 = 0.5 x EC50) showed normal viability and development. We therefore recommended using a dosage of 10µM Lu0128 for Step II. This recommendation was approved by ABC Aging.

Step II. Does compound Lu0128 extend lifespan and/or healthspan?

2.1. Lifespan Assay

- Conditions tested:
  - Compound Lu0128 at 10µMN-acetyl-L-cysteine (NAC), a class-specific positive control, at 50µM
  - Vehicle control: water
  - 3 biological replicates of 150 worms each derived from independent synchronizations. >450 worms total.
  - 10µM Lu0128 delivered by permeating solid agar media and food.
  - Treatment with Lu0128 began on day 1 of adulthood (day 3 post-hatch)
  - Automated lifespan recording was performed using Life and Death Instrument (LaDI, InVivo Biosystems, Inc)

To understand the physiological action and therapeutic potential of compounds targeting aging, it is crucial to use quantitative models. The analysis of lifespan data is grounded in the study of two mathematical functions: the survival curve and the hazard function. The survival curve (Figure 2.1.A.) describes the fraction of a tested population that remains alive over time. The hazard function (Figure 2.1.B.) is related to the survival curve and provides an intuitive measure of the risk of death. This function describes the probability that a typical individual who is currently alive will soon die, providing a clear visualization of the way a compound may change patterns in mortality.

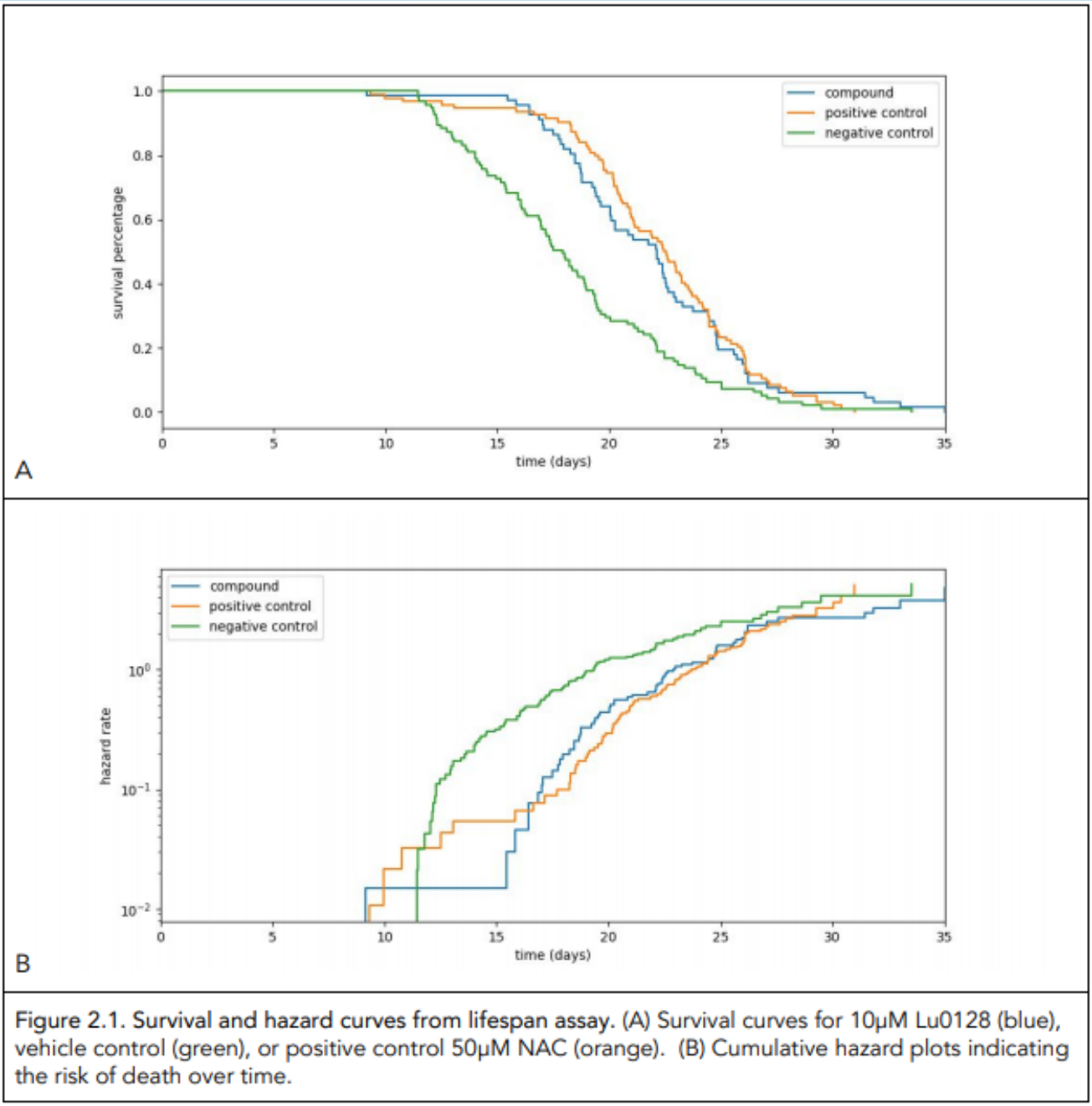

Figure 2.1. Survival and hazard curves from lifespan assay. (A) Survival curves for 10μM Lu0128 (blue), vehicle control (green), or positive control 50μM NAC (orange). (B) Cumulative hazard plots indicating the risk of death over time.

Worms that receive the Lu0128 treatment (blue line) lived significantly longer (maximum lifespan 33 days) than worms that did not (green line, maximum lifespan 28 days). Treatment with positive control NAC (orange line) also increased lifespan as expected. Tables 2.1 to 2.4 detail the raw measurements, calculations and statistical analysis related to the survival curve in Figure 2.1.A.

Lu0128 treatment temporally shifted survival by delaying early deaths (Figure 2.1.A). This is reflected in a delayed increase in the hazard rate (Figure 2.2.B.). Lu0128 increased maximum lifespan (Table 2.1.), with a small subpopulation exceeding the maximum lifespan

of the positive control. The hazard ratio (Table 2.4.) compares two treatments. It is not computed at any one time point, but includes all the data in the survival curve. The hazard ratio between the Lu0128-treated population and the untreated control population is 0.46, indicating that the rate of deaths in the Lu0128-treated group is 54% lower than in the control group.

Table 2.1. Lifespan assay summary

	Lu0128 (10μM)	Vehicle (H_2O)	Positive control
Death events	411	431	428
Censored subjects (#worms)	N/A	N/A	N/A
Median lifespan (days)	24	21	25
Average lifespan (days)	22	19	23
Maximum lifespan (days)	33	28	35

Table 2.2. Mean lifespan and age in days at percent mortality

treatment	Mean (days)	Mean 95 % CI	25% mortality	50%	75%	90%	100%
Lu0128	21.49	20.93 - 22.06	19.03	20.04	21.25	24.14	33
Vehicle (water)	19.81	19.43 - 20.19	17.18	19.16	20.31	20.99	28
NAC	22.46	21.98 - 22.95	18.5	21.57	23.19	25.08	35

Mean is calculated from the area under the survival curve. Age at percent mortality is calculated from a linear interpolation of the survival curve.

Table 2.3. Comparison of survival curves at specific time points using Fisher's exact test.

Treatment	at 25% mortality	at 50%	at 75%	at 90%
Lu0128 vs. Vehicle	0.002 **	3.90E-7 ****	0.0042 **	0.0052 *
Lu0128 vs. NAC	0.6782	0.0293 *	0.0593	0.4316 *
NAC v.s. Vehicle	0.0001***	0.00236 **	0.0044 **	0.00454 **

p-values for Fisher's exact test calculating the probability of obtaining the observed data at different stages of mortality if there is no difference between the two populations. P-values shown.

Table 2.4 Pairwise statistical analysis of survival curves.			
Curve comparison	Hazard Ratio	Log-rank (Mantel-Cox) test†	Gehan-Breslow-Wilcoxon test‡
Lu0128 (10µM) vs vehicle.	0.46	<0.0001 ****	<0.0001 ****
Lu0128 (10µM) vs positive control	1.07	0.17	0.23
The hazard ratio represents the overall rate of death relative to control. It is generalized to all of the data in the survival curve, and will not be consistent over time for survival curves that cross or inflect. †Mantel-Cox test compares groups across the duration of the lifespan. ‡Gehan-Breslow-Wilcoxon test applies more weight to earlier deaths. Numbers and asterisks represent P-value and significance, respectively.			

Worms treated with 10µM Lu0128 showed

- An increased median and maximum lifespan
- A significantly increased lifespan relative to vehicle control (Log-rank test, P-value = <0.0001).
- A significantly lower hazard ratio than untreated worms.

2.2. Healthspan, movement, and morphology analysis

To measure healthspan, precise movement data was collected concurrently with survival data from lifespan assay. Images collected automatically from the LaDI for lifespan recording were analyzed to assess healthspan.

- Aggregate movement data was captured from LaDI analysis

Worms treated with Lu0128 remained in a mobile state significantly longer than those treated with vehicle control and slightly longer than those treated with positive control.

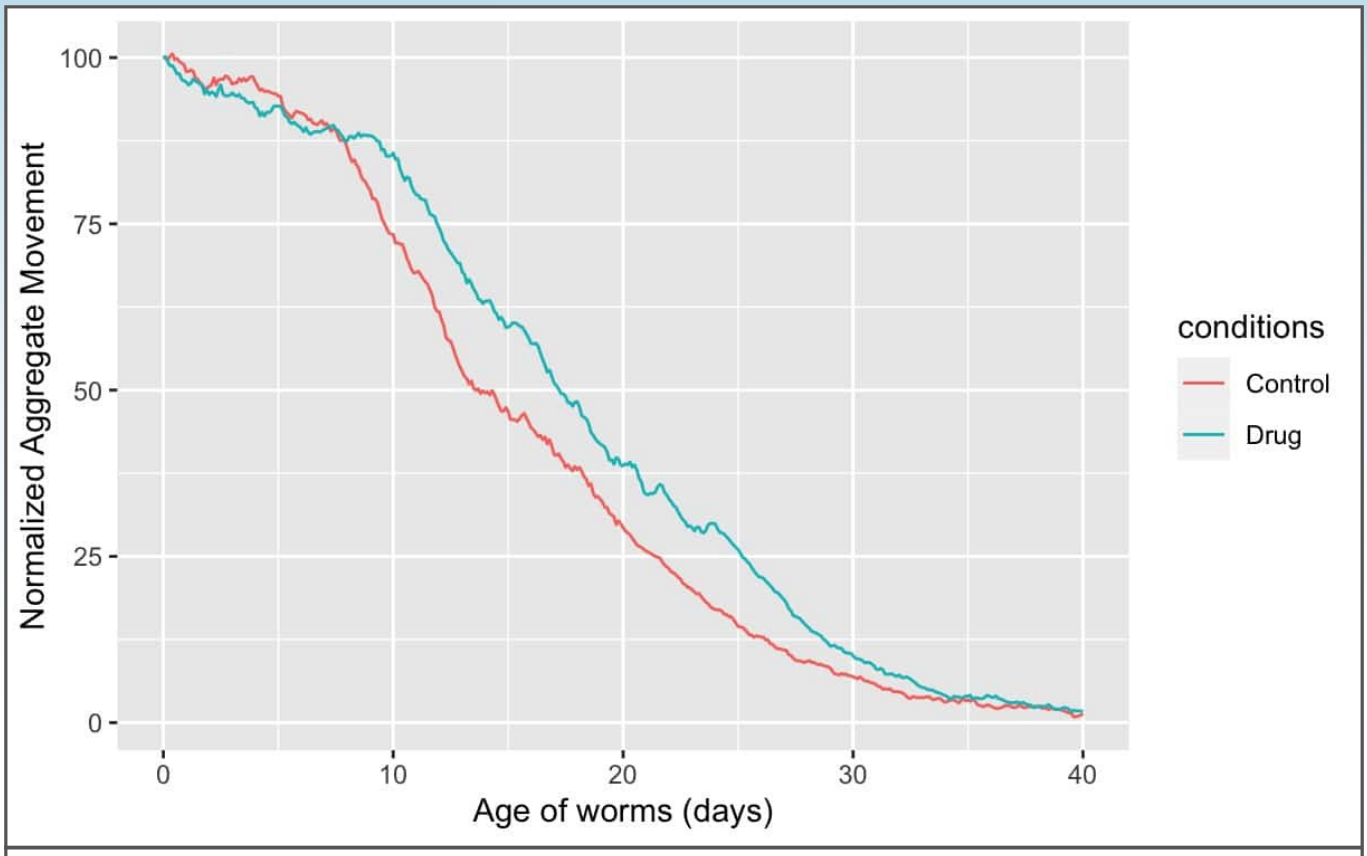

Figure 2.2 Aggregate motility and morphology analysis over duration of lifespan. Representative data from worms treated with either vehicle (Control) or a compound that improves healthspan (Drug). All measures are obtained by averaging data for all worms detected on a plate, then averaging across different replicate plates of the same condition. All measurements are based on worms that are still alive and moving at the time of quantification. Motility measures are normalized to fall between 0 and 100.

Table 2.5. Pairwise statistical analysis of movement/healthspan curves.

Treatment	HS_{50} P-value	Log-rank test
Lu0128 (10μM) vs vehicle	<0.0001 ****	<0.0001 ****
Lu0128 (10μM) vs positive control	0.013 *	0.021 *
Positive control vs vehicle	<0.0001 ****	<0.0001 ****

Healthspan-50 (HS_{50}) is the time (days) at which aggregate movement is reduced to 50% of maximum. HS_{50} = 12.5, 14.5, and 15.0 for vehicle, positive control, and Lu0128, respectively.
Lox-rank test compares the aggregate movement curves over the duration of the lifespan.

Step III. Identifying Aging Pathways

To determine the mode of action for Lu0128-mediated lifespan extension, we analyzed gene transcription in young and aged adults by RNA-Seq (whole transcription analysis). For each condition, >300 worms were treated in parallel with the lifespan assay (Step II) and maintained under identical conditions.

- Experimental conditions:
  - Lu0128 10µM Day 1 adult
  - Lu0128 10µM Day 15 adult
  - Vehicle Day 1 adult
  - Vehicle Day 15 adult

3.1 Gene expression analysis

A total of 74 genes showed significantly ($p<0.00001$) different expressions at day 10 of adulthood in Lu0128 treated vs. untreated animals (Figure 3.1, Table 3.1. and Supplementary Materials).

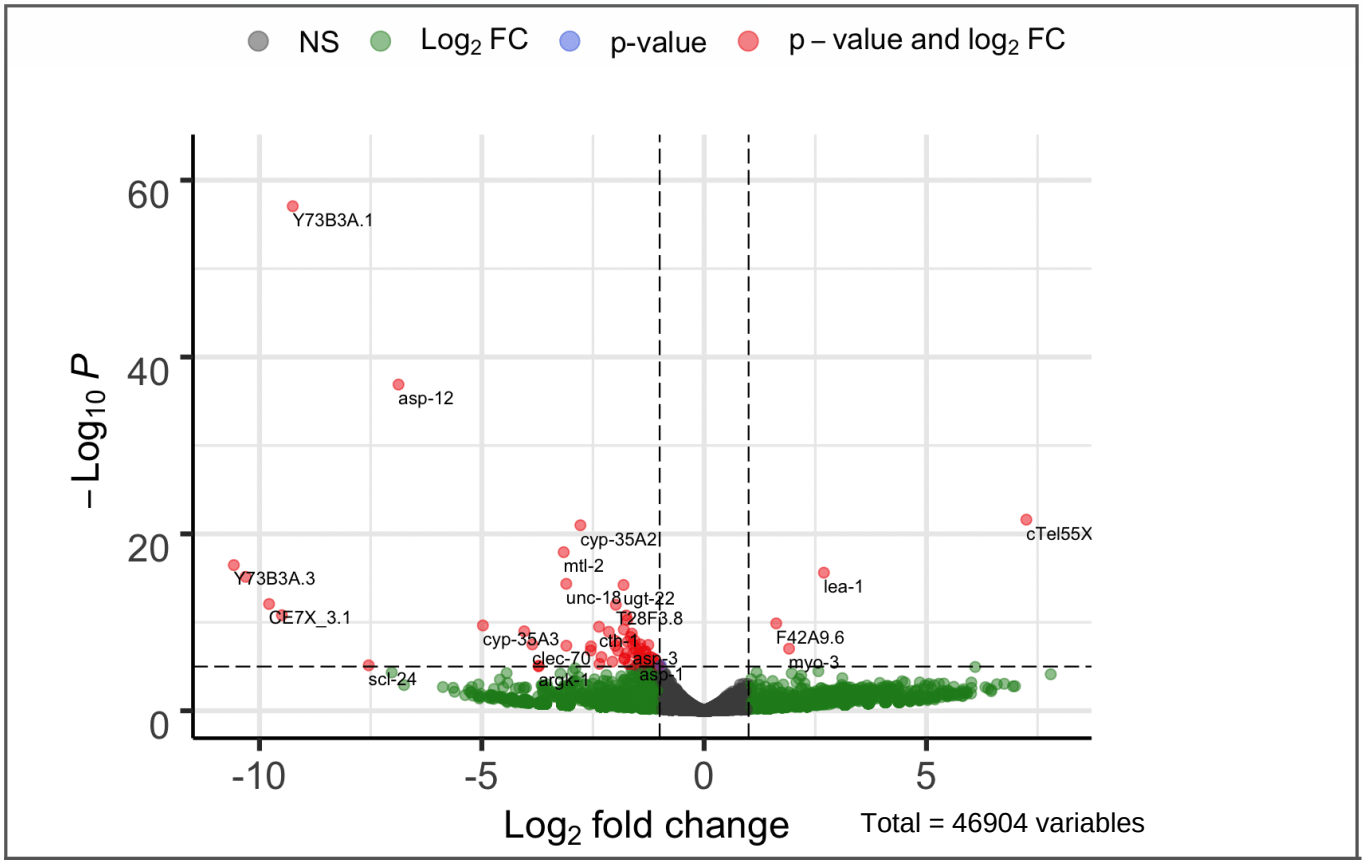

Figure 3.1.1 Volcano plat shows the negative log10 of P-values as a function of log2 fold changes of C. elegans genes for Lu0128 treated vs. untreated day 10 adult animals. Lu0128 treated day 10 animals have gene expression changes compared with untreated day 10 animals. Red dots indicate genes with significant changes

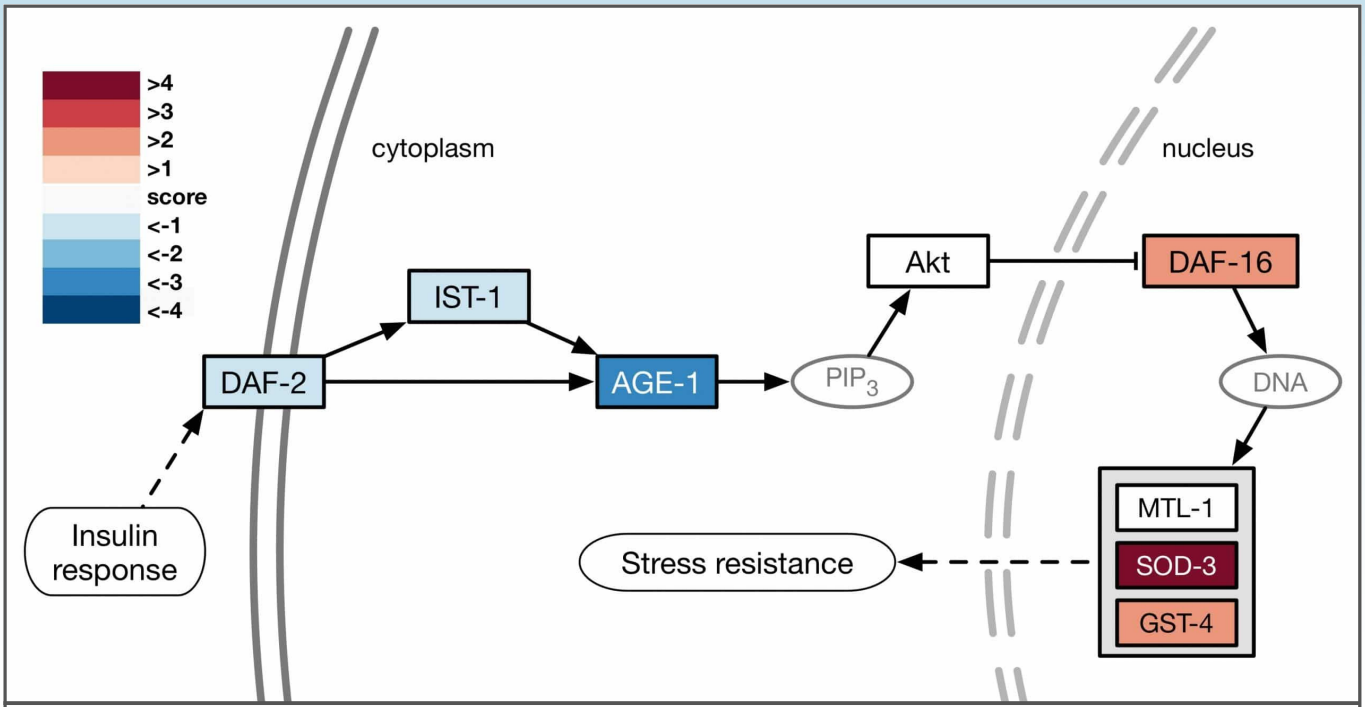

Figure 3.1.2 Mapping of gene expression data to longevity pathways in the KEGG database. Pathway shown represents changes of expression that might be observed under conditions of dietary restriction. Pathway components are color coded based on the level of up or down regulation of treated samples versus control.

Table 3.1 Individual genes highy up-or downregulated on day 10 by Lu0128. Top 20 most significant hits shown: Comprehensive list of genes is enclosed in Supplemental Materials.

gene	logFC	logCPM	LR	PValue
Y73B3A.1	-9.25	5.94	256.75	8.76E-58
asp-12	-6.87	8.85	164.32	1.29E-37
cTel55X.1	7.25	3.82	94.51	2.44E-22
cyp-35A2	-2.78	5.65	91.62	1.05E-21
mtl-2	-3.16	5.77	77.76	1.17E-18
Y73B3A.3	-10.58	2.53	71.12	3.36E-17
lea-1	2.69	9.58	67.21	2.43E-16
Y73B3A.21	-10.31	2.23	65.09	7.16E-16
unc-18	-3.10	4.08	61.51	4.41E-15
ugt-22	-1.81	6.47	60.93	5.93E-15
CE7X_3.1	-9.78	1.80	51.18	8.44E-13
T28F3.8	-1.99	5.41	50.78	1.03E-12
Y73B3A.20	-9.50	1.29	45.46	1.56E-11
F31F7.1	-1.75	6.37	45.32	1.67E-11
F23A7.8	-1.73	5.74	43.06	5.30E-11
F42A9.6	1.62	5.66	41.28	1.32E-10
cyp-35A3	-4.98	2.24	40.23	2.25E-10
cth-1	-2.36	6.33	39.57	3.17E-10
atic-1	-1.81	5.42	38.14	6.58E-10

3.2 Mechanism of Action Report

Our pathway analysis shows that worms treated with Lu0128 exhibit a significant change in the activity of pathways linked to oxidative phosphorylation and oxidative stress.

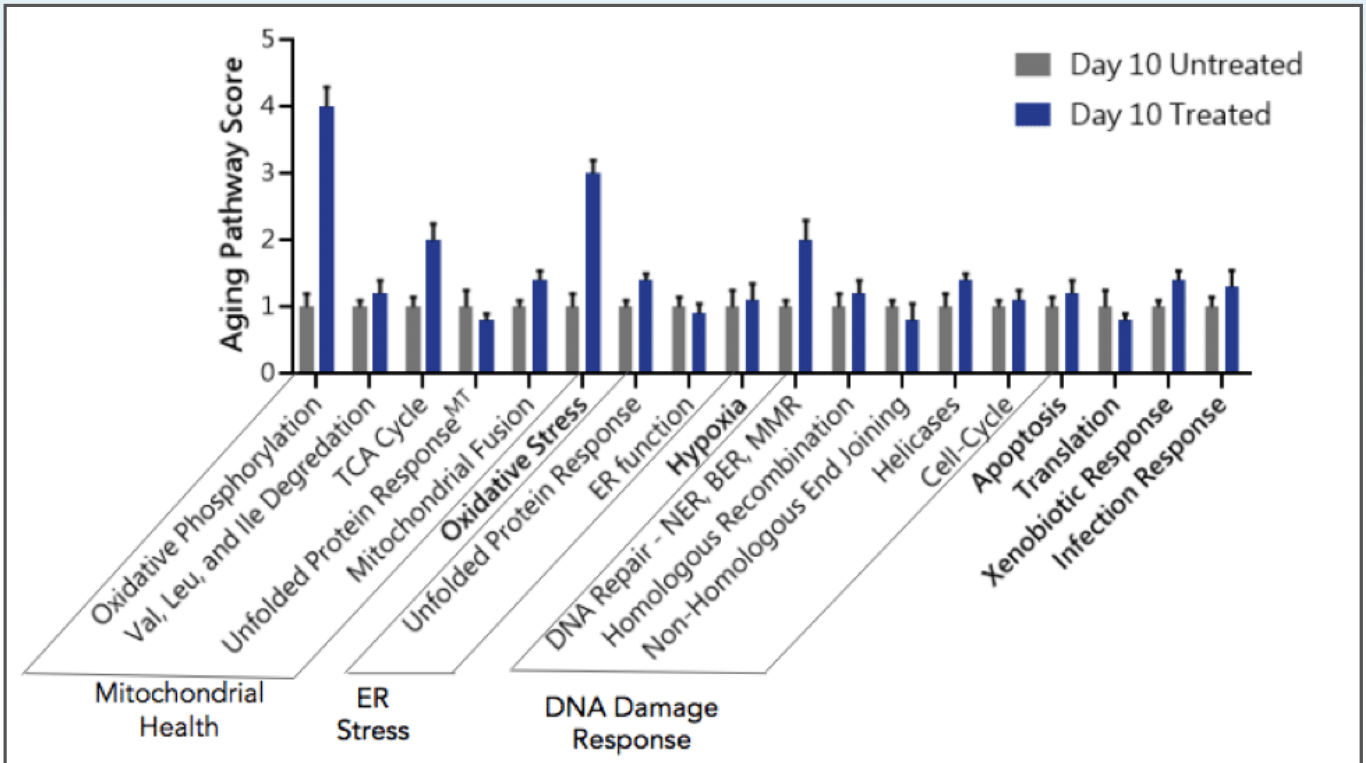

Figure 3.2. Curated longevity pathway analysis. Lu0128 treated 10 day old animals have expression changes in aging pathway genes compared with untreated 10 day old animals. Aging pathway enrichment analysis using our proprietary scoring metric to compare expression changes in day 10 adults treated with L0128 vs untreated.

METHODS, SUPPLEMENTAL MATERIALS, AND RAW DATA

The following files were sent to the customer via email in a .zip folder

- Assay design, materials and methods
  - ABCA-001_Materials_Methods.pdf
- Raw data for feasibility and doseage tests
  - ABCA-001_doseage_avoidance.xlsx
- Raw lifespan/healthspan assay data in spreadsheet.
  - ABCA-001_lifespan_scoring.csv
  - ABCA-001_movement.csv
- Raw RNA-seq data
  - ABCA-001_day1.csv
  - ABCA-001_day8.scv
- Supplemental data
  - ABCA-001_supplement.pdf