

Journal of Visualized Experiments

Quantification of Insoluble Protein Aggregation in *Caenorhabditis elegans* during Aging with a Novel Data-Independent Acquisition Workflow

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61366R1
Full Title:	Quantification of Insoluble Protein Aggregation in <i>Caenorhabditis elegans</i> during Aging with a Novel Data-Independent Acquisition Workflow
Section/Category:	JoVE Biology
Keywords:	protein aggregation, insolublome, aging, <i>Caenorhabditis elegans</i> , mass spectrometry, data-independent acquisition
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Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US\$4,200)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Novato, Marin County, CA 94945, USA



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Xiaoyan Cao, Ph.D.
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Dear Dr. Cao,

In response to the revisions, I would like to re-submit our revised manuscript titled “Quantification of Insoluble Protein Aggregation in *Caenorhabditis elegans* during Aging with a Novel Data-Independent Acquisition Workflow”. This work describes a workflow that we have optimized over years with my colleagues Drs. Gordon Lithgow and Julie Andersen, who are both experts in proteostasis and age-related diseases, and we will be using *C. elegans* models to perform fast and efficient drug screening assays for therapeutic interventions in aging.

We thank the reviewers for their revisions and we believe that we have thoroughly addressed all points mentioned by the reviewers. We have also added in a new Figure 5 in response to both reviewers’ comments to compare our protocol with previously published protocols, which showed as expected great overlap of proteins and pathways. So while our novel protocol provides a highly similar depth and quality of results, we can use greatly reduced amounts of starting material and the proteomic analysis has been demonstrated to be highly quantitative, reproducible and accurate.

Again, we thank the reviewers for the input and suggestions and we think that the revisions have nicely improved this manuscript. We have provided a detailed rebuttal which addresses each point brought up by the reviewers. Please let me know if you have any additional questions and I will be happy to provide anything else that may be needed.

Sincerely,

Birgit Schilling, PhD

Editorial comments:

Changes to be made by the author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: We have thoroughly proofread the manuscript and corrected spelling or grammar issues.

2. Please revise lines 374-376, 378-384, 386-388, 393-398, 400-410, 414-416, 418-427, 429-442, and 457-469 to avoid textual overlap with previously published work.

Response: As suggested we have revised the lines 374-376, 378-384, 386-388, 393-398, 400-410, 414-416, 418-427, 429-442, and 457-469, and we have changed the text accordingly.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by “(Table of Materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Thermo, Spectronaut, Roche, Bioruptor, NuPAGE, Sypro, ZipTips, Millipore, Ultra Plus, etc.

Response: We have removed all commercial language from the manuscript and used generic terms instead. All commercial products are referenced in the Table of Materials.

4. Keywords: Please remove “Spectronaut” (trademarked) to remove commercial language.

Response: We have removed all commercial language from the manuscript and used generic terms instead. Spectronaut specifically was removed from the keywords is now referenced in the Table of Materials. The text has been modified in multiple places, one example as follows:

“Relative quantification of aged and young C. elegans insolublome data was carried out using the ‘DIA Quantitative Analysis Software’ to process the novel DIA dataset and the generated spectral library.”

5. 1.2.3: Please specify the actions being repeated (both adding 10 Ml of S-basal solution and spinning?). Do you remove supernatant after spinning?

Response: This step has been revised to specify the actions being repeated and we also indicate that the supernatant was removed after centrifugation. The text was modified as follows:

“1.2.3. Spin the sample at 520 g for 30 sec. Remove supernatant after spinning. To eliminate any OP50 E. coli, add 10 mL of S-basal solution to the pellet and spin again followed by removal of the supernatant. Repeat this step.”

6. 3.17: What happens after centrifugation? Do you discard the supernatant? What is desalted, the supernatant?

Response: The supernatant containing the peptides was moved into a new 0.65 mL siliconized tube, then this supernatant containing the peptides was desalted. We have revised the text for this step to clarify:

“Spin the samples at 1850 g at room temperature for 5 min, aspirate the peptide solution and move into a new clean 0.65 mL siliconized tube, then desalt the peptides solution using C18 desalting tips (see below).”

7. Sections 5 and 6: Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Response: Thank you for this comment. We have implemented this simplification as suggested.

8. After you have made all the recommended changes to your Protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Response: Thank you. We now have 2.75 pages and no less than 1 page of the protocol text (including header and spacing) highlighted in yellow which can be featured in the video.

9. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Response: Thank you for this comment we now always highlight complete sentences, and we have removed highlights from the notes. We also have double checked that all highlighted sentences show a cohesive narrative.

10. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: Thank you for the comment. We have now provided all relevant details for the steps mentioned and highlighted all relevant information for filming. All sub-steps with relevant details have been highlighted.

11. Figure 2: Please mark the fragment sizes.

Response: We have generated a new Figure 2 now showing a gel that also contains the molecular weight markers (MW) with their sizes.

12. Table of Materials: Please remove any TM/[®]/[©] symbols. Please sort the materials alphabetically by material name.

Response: All TM/[®]/[©] symbols have been moved. The materials have been sorted alphabetically by material name.

13. References: Please do not abbreviate journal titles; use full journal name.

Response: Thank you – we have adjusted our Reference section accordingly. Full journal names are now used in the References section.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The method reported is important, timely and novel. This should be published to encourage further detailed analysis of *C. elegans* proteome. A few minor points need to be addressed for making the method clearer to novices in the field.

Response: We thank the reviewer for the positive feedback and the detailed and helpful suggestions below.

Major Concerns:
none

Minor Concerns:

Line 172: please indicate that the supernatant was taken after centrifugation.

Response: The text of this step (1.2.3) has been revised to indicate that the supernatant was removed after centrifugation. The text now is as follows:

“1.2.3. Spin the sample at 520 g for 30 sec. Remove supernatant after spinning. To eliminate any OP50 E. coli, add 10 mL of S-basal solution to the pellet and spin again followed by removal of the supernatant. Repeat this step.”

1.2.4: Room temperature or 4DC? Briefly would be ~5s? Any special tube?

Response: We have modified the text for this step accordingly to provide the requested details:

“Add 10 mL of sodium hypochlorite bleaching solution (containing 0.5 M KOH and 0.48% sodium hypochlorite in water) to the worm pellet in a 15 mL conical tube and vortex vigorously for 2 min at room temperature.”

1.2.7: how was the pellet obtained?

Response: We have added additional details. In fact, the egg pellet in step 1.2.7. was obtained by spinning at 520 g for 30 sec as described in step 1.2.4 and step 1.2.5

1.2.11: plates were kept inverted or upright?

Response: We have added “inverted” in the text to indicate that plates were kept inverted in this step. The text was modified as follows:

“1.2.11. Allow the S-basal solution to dry and then place plates inverted in a 20 °C incubator for 48 h.”

2.2: Any tubes recommended?

Response: We use and recommend 15 mL Falcon tubes in this step. 1.5

This is now also specified in steps 1.2.14 and 2.2.

2.4 what is the approximate protein concentration expected from the recommended worm numbers of N2?

Response: The approximate protein concentration from the recommended worm numbers of N2 in our protocol at this step is around 1.0-1.5 mg/mL. The text was modified as follows:

“To obtain the required input of protein lysate (1.0–1.5 mg) for isolation of the SDS-insoluble fraction, use approximately 3,000 synchronized worms per sample.”

2.7: Should we follow the note below while washing with SDS?

Response: This note was removed as we realized that we currently do not break up the pellet anymore using pipet tips as part of our protocol. The note was removed, but the steps are clearly described in 2.7.

2.9 Do we expect the whole pellet top dissolve?

Response: Yes, the entire pellet should be dissolved in this step. To clarify this, we have added a note after step 2.9 that reads as follows:

“NOTE: The SDS- insoluble pellet is typically hard to dissolve in the buffer. However, in the end the entire pellet should be dissolved in this step.”

2.11,12: what percentage gel is recommended for obtaining a good coverage?

Response: In this protocol, we have used a NUPAGE Bis-Tris gel with a gradient of 4-12% which has shown good coverage. We mention this detail now in the manuscript as follows:

“Load 13 μ L onto a 4-12% NUPAGE Bis-Tris gel and run the gel.”

3.12 Please mention the details of sonication.

Response: We are using a VWR M8800 Ultrasonic bath (117 V, holds 5.5 gallons). The sonicator details have now been added to the material table.

3.14 Please mention if the extract from 3.13 was pooled.

Response: The description of step 3.14 has been revised to indicate that the extract from both steps 3.13 and 3.14 were pooled. We added the following sentence:

“Pool all the solutions containing the extracted peptides from this step and the previous step 3.13 together into a single tube.”

4.4: Was 30 μ L of the solution pipetted up?

Response: Even though the volume of the solution is 30 μ L we are using a pipet set to 10 μ L and we pipet a volume of 10 μ L up and down 15 times. This action will ensure binding of all peptides to the resin of the desalting tips. The text was changed accordingly and a note was added:

“4.4. Set the pipet to 10 μ L and load the peptides from the solution to the resin by pipetting the digested peptides through the resin up and down 15x.

NOTE: The repeated action will ensure binding of all peptides to the resin of the desalting tips.”

4.5 Did you mean washing the zip tip?

Response: We used the word wash – but it would have been better to say “desalt”, in this step the peptides bound to the C18 resin in the Ziptip column were desalted here. We modified the text to the following:

“4.5. Desalt the digested peptides bound to the resin by pipetting up 10 μ L of 0.2% FA in water and then discarding it. Repeat this step 4x to complete the desalting step.”

4.8 Please define iRT peptide.

Response: The definition of iRT peptides “indexed Retention Time” has been added in this step. The sentence now reads:

“Resuspend peptides in 15 μ L of 0.2% FA + 1 μ L iRT (indexed Retention Time) peptides.”

Line 512: “..., even with a 592 fold enrichment...” - the statement is not clear.

Response: This sentence has been revised to be more clear and to read as follows:

“Aged worms were demonstrated to be significantly enriched for the insolublome: The largest change observed showed the relative protein abundance in the insolublome to be 592 times higher in the old versus young worms; and for 32 proteins the relative protein abundance in the insolublome was >250 times higher in the old versus young worms indicating dramatic insolublome changes with age.”

Comparison of data obtained this technique with the one using 40000 worms from previous reports will be helpful in convincing readers and future reviewers.

Response: We have compared the data obtained from this current protocol (this study) with previously published reports by David et al. 2010, Plos Biology and Mark et al. 2016, Cell Reports. This analysis is presented in the new Figure 5, and we also provide a new Supplemental Table 1 B which shows greatly overlapping gene ontologies. We have also added the following sentences to describe this comparison: *"We then compared the list of proteins identified in this study with previously published work from David et al.² and Mark et al.¹¹ as demonstrated in Venn diagrams (Figure 5A and B). The comparison showed significant overlap of identified proteins 394/721 and 444/721 with David et al (Figure 5A) and Mark et al. (Figure 5B) study, respectively. The biological pathways revealed by the KEGG analysis of insolublome from this study have also been identified in past thus validating our methodology (Supplementary Table 1B). Identification of these pathways and proteins suggests that they may serve as candidates for further biological investigation in regards to their function in the context of aging."*

Reviewer #2:

Manuscript Summary:

Aging is accompanied by the accumulation of toxic insoluble protein aggregates, which in turn further accelerate the aging process. Identifying the nature of these proteins is therefore of fundamental importance to better understand molecular mechanisms underlying the aging process. *C. elegans* is a powerful model organisms for aging studies, which the authors already used in the past to investigate the contribution of insoluble proteins to aging and associated disorders. In this paper, the authors followed up on their previous studies and describe in details a protocol that they optimized in *C. elegans* to improve isolation and further characterization of insoluble proteins. Specifically, they use a Bioruptor sonicator to enhance protein extraction from a substantially reduced number of worms as compared to previous protocols. Subsequent proteomic and bioinformatics analysis confirmed that wild type older animals accumulate increased amount of insoluble proteins as compared to young worms. The author developed a great extraction method that coupled with the following straightforward pipeline analysis, will certainly help improving our knowledge on the role of insoluble proteins in different pathophysiological context. Moreover, this improved protocol will represent an excellent tool for many other scientists working with *C. elegans*.

Response: We very much thank the reviewer for the encouraging words.

Major Concerns:

To further support the power and efficacy of their improved protocol it is recommended that the authors first validate their results against data already available in the literature. Namely, other works in the past, also from the authors, have already quantified and identified a series of insoluble proteins significantly enriched during aging. They should therefore compare results from these studies with those obtained from their newly developed protocol to ensure that the latter does not introduce biases due to the small amount of starting materials. Yielding different types or amount of proteins may indeed dramatically impact on the interpretation of the data of relevance for any biological process under investigation.

Response: We have compared the data obtained from the current protocol with previously published reports by David et al. in Plos Biology 2010 and Mark et al. in Cell Reports 2016. This analysis is presented in the new Figure 5. We also provide a new Supplemental Table 1 B which shows greatly overlapping gene ontologies. We have also added the following sentences to describe this comparison:

“We then compared the list of proteins identified in this study with previously published work from David et al.² and Mark et al.¹¹ as demonstrated by Venn diagrams (Figure 5A and B). The comparison showed significant overlap of identified proteins 394/721 and 444/721 with David et al (Figure 5A) and Mark et al. (Figure 5B) study, respectively. The biological pathway revealed by the KEGG analysis of insolublome from this study has also been identified in past thus validating our methodology (Supplementary Table 1B). Identification of these pathways and proteins suggests that they serve as candidates for further biological investigation in regards to their function in the context of aging.”

TITLE:

Quantification of Insoluble Protein Aggregation in *Caenorhabditis elegans* during Aging with a Novel Data-Independent Acquisition Workflow

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KEYWORDS:

protein aggregation, insolublome, aging, *Caenorhabditis elegans*, mass spectrometry, data-independent acquisition

SUMMARY:

This novel workflow efficiently extracts and isolates SDS-insoluble proteins (insolublome) from *Caenorhabditis elegans* with minimal starting material for quantitative differential proteomic analysis. The protocol uses a comprehensive data-independent acquisition mass spectrometry analysis to quantify the insolublome and bioinformatic analysis to gain biological insights into aging mechanisms and pathologies.

ABSTRACT:

We and others have shown that the aging process results in a proteome-wide accumulation of insoluble proteins. Knocking down genes encoding the insoluble proteins over 40% of the time results in an extension of the lifespan in *C. elegans*, suggesting that many of these proteins are key determinants of the aging process. Isolation and quantitative identification of these insoluble proteins are crucial to understand key biological processes that occur during aging. Here, we present a modified and improved protocol that details how to extract and isolate the SDS-insoluble proteins (insolublome) from *C. elegans* more efficiently to streamline mass spectrometric workflows via a novel label-free quantitative proteomics analysis. This improved protocol utilizes a highly efficient sonicator for worm lysis that greatly increases efficiency for

protein extraction and allows us to use significantly less starting material (approximately 3,000 worms) than in previous protocols (typically using at least 40,000 worms). Subsequent quantitative proteomic analysis of the insolublome was performed using data-dependent acquisition (DDA) for protein discovery and identification and data-independent acquisition (DIA) for comprehensive and more accurate protein quantification. Bioinformatic analysis of quantified proteins provides potential candidates that can be easily followed up with other molecular methods in *C. elegans*. With this workflow, we routinely identify more than 1000 proteins and quantify more than 500 proteins. This new protocol enables efficient compound screening with *C. elegans*. Here, we validated and applied this improved protocol to wild-type *C. elegans* N2-Bristol strain and confirmed that aged day-10 N2 worms showed greater accumulation of the insolublome than day-2 young worms.

INTRODUCTION:

Protein homeostasis progressively declines with aging and results in increased protein aggregation¹⁻³. Protein aggregation is associated with several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis⁴. Aging is considered a principal risk factor for the onset of neurodegenerative disorders associated with protein aggregation. Proteins that are prone to form insoluble aggregates are often linked with cellular toxicity and tissue dysfunction, which might further accelerate aggregation of other proteins⁵⁻⁷. Alternatively, insoluble protein aggregates may activate cellular defense mechanisms to remove the toxic oligomeric forms of the protein from the system. Knocking down selected genes encoding insoluble proteins modulates the lifespan of *Caenorhabditis elegans* in the context of both age-related disease and normal aging^{5,8,9}. Thus, studying the cellular and molecular mechanisms of protein aggregation is crucial to understanding aging and ultimately may lead to approaches for treating neurodegenerative diseases.

The nematode *C. elegans* has become one of the most extensively used model organism to study protein aggregation in aging and age-related diseases due to its unique characteristics, such as a relative short lifespan (around 2 weeks), ease of cultivation and genetic manipulation.

The ability to extract and characterize insoluble proteins has played a critical role in determining the age-related changes associated with protein aggregation in *C. elegans* models. To investigate the contribution of protein aggregation to normal aging processes, we⁵ and others² previously extracted and proteolytically digested the insolublome of young versus aged *C. elegans*, chemically labeled using iTRAQ reagents ('isobaric tagging for relative and absolute quantification'), and then quantified using MS-based methods. Using an isobaric labeling method and 120 mg of wet worms (about 40,000 worms), we were able to gain significant protein insolublome depth and coverage⁵. Quantitative analysis demonstrated that 203 out of 1200 proteins identified were significantly enriched in the insolublome of aged *C. elegans* compared to similar insolublome fractions from young worms⁵. Independently, David et al. also utilized an iTRAQ LC-MS/MS workflow to examine alterations in protein aggregates with normal aging². Starting with about 300 mg of worms, they identified ~1000 insoluble proteins using two biological replicates and determined that ~700 out of about 1000 proteins accumulated by 1.5-

fold or more with age compared to young worms². Overall, these independent results indicate that widespread protein insolubility and aggregation are an inherent part of normal aging and may affect both lifespan and the incidence of neurodegenerative disease^{2,5}.

Studying the insolublome had allowed us to determine how environmental influences can accelerate or decelerate the aging process. Klang et al. established label-free proteomic workflows in *C. elegans* to investigate the role of metallostasis in longevity¹⁰. In this study, at least 40,000 worms were used to extract the insolublome¹⁰. Data showed that iron, copper, calcium and manganese levels increase with aging and that feeding worms a diet with elevated iron significantly accelerated the age-related accumulation of insoluble proteins¹⁰. Using the same workflow to examine the effects of vitamin D on the insolublome of *C. elegans*, 38 proteins were quantified in young worms (Day 2) and 721 proteins in aged worms (Day 8). Vitamin D feeding significantly reduced the insolublome of aged worms from 721 to 371 proteins¹¹. Further investigation revealed that feeding vitamin D suppressed protein insolubility with age, promoted protein homeostasis, and extended the lifespan in *C. elegans* N2 wild type worms¹¹. Thus, studying the insolublome can help identify novel modulators of aging and age-related diseases.

While studying the insolublome has been invaluable in progressing the understanding of the aging process, it has been hindered by the requirement for collecting large amounts of starting sample material. Groh et al. recently introduced a label-free proteomic quantification workflow to study inherent protein aggregation changes in *C. elegans* with aging; however, it required large amounts of starting material (350 mg of ground worms)¹². In the present report, we established an improved new extraction and isolation protocol (**Figure 1**). Use of the highly efficient sonicator during worm lysis significantly improved the extraction efficiency and subsequently reduced the amount of starting material needed, from 40,000 to 3,000 worms. Combining this novel insolublome isolation protocol with a label-free data-independent acquisition (DIA) mass spectrometric workflow significantly improved protein depth and coverage. The protocol presented here is cost-effective and easily modified to allow the performance of insolublome analyses in other model systems.

PROTOCOL:

NOTE: For a better understanding of the experimental procedure, see **Figure 1** for a schematic of the workflow.

1. Mass culture of synchronized aging *C. elegans*

1.1. Plate preparation

1.1.1. Pour 30 mL of NGM agar with or without 50 μ M fluorodeoxyuridine (FUdR) into each 90 mm plate and let the plates dry for 2 days in culture hoods.

1.1.2. On the day before seeding the NGM agar plate, inoculate a 50 μ L aliquot of frozen glycerol stock of the OP50 bacterial strain into 1 L of pre-autoclaved LB medium in a 2 L flask.

1.1.3. Grow the bacteria at 37 °C in a shaking incubator at 250 rpm for 16 h.

1.1.4. After 16 h, centrifuge the bacterial cultures at a speed of 5,000 x *g* for 10 min.

1.1.5. Discard the supernatant and re-suspend the bacterial pellet completely in 40 mL of autoclaved S-basal solution (containing 50 mM K₃PO₄, 100 mM NaCl in water) to generate the 25x concentrated OP50 *E. coli* culture.

1.1.6. Dispense 2 mL of the 25x concentrated bacterial culture onto each NGM agar plate and spread evenly so that the bacterial culture covers the entire agar surface.

1.1.7. Remove the lids from the plates in a bacterial culture hood for 2–3 h until the plates are properly dried.

NOTE: Make sure to evenly distribute the bacteria by swirling the plates every 15 min. This is important as bacteria tend to concentrate on one side of the plate resulting in regions with low bacterial density.

1.1.8. After the plates are dried, close the lids and leave the plates at room temperature for 48 h to ensure complete drying of the OP50 *E. coli* food source.

NOTE: Plates can be prepared 2 weeks in advance and stored at 4 °C before use.

1.2. Maintenance and preparation of synchronized cohorts of *C. elegans*

1.2.1. To obtain the required input of protein lysate (1.0–1.5 mg) for isolation of the SDS-insoluble fraction, use approximately 3,000 synchronized worms per sample.

1.2.2. Transfer ~100 N2 wild-type gravid adult worms to a NGM 25x OP50 *E. coli*-seeded plate and maintain the worms at 20 °C for ~72 h until the plate is full with gravid adult worms.

1.2.3. Pipette ~10 mL of S-basal solution onto each plate and collect all the worms into a 15 mL tube.

NOTE: It is important to use low-retention tips or Pasteur pipettes during this and all future steps involving worm collection to prevent any loss of worms due to attachment to tip surfaces.

1.2.4. Spin the sample at 520 x *g* for 30 s. Remove the supernatant after spinning. To eliminate any OP50 *E. coli*, add 10 mL of S-basal solution to the pellet and spin again followed by removal of the supernatant. Repeat this step.

1.2.5. Add 10 mL of sodium hypochlorite bleaching solution (containing 0.5 M KOH and 0.48% sodium hypochlorite in water) to the worm pellet in a 15 mL conical tube and vortex vigorously for 2 min at room temperature.

1.2.6. Centrifuge at 520 x *g* for 30 s at room temperature to spin down any incompletely dissolved worm bodies and eggs. Then remove the supernatant.

1.2.7. Repeat steps 1.2.5 and 1.2.6 to ensure complete removal of all worm bodies and release of eggs (view tube under microscope to assure).

1.2.8. Wash the egg pellet at least 4 times with 10 mL of S-basal solution to ensure complete removal of bleach solution.

NOTE: Complete removal of bleach is important as remaining bleach may hinder the hatching of eggs.

1.2.9. Leave eggs resuspended in 5 mL of S-basal media in a 15 mL tube and place in rotor at 20 °C for 24 h to allow eggs to hatch.

1.2.10. After 24 h, take 10 µL of S-basal larvae solution to count the number of worms in it. Use at least three separate aliquots of 10 µL of S-basal larvae solution for replication of the worm count. Calculate the average of the three larval concentrations. Based on the average concentration, dilute the worm solution to obtain ~3,000 larvae per 200 µL of solution.

NOTE: Make sure to shake the tube gently by inverting as the larvae tend to settle if left undisturbed and that can affect the larva count.

1.2.11. Mix well and add 200 µL of S-basal larvae solution onto each of the 90-mm NGM bacteria-seeded plates.

NOTE: Add drops of S-basal larvae solution throughout the plate for even distribution of larvae to avoid bacterial depletion from certain spots.

1.2.12. Allow the S-basal solution to dry and then place plates inverted in a 20 °C incubator for 48 h.

1.2.13. After 48 h, collect the worms from the plate in S-basal solution and transfer them to a fresh NGM-seeded plate containing 50 µM FUDR and incubate plates at 20 °C.

NOTE: It is important to transfer worms early in the L4 stage to ensure FUDR efficiency.

1.2.14. Collect day-2 young adult samples after 48 h by adding 10 mL of S-basal solution onto the plate and transferring the solution into a 15 mL tube. Allow worms to settle by gravity and remove the S-basal solution after it becomes clear.

1.2.15. Wash the worm pellet 2-3x in 5 mL of S-basal solution to remove any attached bacteria. Remove as much S-basal solution as possible and freeze the worm pellet (in 15 mL tubes) in a dry ice/ethanol bath. Frozen samples are stored at -80 °C.

1.2.16. Transfer sample worms to a fresh NGM OP50 *E. coli* plate containing FUDR every alternate day until day 10. Collect and freeze worms as described in steps 1.2.14 and 1.2.15.

NOTE: Start with a higher number of worms for day-10 samples to account for any loss in worm number due to transfer and burrowing.

2. Extraction of SDS-insoluble fraction from worms

2.1. Thaw the frozen worm pellet on ice in the presence of 900 µL of ice-cold worm lysis buffer cocktail containing 20 mM Tris base, pH 7.4, 100 mM NaCl, 1 mM MgCl₂ and EDTA-free protease inhibitor. Briefly vortex samples to ensure complete resuspension of worm pellet in the lysis buffer.

2.2. Place the 15 mL tubes in the sonication bath and set for 10 sonication cycles (30 s on and 30 s off for each cycle) at high intensity. Repeat for up to five cycles (make sure worms are fully lysed).

2.3. Spin the lysate at 3,000 x *g* for 4 min in the cold room. After centrifugation, discard the pellet containing any worm debris and carefully transfer the supernatant to fresh pre-cooled 1.5 mL centrifuge tubes on ice.

2.4. Quantify the protein concentration using the BCA assay. Transfer lysate aliquots, typically containing about 1 mg of protein, into fresh pre-cooled 1.5 mL centrifuge tubes.

2.5. Centrifuge the protein lysate for 15 min at 20,000 x *g* in the cold room.

2.6. Without disturbing the pellet, transfer the supernatant into a fresh 1.5 mL centrifuge tube and save it as the aqueous-soluble protein fraction.

2.7. Wash the pellet from the previous step in 500 µL of worm lysis buffer containing 1% SDS at room temperature and centrifuge at 20,000 x *g* for 15 min at room temperature. Remove the supernatant and save as the SDS-soluble fraction. Repeat this washing step two times to remove any SDS-soluble fraction. The remaining pellet after the third round of washing is defined as the 1% SDS-insoluble protein fraction.

260 2.8. Resuspend the SDS-insoluble protein pellet in 60 μ L of 70% formic acid and vortex vigorously
261 to dissolve the proteins. Repeat vigorous vortexing as many times as necessary to dissolve the
262 pellet.

263
264 2.9. Sonicate the pellet for 30 min in an ultrasonicator water bath at room temperature.

265
266 NOTE: The SDS- insoluble pellet is typically hard to dissolve in the buffer. However, in the end the
267 entire pellet should be dissolved in this step.

268
269 2.10. Dry the samples in a vacuum concentrator for 1 h to completely remove the formic acid
270 solution.

271
272 2.11. Add 40 μ L of 1x LDS sample gel buffer to the dried pellet and heat the sample to 95 $^{\circ}$ C for
273 10 min. Briefly vortex and spin down the samples. Load 13 μ L onto a 4-12% NUPAGE Bis-Tris gel
274 and run the gel. Stain the gel with a fluorescent protein stain for imaging. Save the rest of the
275 sample for mass spectrometry (MS) analysis.

276
277 2.12. Load the remaining sample into a 4-12% Bis-Tris gel and run for about 20 min for MS in-gel
278 digestion.

279 **3. In-gel digestion with trypsin protease to isolate proteins for MS analysis**

280
281
282 3.1. Prepare the following solutions (fresh): 25 mM NH_4HCO_3 (pH 7–8), 25 mM NH_4HCO_3 in 50%
283 acetonitrile (ACN, pH 7–8), and 50% ACN in 5% formic acid.

284
285 3.2. Dice each gel slice into small pieces (typically $<1 \text{ mm}^2$) and place these into 0.65 mL
286 siliconized tubes.

287
288 3.3. Add about 100 μ L (or enough to cover) of a 25 mM NH_4HCO_3 /50% ACN solution and vortex
289 at room temp for 10 min. Extract the supernatant and transfer to a separate tube (to be
290 discarded). Repeat this step two more times.

291
292 3.4. Dry the gel pieces completely in a vacuum concentrator (~20 min).

293
294 3.5. Prepare fresh solutions and add ~100 μ L (or enough to cover) of 10 mM DTT in 25 mM
295 NH_4HCO_3 to the dried gel pieces (use freshly prepared NH_4HCO_3). Vortex and briefly spin. Allow
296 the reaction to proceed at 56 $^{\circ}$ C for 1 h and with shaking at 1400 rpm on a mixer.

297
298 3.6. Remove the supernatant and add 100 μ L of 55 mM iodoacetamide (IAA) in 25 mM NH_4HCO_3
299 to the gel pieces. Vortex and briefly spin. Allow reaction to proceed in the dark for 45 min at RT.

300
301 3.7. Remove the supernatant and discard. Wash the gel pieces by adding ~100 μ L of 25mM
302 NH_4HCO_3 and vortexing for 10 min. Briefly spin and remove the supernatant, discarding the latter.

303

3.8. Dehydrate the gel pieces by adding ~100 μ L (or enough to cover) of 25 mM NH_4HCO_3 in 50% ACN to the gel pieces and vortex for 10 min. Then briefly spin to remove and discard the supernatant. Repeat this step twice or even a third time if the gel pieces are not quite dry as indicated by smaller size of the gel pieces and cloudy white color.

3.9. Dry the gel pieces completely in a vacuum concentrator (~20 min).

3.10. Initially add 15 μ L of trypsin solution (250 ng trypsin) to each sample, and then add enough 25 mM NH_4HCO_3 to cover the gel pieces (~100 μ L). Vortex for 10 min. Then briefly spin and incubate at 4 $^\circ\text{C}$ in the cold room for 30 min without mixing.

3.11. Add 25 mM NH_4HCO_3 as needed to completely cover the gel pieces. Spin and incubate at 37 $^\circ\text{C}$ overnight for 16–20 h at 1400 rpm on a mixer.

3.12. The next day, briefly vortex and spin the digestion. Add about 100 μ L of HPLC grade water, spin, parafilm the tube and sonicate continuously for 10 min. Spin briefly after sonication.

3.13. Transfer the digestion solution, representing the aqueous extraction, into a clean 0.65 mL siliconized tube.

3.14. Add ~100 μ L of 50% ACN/5% formic acid to the gel pieces (enough to cover them), vortex 10 min at room temperature, spin briefly and collect the solution, and repeat once. Pool all solutions containing the extracted peptides from this step and the previous step 3.13 together into a single tube.

3.15. Vortex the extracted digestions. Dry the peptides completely in a vacuum concentrator (~2 h).

3.16. Add 30 μ L of 0.2% formic acid to resuspend the peptides on a mixer in the cold room for 10 min.

3.17. Spin the samples at 1850 $\times g$ at room temperature for 5 min. Aspirate the peptide solution and move into a new clean 0.65 mL siliconized tube. Then desalt the peptides solution using C18 desalting tips (see below).

4. Desalting digested peptides with C18 desalting tip

4.1. Set the pipette to 10 μ L and attach the C18 desalting tip. Wet the desalting tip by pipetting up 10 μ L of 100% ACN and then discarding it. Repeat this step 2x.

4.2. Wash the C18 desalting tip by pipetting up 10 μ L of 50% ACN, 49.8% water, and 0.2% FA and then discarding it. Repeat this step 2x.

4.3. Equilibrate the C18 desalting tip by pipetting up 10 μ L of 0.2% FA in water and then discarding it. Repeat this step 2x.

4.4. Set the pipet to 10 μ L and load the peptides from the solution to the resin by pipetting the digested peptides through the resin up and down 15x.

NOTE: The repeated action will ensure binding of all peptides to the resin of the desalting tips.

4.5. Desalt the digested peptides bound to the resin by pipetting up 10 μ L of 0.2% FA in water and then discarding it. Repeat this step 4x to complete the desalting step.

4.6. In a new tube, elute the peptides with 10 μ L of 50% ACN, 49.8% water, and 0.2% FA by pipetting up and down 10x. Repeat this step once to elute the protein digest for a second time in the same tube.

4.7. Dry the desalted peptides completely in a vacuum concentrator (~20 min).

4.8. Resuspend peptides in 15 μ L of 0.2% FA + 1 μ L iRT (indexed Retention Time) peptides. Vortex for 10 min, centrifuge at 12,000 $\times g$ for 2 min, and then transfer to an autosampler vial for MS analysis (see below).

5. Mass spectrometry analysis of digested peptides using DDA and DIA

NOTE: Samples can be analyzed using either DDA or DIA LC-MS/MS methods. In this study, the samples were analyzed using a nano-LC 2D HPLC system coupled to a high-resolution mass spectrometer.

5.1. Use a HPLC system combined with a chip-based HPLC system directly connected to a quadrupole time-of-flight mass spectrometer (other LC-MS configurations and systems can also be used).

5.2. Analyze samples using reverse-phase HPLC-ESI-MS/MS.

5.2.1. Build the chromatography protocol to load peptides sample onto a C18 pre-column chip. Wash and desalt the loaded peptides = with loading solvent (0.1% formic acid) for 10 min at a flow rate of 2 μ L/min.

5.2.2. Transfer the peptides to an analytical column C18 chip and elute at a flow rate of 300 nL/min with a 3 hour gradient using mobile phase A (2% acetonitrile, 0.1% formic acid) and B (98% acetonitrile, 0.1% formic acid). The first elution step consists of a linear gradient from 5% B to 35% B over a period of 80 min.

5.2.3. Ramp the mobile phase B to 80% over 5 min, and then maintain the mobile phase B at 80% for 8 min before changing into 5% B to re-equilibrate the column for 25 min.

5.3. Set up a MS instrument method for DDA and define the instrument parameters as following.

5.3.1. Use the following parameters for Experiment 1: MS1 precursor ion scan from m/z 400–1500 (accumulation time of 250 ms); set the intensity threshold to trigger MS/MS scans for ions of charge states 2–5 to 200 counts; set the dynamic exclusion of precursor ions to 60 s).

5.3.2. Use the following parameters for Experiment 2: MS/MS product ion scans with a MS2 scan range from m/z 100–1500 (accumulation time of 100 ms per each of the 30 product ion scans per cycle); set the collision energy spread to CES=5, and checkmark ‘high sensitivity product ion scan mode’.

NOTE: The DDA acquisition method is used to build spectral libraries as described in step 6.3 (see below). Here, it will acquire MS/MS spectra for the 30 most abundant precursor ions after each MS1 scan per cycle; the total cycle time is about 3.3 s.

5.4. Obtain DIA. Set up a MS instrument method for DIA and define the instrumental parameters as follows.

5.4.1. Use the following parameters for Experiment 1: MS1 precursor ion scan from m/z 400–1250 (accumulation time of 250 ms).

5.4.2. Use the following parameters for Experiment 2: MS/MS product ion scans for 64 variable SWATH segments with a MS2 scan range from m/z 100–1500 (accumulation time of 45 msec per each of the 64 product ion scans per cycle); set the collision energy spread to CES=10, checkmark ‘high sensitivity product ion scan mode’.

NOTE: Use the 64-variable window DIA/SWATH acquisition method as described by Schilling et al.¹³ to perform label-free quantification with a total cycle time of about 3.2 s. For DDA acquisition, instead of using the Q1 quadrupole to transmit precursor ion of a narrow mass range to the collision cell, a range of variable window width (for example m/z 5–90) is used to incrementally step over the full m/z range (m/z 400–1250) with 64 SWATH segments, 45 ms accumulation time for each segment, resulting in a cycle time of 3.2 sec, which includes one MS1 scan with 250 ms accumulation time. The variable window width is adjusted according to the complexity of the typical MS1 ion current observed within a certain m/z range using a SCIEX ‘variable window calculator’ algorithm¹³ (narrower windows are applied to ‘busy’ m/z ranges, wider windows are applied to m/z ranges with few eluting precursor ions). On other MS platforms, alternative DIA window selection strategies can also be implemented.

6. Data analysis

NOTE: Certain data analysis settings should be tailored to specific experimental conditions. For example, the protein database (fasta file) selected will depend on the species that the sample was prepared from (in this protocol we used *C. elegans*).

6.1. Use a **MS database search engine** to analyze DDA acquisitions and identify proteins. Generate a database search engine method as follows:

6.1.1. For Sample Description Parameters, select **Identification**; under **Sample Type**, select **Iodoacetic Acid**; under **Cysteine Alkylation**, select **Trypsin**; under **Digestion** (assuming C-terminal cleavage at lysine and arginine), select the name of the mass spectrometer under **Instrument**; and select ***Caenorhabditis elegans*** under **Species**.

6.1.2. For **Specific Processing** parameters, check **Biological modifications**; under **ID Focus**, select **SwissProt**; under **Database**, check **Thorough ID**; under **Search Effort**, select **0.05 (10%)** under **Detected Protein Threshold**; and check **Run False Discovery Rate Analysis** under **Results Quality**. Save the search engine method and submit the MS raw files to the database search engine for processing, using the generated method.

NOTE: In an iterative process, all MS and MS/MS scans were automatically recalibrated by the search engine based on initial annotations and results.

6.2. Click on **Export Peptide Summary** upon completion of the search and filter all peptide identification results by the confidence threshold of 99 in Excel (false discovery rate (FDR) = 1%).

6.3. Build MS/MS spectral libraries for further processing of the DIA raw data file, and for further relative data quantification.

6.3.1. Open the **DIA Quantitative Analysis Software**. Select **Library** tab and then (at the bottom of the page) click **Generate Spectral Library from "Database Search Engine"** and open a **"Database Search Engine"** FDR report (the *FDR.xlsx spreadsheet file) that was automatically generated as part of the DDA raw data file database search process.

6.3.2. Click **next**, select the **Library Settings Schema** and click **Next**. Select **Uniprot_*Caenorhabditis elegans* _proteome** as the database then click **Finish**, the spectral library will be generated.

6.4. Use the **DIA Quantitative Analysis Software** to analyze DIA acquisitions for comprehensive relative protein quantification. DIA data analysis pipeline is described as follows.

6.4.1. To analyze and quantify peptides, open the **DIA Quantitative Analysis Software** to set up an analysis schema by using the template schema. The template schema is available in the software by clicking **Settings**, then **DIA Analysis**, and finally **BGS Factory Settings**.

6.4.2. Set up the parameters in the template schema as the following:
1) under identification select PTM localization (probability cutoff = 0.75).

2) under quantification, select Major Group Quantity as Sum peptide quantity, set up Major Group N as Max 7 and Min 1, select Minor Group Quantity as Sum precursor quantity, and set up Minor Group N as Max 10 and Min 1, and selected Data Filtering as Qvalue sparse and do not select Cross Run Normalization.

6.5. Perform Relative Quantification Analysis.

6.5.1. Select the **Pipeline** tab, click **Set up a DIA Analysis from File**, open the MS DIA raw files of interest (for example: sample and control), click **Assign Spectral Library**, select the library that was generated in 6.3, click **Load**, and lastly click **Next**.

6.5.2. Select the analysis schema set up in 6.5.1 and click **Next**. Select the appropriate database fasta file (in this protocol: Uniprot_*Caenorhabditis elegans*_proteome) and click **Next**. Define the condition setup, and assign the different conditions according to the samples and click **Next**.

6.5.3. Review the analysis overview (summary of the experiment set-up) and browse to the **Output Directory** and click **Finish**. Finally click **Run Pipeline** to perform the label-free quantitative analysis.

NOTE: Statistical modules in the 'DIA Quantitative Analysis Software' automatically perform FDR analysis, generate heat maps and volcano plots comparing the different conditions, generate lists of identified and quantified peptides and proteins and provide Q-values along with relative fold-changes comparing different conditions. Here sample conditions set-up included Day 10 aged worms with two biological replicates and Day 2 young worms with two biological replicates as control.

REPRESENTATIVE RESULTS:

Traditional worm lysis methods have various disadvantages. For example, probe-based sonication and bead-beater methods produce excessive heat by allowing contact of the metal tip or beads directly with the samples, resulting in variable protein recoveries and protein denaturation. Liquid nitrogen grinding followed by sonication in lysis buffer, can be time-consuming and requires a large number of worms. Due to the limitations of traditional worm lysis methods, previous MS workflows, such as the labeling methods iTRAQ or label-free methods that have been historically used in the *C. elegans* model system to gain quantitative information about the insolublome, require large input of starting material (at least 40,000 worms). Laborious worm culture work is required to obtain these numbers of worms. Moreover, the labelling methods require expensive isobaric chemical labels. Label-free quantification methods are cost-effective and have easier and more straightforward sample preparation and labeling methods, but require significantly large numbers of worms to achieve sufficient MS analysis coverage.

The sonicator that we used greatly increases the efficiency and reproducibility of worm lysis by lysing multiple worms samples simultaneously in a temperature-controlled water bath sonicator without cross-contamination¹⁴, thus significantly reducing the amount of starting worm material required. Combining the highly efficient sonication method and the quantitative DIA label-free

MS approach, we were able to robustly quantify the insolublome of aged and young worms using ~3,000 worms. Here we tested and validated the efficiency of the protocol and compared the insolublome of aged and young worms from a wild-type worm strain, N2-Bristol *C. elegans*. We applied this protocol to extract and isolate the insolublome from ~3,000 aged and young N2 *C. elegans* (two biological replicates for each condition), followed by MS analysis with a quadrupole time-of-flight mass spectrometer or other MS systems using a combination of data-dependent acquisition (DDA) and data-independent acquisitions (DIA/SWATH) for protein identification and quantification. The insoluble proteins were first analyzed on a Bis-Tris 4-12% gradient gel to determine the amount of protein in each insolublome sample. As demonstrated in **Figure 2**, insolublome sample from N2 aged worms (lanes 2 and 3, biological replicate experiments) has significantly more protein than samples from the N2 young worms (lanes 1 and 4, biological replicate experiments).

After in-gel digestion, the protein profiles of the insolublome were analyzed by HPLC-MS. Using this workflow, we can generally identify 1000–1500 proteins and quantify 500–1,000 proteins from the SDS-insoluble fraction with high reproducibility (unpublished data). Here we were able to quantify 989 proteins from the insolublome of N2-Bristol *C. elegans* by analyzing the DIA data and removing redundancy: 768 proteins were significantly enriched and 27 proteins were significantly decreased in the insolublome of aged N2 worm (Day 10) compared with young (day 2) using a fold-change of at least 1.5 and a Q value of less than 0.01 (**Figure 3A**). As seen on the histogram plot (**Figure 3B**), the fold-change of significantly altered proteins shows a normal distribution. Aged worms were demonstrated to be significantly enriched for the insolublome: The largest change observed showed the relative protein abundance in the insolublome to be 592 times higher in the old versus young worms; and for 32 proteins the relative protein abundance in the insolublome was >250 times higher in the old versus young worms indicating dramatic insolublome changes with age.

After extracting the list of insoluble proteins that are significantly increased in the aged worms and identified by the wormbase (WS271), KEGG pathway and Gene Ontology (GO) analysis were performed to determine the pathways that are enriched in the aged insolublome to gain biological insights into how these relate to aging. The KEGG pathway analysis of proteins identified in this study shows enrichment of several pathways involving ribosomes, mitochondria, proteasome and spliceosome (**Figure 4A**). The gene ontology analysis shows that the insolublome from aged worms comprises many proteins in particular categories including mitochondrial, developmental, determinants of adult lifespan, and ribosomal proteins (**Figure 4B** and **Supplementary Table 1A**). We then compared the list of proteins identified in this study with previously published work from David et al.² and Mark et al.¹¹ as demonstrated in Venn diagrams (**Figure 5A,5B**). The comparison showed significant overlap of identified proteins 394/721 and 444/721 with David et al (**Figure 5A**) and Mark et al. (**Figure 5B**) study, respectively. The biological pathways revealed by the KEGG analysis of insolublome from this study have also been identified in past thus validating our methodology (**Supplementary Table 1B**). Identification of these pathways and proteins suggests that they may serve as candidates for further biological investigation in regards to their function in the context of aging.

In summary, the use of the efficient sonication method enables the lysis of multiple worm samples at the same time in an environment with well-controlled temperatures and reduced cross-contamination to achieve high protein coverage with significantly less starting worm material. Combining the efficient sonication method with a DIA label-free protein quantification workflow has provided reliable and reproducible results for the quantification of insoluble worm proteins.

FIGURE AND TABLE LEGENDS:

Figure 1. Experimental workflow of the protocol. *C. elegans* were cultured and collected on different days. After worm lysis with a sonicator, the 1% SDS-insoluble protein fraction (insolublome) was extracted and isolated from the lysate. The insolublome was then digested via in-gel trypsin digestion and quantified via DIA mass spectrometry, followed by bioinformatic analysis.

Figure 2. SDS-PAGE gel of the insolublome isolated young versus old worms of the N2-Bristol strain. The insolublomes of young versus old worms of the N2-Bristol strain were analyzed by SDS-PAGE to determine the amount of protein present. The SDS-PAGE gel was stained with a fluorescent protein stain to visualize protein bands. Lanes 1 and 4: Insolublome from two biological replicate experiments of young N2 worms (Day 2). Lanes 2 and 3: Insolublome from two biological replicate experiments of aged N2 worms (Day 10).

Figure 3. Protein candidates identified as showing significant alteration in the aged versus young insolublome and their fold-change distributions. (A) Volcano plot for quantification of the insolublome of aged versus young N2-Bristol worms. Candidates with an absolute fold change ≥ 1.5 and Q value < 0.01 are shown as red dots. (B) Histogram plot for fold-change distribution of significantly enriched SDS-insoluble proteins in aged versus young worm samples.

Figure 4. KEGG pathway and Gene Ontology (GO) analysis. (A) KEGG pathway analysis of the day-10 insolublome arranged according to *p*-value with highly significant pathway shown at the top. (B) Gene Ontology analysis shows that insolublome of aged worms is enriched for many proteins in particular categories including mitochondrial, developmental, determinants of adult lifespan, and ribosomal proteins. The scatterplot view visualizes the GO terms in a “semantic space” where the more similar terms are positioned closer together. The color of the bubble reflects the *p*-value obtained in the STRING analysis, while its size reflects the generality of the GO term in the UniProt-GOA database.

Figure 5. Insolublome protein overlap identified in day-10 insolublome comparing this study with (A) David et al.² and (B) Mark et al.¹¹ studies.

Supplementary Table 1 (related to Figure 4 and Figure 5). (A) Gene ontology (biological process) analyzed with STRING database. (B) Detailed list of proteins and KEGG pathways identified in this study with color codes depicting their overlap with the published work.

DISCUSSION:

In this protocol, we report an improved sample preparation method for the extraction of insoluble proteins from *C. elegans*. By replacing traditional worm lysis (e.g., probe sonication or bead beater techniques) with the efficient sonicator, we increased the yield of the insoluble protein extraction and reduced the number of worms needed for label-free MS analysis from 40,000 worms to 3,000 worms. A database search engine was used for protein identification from DDA data and the *C. elegans* spectral library was built using the 'DIA Quantitative Analysis Software' and the corresponding DDA database search results (importing FDR file reports generated by the database search engine). Relative quantification of aged and young *C. elegans* insolublome data was carried out using the 'DIA Quantitative Analysis Software' to process the novel DIA dataset and the generated spectral library.

Several steps are critical in the protocol. The short lifespan of *C. elegans* makes it an ideal system to study aging, compared to other eukaryotes such as mammalian cells, but it is crucial to isolate a homogenous population of worms when studying aging-related phenomenon. FUDR was used in this protocol to obtain synchronized aging worms. It is important to transfer worms early in their L4 stage onto the NGM-seeded plate containing FUDR to ensure its effectiveness. During worm lysis using the efficient sonicator, water bath temperature must be set at 4 °C and sonication at 30 s ON and 30 s OFF to prevent overheating of the samples. After the first round of sonication for 10 cycles (10 min), it is important to check under the microscope to assure that all worms were efficiently lysed. If not, more cycles of sonication are needed. During the process of in-gel digestion, each gel slice must be diced into pieces of the proper size (<1 mm²)—if too small, they may be lost in the sample preparation process, and if too large, the digestion may be inadequate.

The need for much less starting material significantly reduces the laborious work associated with worm culture to obtain samples for insolublome analyses. However, extraction and isolation of the 1% SDS-insoluble protein fraction involves multiple washing steps and careful sample handling is required to avoid sample loss and to ensure reproducible results. The amount of material generated for MS analysis is sufficient for ~3 injections for subsequent DDA and DIA analysis but not to save for future experiments. Furthermore, despite its potentially confounding effects¹⁵, we used the lowest possible concentration of FUDR to sterilize worms during the aging process. Future studies may circumvent the use of FUDR by using sterile mutants or by manually transferring and collecting worms.

Use of the highly efficient sonicator for worm lysis allows efficient extraction of the insolublome allowing good protein coverage and cost-effective label-free DIA MS analysis for quantify the insolublome using a greatly reduced number of worms. It significantly reduces the workload allowing for the screening for more conditions per experiment. In addition, the label-free MS DIA workflow is cost-effective and provides protein depth and coverage at comparable levels to labeling methods including iTRAQ, TMT or SILAC. The *C. elegans* model is a fast screening system for aging research. The workflow can be easily modified and applied to study aging and age-related disease research in this and other organisms. For example, in ongoing studies we are applying this workflow to investigate protein profiles of insolublome and proteostasis in various

Alzheimer's disease (AD) *C. elegans* models including A β , tau, and dual A β /tau worms with or without different drug interventions for future high-throughput drug screening.

ACKNOWLEDGMENTS:

This work was supported by a NIH shared instrumentation grant for a TripleTOF system (1S10 OD016281, Buck Institute), NIH grant, RF1 AG057358 (GJL, JKA) and NIH grant U01AG045844 (GJL). XX is supported by a T32 postdoctoral fellowship (NIH grant 5T32AG000266, PI: Judith Campisi and Lisa Ellerby). MC is supported by a postdoctoral fellowship from the Larry L. Hillblom Foundation.

DISCLOSURES:

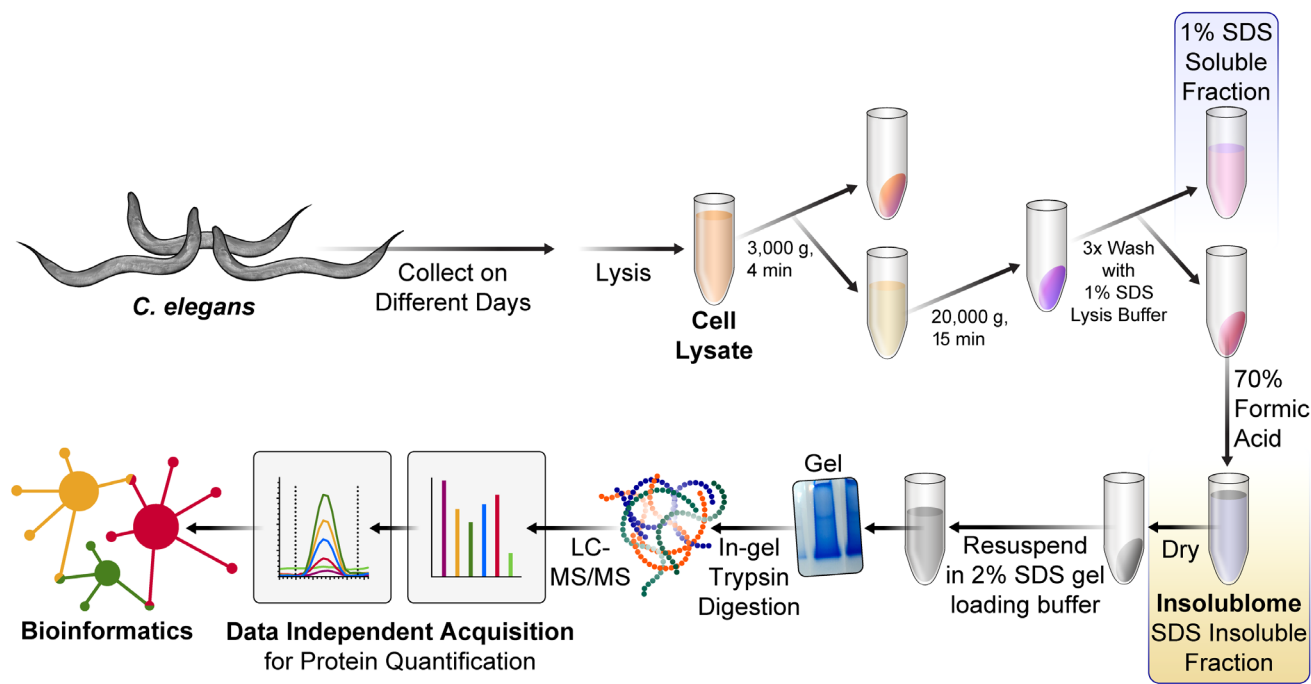
The authors have nothing to disclose.

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701

Figure 1



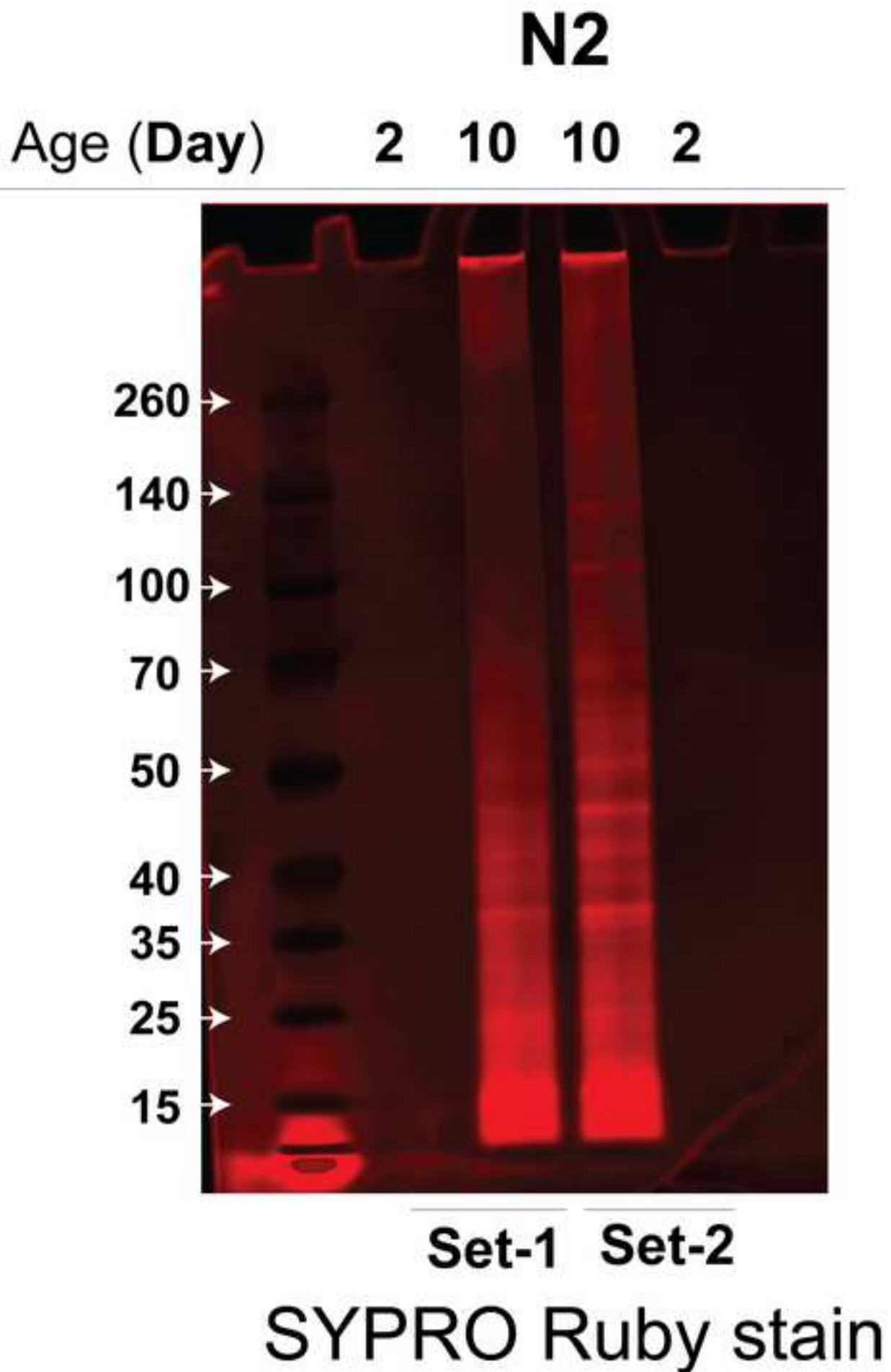
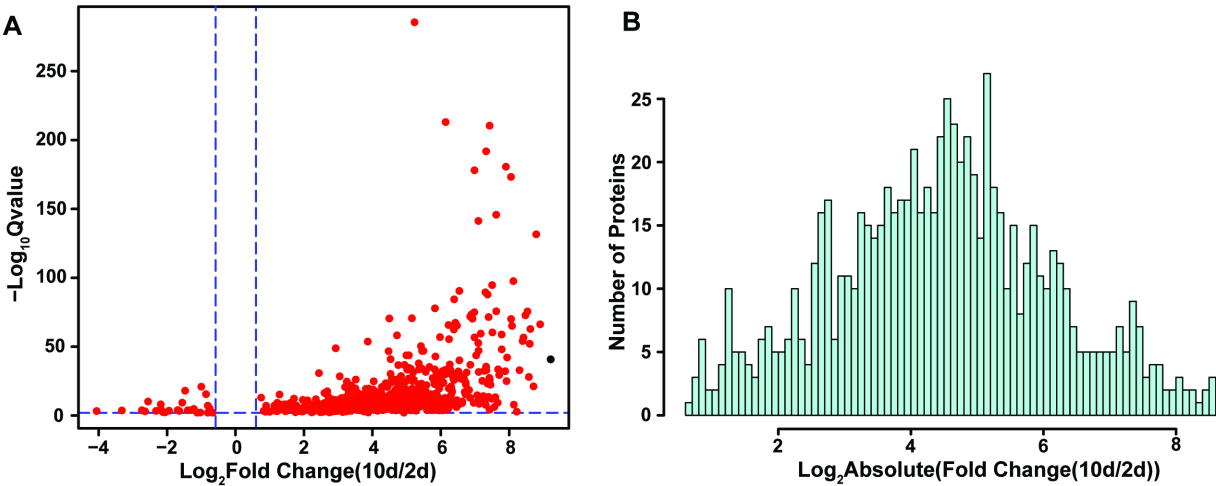


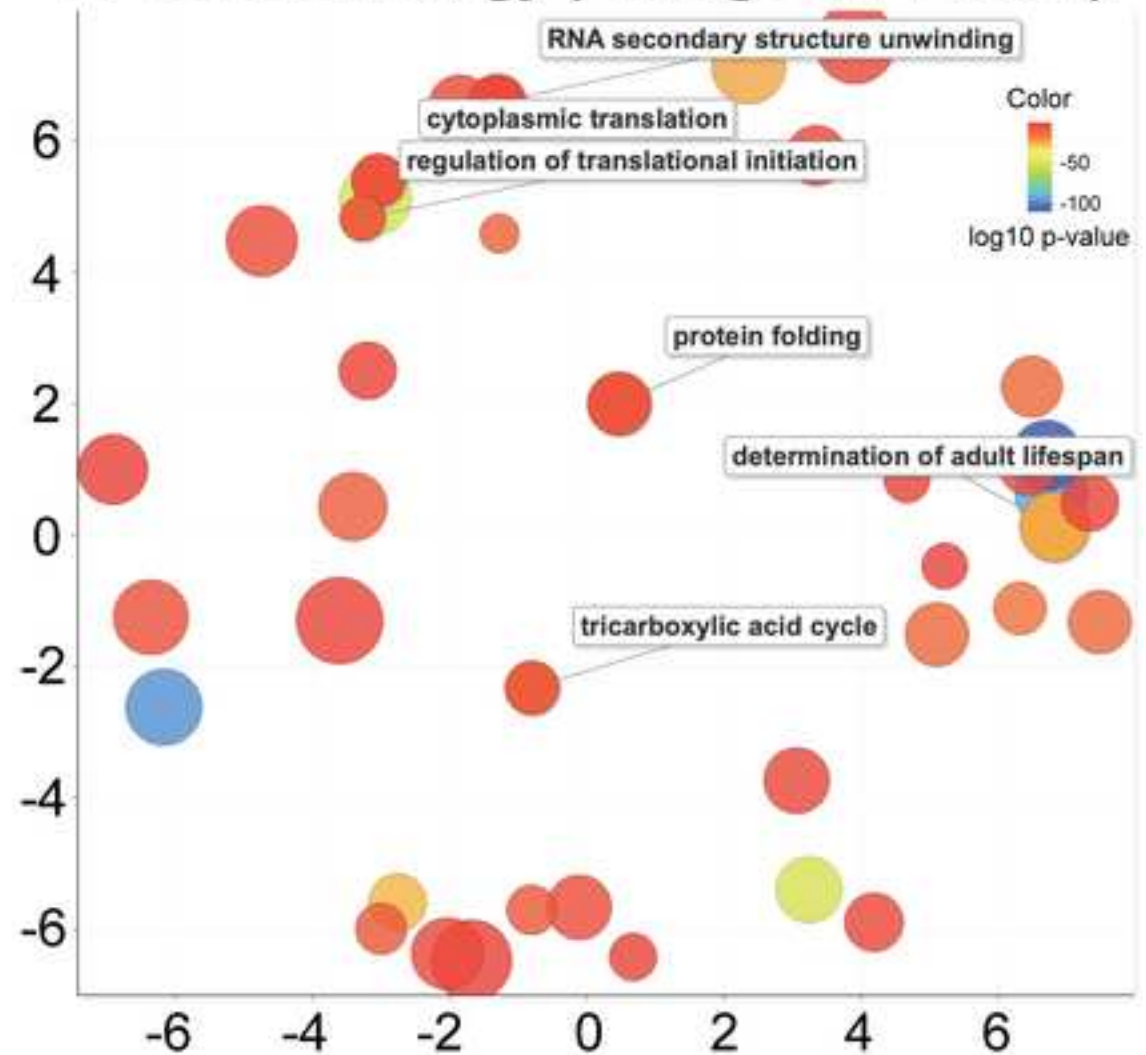
Figure 3



A Insolublome pathways

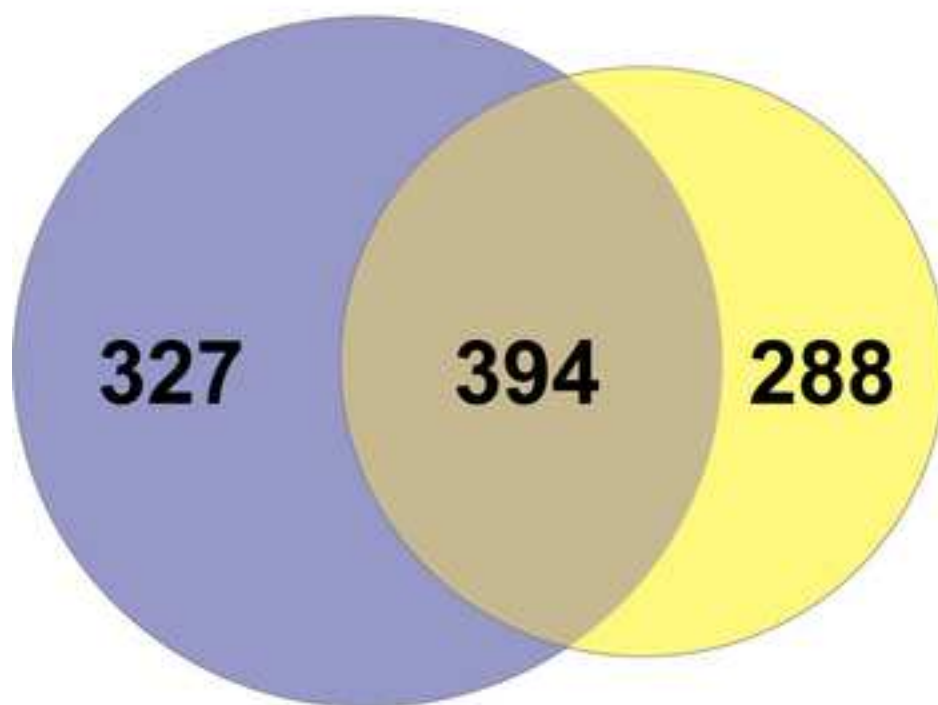
KEGG Pathway	Gene count	FDR
Ribosome	63	3.46E-43
Metabolic pathways	115	2.29E-33
Oxidative phosphorylation	43	2.67E-26
Phagosome	26	1.32E-16
Carbon metabolism	30	5.09E-15
Citrate cycle (TCA cycle)	18	4.22E-13
RNA transport	28	4.97E-13
Protein processing in endoplasmic reticulum	26	4.18E-10
Proteasome	13	2.18E-07
Valine, leucine and isoleucine degradation	14	2.61E-07
Glycolysis / Gluconeogenesis	12	1.29E-06
DNA replication	11	1.69E-06
Fatty acid metabolism	13	2.75E-06
Pyruvate metabolism	10	3.18E-06
Propanoate metabolism	10	3.18E-06
Fatty acid degradation	11	3.75E-05
Endocytosis	15	5.30E-05
Protein export	7	2.00E-04
ECM-receptor interaction	5	2.20E-04
Biosynthesis of amino acids	11	4.00E-04
Mismatch repair	6	5.10E-04
mTOR signaling pathway	11	7.70E-04
Fatty acid elongation	6	9.40E-04
Lysosome	12	1.30E-03
beta-Alanine metabolism	6	1.60E-03
N-Glycan biosynthesis	7	2.20E-03
Aminoacyl-tRNA biosynthesis	7	2.50E-03
2-Oxocarboxylic acid metabolism	5	3.50E-03
Amino sugar and nucleotide sugar metabolism	6	7.60E-03
Spliceosome	11	8.80E-03
Nucleotide excision repair	6	9.10E-03
RNA degradation	6	2.97E-02
Glyoxylate and dicarboxylate metabolism	5	3.30E-02
Fatty acid biosynthesis	3	4.14E-02

B Gene Ontology (Biological Process)

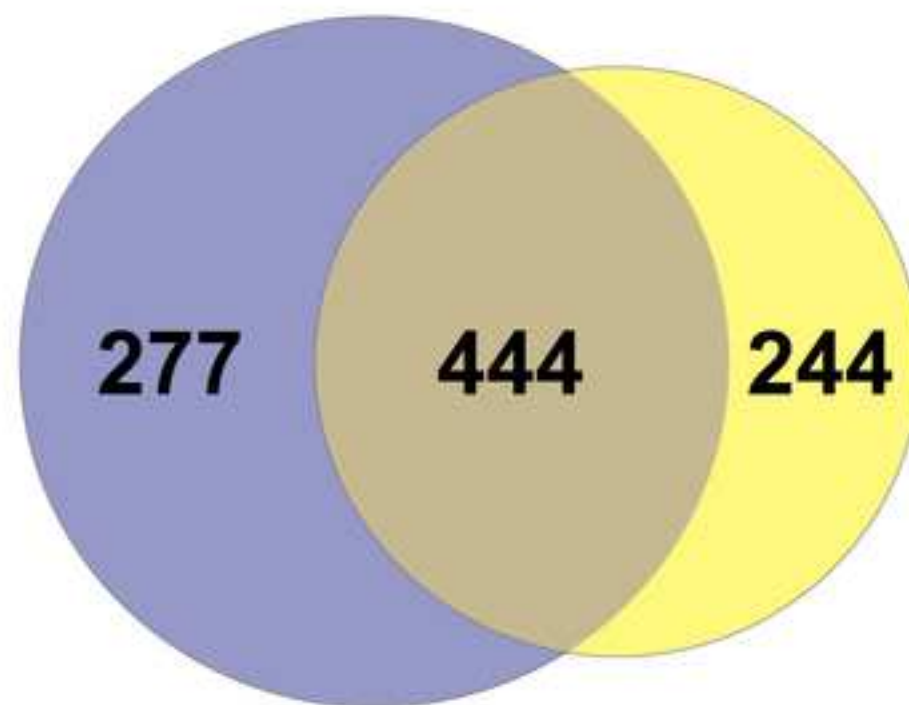


Insolublome protein overlap

A This study David et al.



B This study Mark et al.



Strains used*Escherichia coli* OP50

N2 (Bristol)

Buffer/Solution

NGM (Nematode Growth Media)

S-basal solution

Sodium hypochlorite bleach solution

Name of Material/ Equipment

Agar

Bioruptor Plus sonication device

Cholesterol

2'-deoxy-5-fluorouridine

Glycerol

LB broth, Miller

Sodium dodecyl sulfate (SDS)

Sodium chloride

M880 Ultrasonic bath, 117 V, holds 5.5 gallons

Magnesium sulphate

Magnesium chloride

NGM agar plate

NuPAGE LDS Sample Buffer (4X)

NuPAGE protein gels, 4-12%

Protease inhibitor cocktail (PIC)

Pierce BCA Assay

Sodium hypochlorite 5%

SYPRO Ruby Protein Gel Stain

MS Section

Acetonitrile, Burdick and Jackson LC-MS

Agilent Zorbax 300Extend C18 column

Ammonium bicarbonate

Dithiothreitol (DTT)

Eppendorf Thermomixer Compact

Formic acid

Indexed retention time (iRT) normalization peptide standard

Iodoacetamide (IAA)

Methanol, HPLC Grade

Nano cHiPLC Trap ChromXP C18-CL, 200 μ m x 6 mm, 3 μ m, 120A.(pre-column chip) (200 μ m x 6 mm ChromXP C18-CL chip, 3 μ m, 300 A)Nano cHiPLC ChromXP 75 μ m by 15cm, C18-CL, 3 μ m, 120 A

(analytical column chip)

Orthogonal quadrupole time-of-flight (QqTOF) TripleTOP 6600 mass spectrometer

ProteinPilot 5.0

Savant SPD131DDA Speedvac Concentrator
Sequencing-grade lyophilized trypsin
Spectronaut
SWATH 2.0 plugin into PeakView 2.2
Ultra Plus nano-LC 2D HPLC system
Water, Burdick and Jackson LC-MS
Waters 1525 binary HPLC pump system
Waters 2487 Dual Wavelength UV detector
Waters 717plus Autosampler
Waters Fraction Collector III

Caenorhabditis Genetics Center (CGC)
 Caenorhabditis Genetics Center (CGC)

Recipe

3 g/L NaCl, 23 g/L agar; 2.5 g/L peptone; 1 mM CaCl₂, 5 mg/L cholesterol, 1 mM MgSO₄, 25 mM KH₂PO₄
 5.85 g/L NaCl, 1g/L K₂HPO₄, 6 g/L KH₂PO₄, H₂O to 1 L
 Mix 0.5 mL 5 N NaOH with 1 ml Sodium hypochlorite (5%) and make volume to 5 mL with H₂O.

Company/Source	Catalogue Number	Comments/Description
Difco Granulated Agar, BD Biosciences	90000-782	
Diagenode, USA	B01020001	
Sigma	c8503	
VWR	TCD2235	
Millipore Sigma	356350-1000ML	
Millipore Sigma	60801-450	
Sigma	L4509-250G	
Sigma	59888	
VWR, USA	89375-458	
Sigma	M506	
Sigma	208337	
VWR Disposable Petri Dishes	25384-342	
Thermo Fisher Scientific	NP0007	
Invitrogen	NP 0335BOX	
Roche	11836170001	
Thermo Fisher Scientific	23225	
VWR	JT9416-1	
Thermo Fisher Scientific	S12000	
Honeywell International Inc., Charlotte, NC, USA	36XL66	
Agilent Technologies Inc., Santa Clara, CA, USA	770995-902	
Sigma Aldrich, St. Louis, MO, USA	9830 (1 kg)	
Sigma Aldrich, St. Louis, MO, USA	D9779-5G	
Eppendorf AG, Hamburg, Germany	T1317-1EA	
Sigma Aldrich, St. Louis, MO, USA	F0507-500ML	
Biognosys AG, Schlieren, Zurich, Switzerland	Ki-3002-2	
Sigma Aldrich, St. Louis, MO, USA	I1149-25G	
Honeywell International Inc., Charlotte, NC, USA	34885	
Sciex LLC, Framingham, MA, USA	804-00006	
Sciex LLC, Framingham, MA, USA	804-00001	
Sciex LLC, Framingham, MA, USA	Per quote	
Sciex LLC, Framingham, MA, USA	software download Sciex	

Thermo Fisher Scientific, Waltham, MA, USA
Life Technologies
Biognosys AG, Schlieren, Zurich, Switzerland
Sciex LLC, Framingham, MA, USA
Sciex LLC, Eksigent Division, Framingham, MA, USA
Honeywell International Inc., Charlotte, NC, USA
Waters Corp., Milford, MA, USA
Waters Corp., Milford, MA, USA
Waters Corp., Milford, MA, USA
Waters Corp., Milford, MA, USA

SPD131DDA-115
23225
Sw-3001
software download Sciex
Model # 845
600-30-76
WAT022939
WAT081110
WAT022939
186001878

on

Editorial comments:

Changes to be made by the author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: We have thoroughly proofread the manuscript and corrected spelling or grammar issues.

2. Please revise lines 374-376, 378-384, 386-388, 393-398, 400-410, 414-416, 418-427, 429-442, and 457-469 to avoid textual overlap with previously published work.

Response: As suggested we have revised the lines 374-376, 378-384, 386-388, 393-398, 400-410, 414-416, 418-427, 429-442, and 457-469, and we have changed the text accordingly.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by “(Table of Materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Thermo, Spectronaut, Roche, Bioruptor, NuPAGE, Sypro, ZipTips, Millipore, Ultra Plus, etc.

Response: We have removed all commercial language from the manuscript and used generic terms instead. All commercial products are referenced in the Table of Materials.

4. Keywords: Please remove “Spectronaut” (trademarked) to remove commercial language.

Response: We have removed all commercial language from the manuscript and used generic terms instead. Spectronaut specifically was removed from the keywords is now referenced in the Table of Materials. The text has been modified in multiple places, one example as follows:

“Relative quantification of aged and young C. elegans insolublome data was carried out using the ‘DIA Quantitative Analysis Software’ to process the novel DIA dataset and the generated spectral library.”

5. 1.2.3: Please specify the actions being repeated (both adding 10 ML of S-basal solution and spinning?). Do you remove supernatant after spinning?

Response: This step has been revised to specify the actions being repeated and we also indicate that the supernatant was removed after centrifugation. The text was modified as follows:

“1.2.3. Spin the sample at 520 g for 30 sec. Remove supernatant after spinning. To eliminate any OP50 E. coli, add 10 mL of S-basal solution to the pellet and spin again followed by removal of the supernatant. Repeat this step.”

6. 3.17: What happens after centrifugation? Do you discard the supernatant? What is desalted, the supernatant?

Response: The supernatant containing the peptides was moved into a new 0.65 mL siliconized tube, then this supernatant containing the peptides was desalted. We have revised the text for this step to clarify:

“Spin the samples at 1850 g at room temperature for 5 min, aspirate the peptide solution and move into a new clean 0.65 mL siliconized tube, then desalt the peptides solution using C18 desalting tips (see below).”

7. Sections 5 and 6: Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Response: Thank you for this comment. We have implemented this simplification as suggested.

8. After you have made all the recommended changes to your Protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Response: Thank you. We now have 2.75 pages and no less than 1 page of the protocol text (including header and spacing) highlighted in yellow which can be featured in the video.

9. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Response: Thank you for this comment we now always highlight complete sentences, and we have removed highlights from the notes. We also have double checked that all highlighted sentences show a cohesive narrative.

10. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: Thank you for the comment. We have now provided all relevant details for the steps mentioned and highlighted all relevant information for filming. All sub-steps with relevant details have been highlighted.

11. Figure 2: Please mark the fragment sizes.

Response: We have generated a new Figure 2 now showing a gel that also contains the molecular weight markers (MW) with their sizes.

12. Table of Materials: Please remove any [™]/[®]/© symbols. Please sort the materials alphabetically by material name.

Response: All [™]/[®]/© symbols have been moved. The materials have been sorted alphabetically by material name.

13. References: Please do not abbreviate journal titles; use full journal name.

Response: Thank you – we have adjusted our Reference section accordingly. Full journal names are now used in the References section.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The method reported is important, timely and novel. This should be published to encourage further detailed analysis of *C. elegans* proteome. A few minor points need to be addressed for making the method clearer to novices in the field.

Response: We thank the reviewer for the positive feedback and the detailed and helpful suggestions below.

Major Concerns:

none

Minor Concerns:

Line 172: please indicate that the supernatant was taken after centrifugation.

Response: The text of this step (1.2.3) has been revised to indicate that the supernatant was removed after centrifugation. The text now is as follows:

*“1.2.3. Spin the sample at 520 g for 30 sec. Remove supernatant after spinning. To eliminate any OP50 *E. coli*, add 10 mL of S-basal solution to the pellet and spin again followed by removal of the supernatant. Repeat this step.”*

1.2.4: Room temperature or 4DC? Briefly would be ~5s? Any special tube?

Response: We have modified the text for this step accordingly to provide the requested details:

“Add 10 mL of sodium hypochlorite bleaching solution (containing 0.5 M KOH and 0.48% sodium hypochlorite in water) to the worm pellet in a 15 mL conical tube and vortex vigorously for 2 min at room temperature.”

1.2.7: how was the pellet obtained?

Response: We have added additional details. In fact, the egg pellet in step 1.2.7. was obtained by spinning at 520 g for 30 sec as described in step 1.2.4 and step 1.2.5

1.2.11: plates were kept inverted or upright?

Response: We have added “inverted” in the text to indicate that plates were kept inverted in this step. The text was modified as follows:

“1.2.11. Allow the S-basal solution to dry and then place plates inverted in a 20 °C incubator for 48 h.”

2.2: Any tubes recommended?

Response: We use and recommend 15 mL Falcon tubes in this step. 1.5

This is now also specified in steps 1.2.14 and 2.2.

2.4 what is the approximate protein concentration expected from the recommended worm numbers of N2?

Response: The approximate protein concentration from the recommended worm numbers of N2 in our protocol at this step is around 1.0-1.5 mg/mL. The text was modified as follows:

“To obtain the required input of protein lysate (1.0–1.5 mg) for isolation of the SDS-insoluble fraction, use approximately 3,000 synchronized worms per sample.”

2.7: Should we follow the note below while washing with SDS?

Response: This note was removed as we realized that we currently do not break up the pellet anymore using pipet tips as part of our protocol. The note was removed, but the steps are clearly described in 2.7.

2.9 Do we expect the whole pellet top dissolve?

Response: Yes, the entire pellet should be dissolved in this step. To clarify this, we have added a note after step 2.9 that reads as follows:

“NOTE: The SDS- insoluble pellet is typically hard to dissolve in the buffer. However, in the end the entire pellet should be dissolved in this step.”

2.11,12: what percentage gel is recommended for obtaining a good coverage?

Response: In this protocol, we have used a NUPAGE Bis-Tris gel with a gradient of 4-12% which has shown good coverage. We mention this detail now in the manuscript as follows:

“Load 13 μ L onto a 4-12% NUPAGE Bis-Tris gel and run the gel.”

3.12 Please mention the details of sonication.

Response: We are using a VWR M8800 Ultrasonic bath (117 V, holds 5.5 gallons). The sonicator details have now been added to the material table.

3.14 Please mention if the extract from 3.13 was pooled.

Response: The description of step 3.14 has been revised to indicate that the extract from both steps 3.13 and 3.14 were pooled. We added the following sentence:

“Pool all the solutions containing the extracted peptides from this step and the previous step 3.13 together into a single tube.”

4.4: Was 30 μ L of the solution pipetted up?

Response: Even though the volume of the solution is 30 μ L we are using a pipet set to 10 μ L and we pipet a volume of 10 μ L up and down 15 times. This action will ensure binding of all peptides to the resin of the desalting tips. The text was changed accordingly and a note was added:

“4.4. Set the pipet to 10 μ L and load the peptides from the solution to the resin by pipetting the digested peptides through the resin up and down 15x.

NOTE: The repeated action will ensure binding of all peptides to the resin of the desalting tips.”

4.5 Did you mean washing the zip tip?

Response: We used the word wash – but it would have been better to say “desalt”, in this step the peptides bound to the C18 resin in the Ziptip column were desalted here. We modified the text to the following:

“4.5. Desalt the digested peptides bound to the resin by pipetting up 10 μ L of 0.2% FA in water and then discarding it. Repeat this step 4x to complete the desalting step.”

4.8 Please define iRT peptide.

Response: The definition of iRT peptides “indexed Retention Time” has been added in this step. The sentence now reads:

“Resuspend peptides in 15 µL of 0.2% FA + 1 µL iRT (indexed Retention Time) peptides.”

Line 512: "..., even with a 592 fold enrichment..."- the statement is not clear.

Response: This sentence has been revised to be more clear and to read as follows:

“Aged worms were demonstrated to be significantly enriched for the insolublome: The largest change observed showed the relative protein abundance in the insolublome to be 592 times higher in the old versus young worms; and for 32 proteins the relative protein abundance in the insolublome was >250 times higher in the old versus young worms indicating dramatic insolublome changes with age.”

Comparison of data obtained this technique with the one using 40000 worms from previous reports will be helpful in convincing readers and future reviewers.

Response: We have compared the data obtained from this current protocol (this study) with previously published reports by David et al. 2010, Plos Biology and Mark et al. 2016, Cell Reports. This analysis is presented in the new Figure 5, and we also provide a new Supplemental Table 1 B which shows greatly overlapping gene ontologies. We have also added the following sentences to describe this comparison:

“We then compared the list of proteins identified in this study with previously published work from David et al.² and Mark et al.¹¹ as demonstrated in Venn diagrams (Figure 5A and B). The comparison showed significant overlap of identified proteins 394/721 and 444/721 with David et al (Figure 5A) and Mark et al. (Figure 5B) study, respectively. The biological pathways revealed by the KEGG analysis of insolublome from this study have also been identified in past thus validating our methodology (Supplementary Table 1B). Identification of these pathways and proteins suggests that they may serve as candidates for further biological investigation in regards to their function in the context of aging.”

Reviewer #2:

Manuscript Summary:

Aging is accompanied by the accumulation of toxic insoluble protein aggregates, which in turn further accelerate the aging process. Identifying the nature of these proteins is therefore of fundamental importance to better understand molecular mechanisms underlying the aging process. *C. elegans* is a powerful model organisms for aging studies, which the authors already used in the past to investigate the contribution of insoluble proteins to aging and associated disorders. In this paper, the authors followed up on their previous studies and describe in details a protocol that they optimized in *C. elegans* to improve isolation and further characterization of insoluble proteins. Specifically, they use a Bioruptor sonicator to enhance protein extraction from a substantially reduced number of worms as compared to previous protocols. Subsequent proteomic and bioinformatics analysis confirmed that wild type older animals accumulate increased amount of insoluble proteins as compared to young worms. The author developed a great extraction method that coupled with the following straightforward pipeline analysis, will certainly help improving our knowledge on the role of

insoluble proteins in different pathophysiological context. Moreover, this improved protocol will represent an excellent tool for many other scientists working with *C. elegans*.

Response: We very much thank the reviewer for the encouraging words.

Major Concerns:

To further support the power and efficacy of their improved protocol it is recommended that the authors first validate their results against data already available in the literature. Namely, other works in the past, also from the authors, have already quantified and identified a series of insoluble proteins significantly enriched during aging. They should therefore compare results from these studies with those obtained from their newly developed protocol to ensure that the latter does not introduce biases due to the small amount of starting materials. Yielding different types or amount of proteins may indeed dramatically impact on the interpretation of the data of relevance for any biological process under investigation.

Response: We have compared the data obtained from the current protocol with previously published reports by David et al. in Plos Biology 2010 and Mark et al. in Cell Reports 2016. This analysis is presented in the new Figure 5. We also provide a new Supplemental Table 1 B which shows greatly overlapping gene ontologies. We have also added the following sentences to describe this comparison:

“We then compared the list of proteins identified in this study with previously published work from David et al.² and Mark et al.¹¹ as demonstrated by Venn diagrams (Figure 5A and B). The comparison showed significant overlap of identified proteins 394/721 and 444/721 with David et al (Figure 5A) and Mark et al. (Figure 5B) study, respectively. The biological pathway revealed by the KEGG analysis of insolublome from this study has also been identified in past thus validating our methodology (Supplementary Table 1B). Identification of these pathways and proteins suggests that they serve as candidates for further biological investigation in regards to their function in the context of aging.”

Supplementary Table 1. (A) Gene ontology (biological process) analyz (related to Figure 4)

term_ID	description	frequency	plot_X
GO:0000003	reproduction	8.29%	-6.166
GO:0006412	translation	3.71%	-3.082
GO:0006413	translational initiation	0.62%	null
GO:0006898	receptor-mediated endocytosis	0.44%	-2.763
GO:0043652	engulfment of apoptotic cell	0.31%	null
GO:0008152	metabolic process	56.96%	-3.608
GO:0009792	embryo development ending in birth or egg hatching	2.80%	6.7
GO:0032502	developmental process	18.76%	3.915
GO:0040007	growth	2.32%	-3.415
GO:0040011	locomotion	5.23%	2.364
GO:0006457	protein folding	1.01%	0.477
GO:0030968	endoplasmic reticulum unfolded protein response	0.66%	3.35
GO:0006915	apoptotic process	1.53%	3.243
GO:0005975	carbohydrate metabolic process	2.84%	-6.906
GO:0006099	tricarboxylic acid cycle	0.28%	-0.809
GO:0007067	mitotic nuclear division	1.52%	3.064
GO:0007126	meiotic nuclear division	1.33%	null
GO:0007052	mitotic spindle organization	0.31%	null
GO:0040039	inductive cell migration	0.17%	-0.814
GO:0016477	cell migration	1.92%	null
GO:0010501	RNA secondary structure unwinding	0.40%	-1.315
GO:0015991	ATP hydrolysis coupled proton transport	0.20%	-3.003
GO:0015992	proton transport	0.90%	null
GO:0015986	ATP synthesis coupled proton transport	0.24%	null
GO:0016246	RNA interference	0.46%	-3.2
GO:0015031	protein transport	3.60%	-2.045
GO:0006886	intracellular protein transport	2.44%	null
GO:0030163	protein catabolic process	3.37%	-4.739
GO:0040018	positive regulation of multicellular organism growth	0.49%	6.43
GO:0055114	oxidation-reduction process	6.61%	-6.355
GO:0040035	hermaphrodite genitalia development	0.25%	6.314
GO:0008406	gonad development	1.19%	null
GO:0010171	body morphogenesis	0.75%	6.484
GO:0040002	collagen and cuticulin-based cuticle development	0.45%	7.332
GO:0006260	DNA replication	0.96%	-1.854
GO:0018996	molting cycle, collagen and cuticulin-based cuticle	1.06%	7.474
GO:0007413	axonal fasciculation	0.11%	5.216
GO:0008340	determination of adult lifespan	2.37%	6.813
GO:0071688	striated muscle myosin thick filament assembly	0.11%	4.668
GO:0032940	secretion by cell	1.24%	-0.121

GO:0002119	nematode larval development	4.47%	6.768
GO:0006810	transport	18.10%	-1.69
GO:0001731	formation of translation preinitiation complex	0.05%	-1.283
GO:0048477	oogenesis	1.08%	5.104
GO:0007281	germ cell development	1.61% null	
GO:0007276	gamete generation	2.11% null	
GO:0035046	pronuclear migration	0.13%	0.682
GO:0000910	cytokinesis	0.53%	4.187
GO:0002181	cytoplasmic translation	0.27%	-3.047
GO:0006446	regulation of translational initiation	0.10%	-3.278

ed with STRING database

plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated	
	-2.624	2.89	-96.2306	1	0	3	0
	5.113	2.542	-48.8386	0.771	0	6412	0
null		1.771	-11.4802	0.796	0.754	6412	1
	-5.588	1.623	-26.6003	0.826	0	6898	0
null		1.477	-3.0301	0.725	0.75	6898	1
	-1.308	3.727	-2.6989	0.983	0	8152	0
	1.221	2.42	-109.2741	0.758	0	9792	0
	7.476	3.245	-4.2418	0.967	0	32502	0
	0.445	2.338	-9.7799	0.961	0	40007	0
	7.115	2.69	-24.0894	0.962	0	40011	0
	2.005	1.978	-7.4078	0.943	0.022	6457	0
	5.773	1.799	-3.8996	0.901	0.044	30968	0
	-5.39	2.158	-50.2518	0.845	0.049	6915	0
	1.008	2.427	-1.3502	0.92	0.111	5975	0
	-2.329	1.431	-8.821	0.818	0.143	6099	0
	-3.736	2.155	-3.762	0.75	0.171	7067	0
null		2.097	-1.7541	0.642	0.846	7067	1
null		1.477	-2.7809	0.768	0.723	7067	1
	-5.7	1.23	-7.9508	0.778	0.196	40039	0
null		2.258	-2.9919	0.738	0.741	40039	1
	6.578	1.58	-4.0301	0.878	0.233	10501	0
	-5.987	1.301	-7.8633	0.768	0.241	15991	0
null		1.929	-5.2411	0.746	0.865	15991	1
null		1.362	-7.1518	0.616	0.798	15991	1
	2.507	1.643	-2.1519	0.781	0.298	16246	0
	-6.363	2.529	-3.7328	0.801	0.32	15031	0
null		2.36	-4.0079	0.796	0.898	15031	1
	4.475	2.5	-5.4559	0.884	0.332	30163	0
	1.053	1.672	-5.0501	0.787	0.332	40018	0
	-1.258	2.792	-6.2426	0.859	0.334	55114	0
	-1.123	1.38	-14.3143	0.705	0.334	40035	0
null		2.049	-11.4622	0.67	0.839	40035	1
	2.263	1.851	-13	0.799	0.34	10171	0
	0.504	1.633	-2.1064	0.79	0.357	40002	0
	6.52	1.959	-4.2226	0.857	0.363	6260	0
	-1.33	2	-12.7825	0.807	0.364	18996	0
	-0.479	1.041	-1.6692	0.705	0.398	7413	0
	0.136	2.348	-24.7352	0.761	0.438	8340	0
	0.85	1.041	-2.6476	0.701	0.44	71688	0
	-5.661	2.068	-5.567	0.722	0.441	32940	0

	0.653	2.622	-92.2676	0.748	0.479	2119	0
	-6.466	3.229	-1.4808	0.8	0.536	6810	0
	4.587	0.778	-10.4473	0.76	0.584	1731	0
	-1.52	2.009	-12.3862	0.621	0.593	48477	0
null		2.182	-6.3233	0.609	0.938	48477	1
null		2.297	-1.557	0.712	0.825	48477	1
	-6.413	1.114	-2.5431	0.706	0.618	35046	0
	-5.888	1.708	-3.2457	0.801	0.666	910	0
	5.392	1.415	-6.2182	0.806	0.685	2181	0
	4.818	1	-10.3125	0.785	0.698	6446	0

Supplementary Table 1. (B) Detailed list of proteins and KEGG p
(related to Figure 5)

Color codes **Description**

	Overlap of this study with previously published wo
	Overlap of this study with previously ppublished w
	Overlap of this study with previously published wo

#term ID	term description
cel03010	Ribosome
cel01100	Metabolic pathways
cel00190	Oxidative phosphorylation
cel04145	Phagosome
cel01200	Carbon metabolism
cel00020	Citrate cycle (TCA cycle)
cel03013	RNA transport
cel04141	Protein processing in endoplasmic reticulum
cel03050	Proteasome
cel00280	Valine, leucine and isoleucine degradation
cel00010	Glycolysis / Gluconeogenesis
cel03030	DNA replication
cel01212	Fatty acid metabolism
cel00620	Pyruvate metabolism
cel00640	Propanoate metabolism
cel00071	Fatty acid degradation
cel04144	Endocytosis
cel03060	Protein export
cel04512	ECM-receptor interaction
cel01230	Biosynthesis of amino acids
cel03430	Mismatch repair
cel04150	mTOR signaling pathway
cel00062	Fatty acid elongation
cel04142	Lysosome
cel00410	beta-Alanine metabolism

cel00510	N-Glycan biosynthesis
cel00970	Aminoacyl-tRNA biosynthesis
cel01210	2-Oxocarboxylic acid metabolism
cel00520	Amino sugar and nucleotide sugar metabolism
cel03040	Spliceosome
cel03420	Nucleotide excision repair
cel03018	RNA degradation
cel00630	Glyoxylate and dicarboxylate metabolism
cel00061	Fatty acid biosynthesis

pathways identified in this study with color codes depicting

rk^{2,11}

ork²

rk¹¹

observed gene c	background gene c	false discovery rate
63	126	3.46E-43
115	802	2.29E-33
43	109	2.67E-26
26	61	1.32E-16
30	108	5.09E-15
18	33	4.22E-13
28	114	4.97E-13
26	135	4.18E-10
13	41	2.18E-07
14	51	2.61E-07
12	41	1.29E-06
11	34	1.69E-06
13	55	2.75E-06
10	29	3.18E-06
10	29	3.18E-06
11	51	3.75E-05
15	102	5.30E-05
7	22	0.0002
5	8	0.00022
11	70	0.0004
6	18	0.00051
11	77	0.00077
6	21	0.00094
12	98	0.0013
6	24	0.0016

7	37	0.0022
7	38	0.0025
5	19	0.0035
6	35	0.0076
11	111	0.0088
6	37	0.0091
6	49	0.0297
5	36	0.033
3	13	0.0414

g their overlap with the published work.

matching proteins in your network (labels)
Y37E3.8,rla-1,rpl-10,rpl-11.1,rpl-12,rpl-14,rpl-15,rpl-16,rpl-17,rpl-18,rpl-19,rpl-20
B0303.3,C16A3.5,C50D2.7,COX2,CYTB,F07A11.2,F21D5.1,F22B3.4,F38B2.4,F45H
C16A3.5,COX2,CYTB,F45H10.2,F53F4.10,F59C6.5,R04F11.2,R07E4.3,R53.4,T02H6
act-1,act-2,act-5,cnx-1,cpl-1,dhc-1,dli-1,hgrs-1,imp-1,pat-3,rab-7,sec-61,tba-1,tb
C50D2.7,T08B2.7,acd-10,acd-7,aco-2,alh-8,cts-1,dlat-1,dlst-1,ech-6,fbp-1,gdh-
acly-2,aco-2,cts-1,dlat-1,dlst-1,idha-1,idhb-1,idhg-1,mdh-2,mev-1,pck-1,pck-2,pc
egl-45,eif-3.B,eif-3.C,eif-3.D,eif-3.E,eif-3.F,eif-3.G,eif-3.H,eif-3.I,hel-1,ife-1,iffb-1,
ZK686.3,cdc-48.1,cdc-48.2,cnx-1,dad-1,dnj-29,enpl-1,hsp-1,hsp-3,hsp-4,ile-1,let-
pas-5,rpn-1,rpn-2,rpn-3,rpn-5,rpn-6.1,rpn-7,rpt-1,rpt-2,rpt-3,rpt-4,rpt-5,rpt-6
B0303.3,C05C10.3,F53A2.7,T08B2.7,ZK669.4,acd-1,acd-10,acd-7,acd-9,alh-
C50D2.7,R05F9.6,alh-4,dlat-1,fbp-1,gpd-1,gpd-2,gpd-3,pck-1,pck-2,pd-1,pyk-1
F44B9.8,mcm-2,mcm-3,mcm-4,mcm-6,mcm-7,pcn-1,rfc-2,rfc-3,rfc-4,rpa-1
B0303.3,F53A2.7,T08B2.7,acd-1,acd-10,acd-12,acd-7,acs-13,acs-4,art-1,ech
alh-4,dlat-1,icl-1,mdh-2,pck-1,pck-2,pd-1,pyc-1,pyk-1
T08B2.7,ZK669.4,acd-10,acd-7,alh-8,ech-6,mmcm-1,pod-2,suca-1,sucl-1
B0303.3,F53A2.7,T08B2.7,acd-1,acd-10,acd-12,acd-7,acs-13,acs-4,alh-4,ech
alx-1,apb-1,arx-3,cav-1,chg-1,hgrs-1,hsp-1,rab-11.1,rab-7,rho-1,rme-1,snx-1,unc-
F38A1.8,F55C5.8,dnj-29,hsp-3,hsp-4,sec-61,srpa-72
epi-1,lam-1,lam-2,let-2,pat-3
aco-2,cts-1,gpd-1,gpd-2,gpd-3,idha-1,idhb-1,idhg-1,pyc-1,pyk-1,tk-1
F44B9.8,pcn-1,rfc-2,rfc-3,rfc-4,rpa-1
gsk-3,ife-1,npp-20,rho-1,rsks-1,vha-11,vha-12,vha-13,vha-14,vha-15,vha-8
B0303.3,F53A2.7,T08B2.7,art-1,ech-6,let-767
T28H10.3,apg-1,asp-4,chg-1,cpl-1,imp-1,scav-3,vha-15,vha-16,vha-19,vha-5,vha-
T08B2.7,acd-10,acd-7,alh-4,alh-8,ech-6

ZK686.3,dad-1,dpm-1,ostb-1,ostd-1,ribo-1,stt-3
aars-2,ears-1,kars-1,pars-1,qars-1,rars-1,wars-1
aco-2,cts-1,idha-1,idhb-1,idhg-1
F07A11.2,F21D5.1,F22B3.4,K08E3.5,R05F9.6,hpo-19
F26B1.2,F53H1.1,F58E10.3,T08A11.2,emb-4,hel-1,hrp-1,hsp-1,prp-6,prp-8,rsp-3
F44B9.8,pcn-1,rfc-2,rfc-3,rfc-4,rpa-1
cgh-1,hsp-6,hsp-60,let-711,ntl-9,pab-1
aco-2,cts-1,icl-1,mdh-2,mmcm-1
acs-13,acs-4,pod-2

,rpl-21,rpl-22,rpl-23,rpl-24.1,rpl-25.1,rpl-25.2,rpl-26,rpl-27,rpl-3,rp
10.2,F53A2.7,F53F4.10,F59C6.5,K08E3.5,R04F11.2,R05F9.6,R07E4.3
5.11,Y69A2AR.18,ZK809.3,asb-1,asb-2,asg-1,asg-2,atp-2,atp-3,atp-5
a-2,tbb-2,tbb-4,vha-11,vha-12,vha-13,vha-14,vha-15,vha-16,vha-19
-1,gpd-1,gpd-2,gpd-3,icl-1,idha-1,idhb-1,idhg-1,mdh-2,mev-1,mmci
lhb-1,pyc-1,sdha-1,sdha-2,suca-1,sucl-1
ifg-1,imb-1,inf-1,npp-1,npp-12,npp-13,npp-15,npp-20,npp-3,npp-6
70,mek-1,npp-20,ostb-1,ostd-1,pdi-1,pdi-2,pdi-3,ribo-1,sec-24.1,se

4,alh-8,ech-6,ivd-1,mmcm-1

.

i-6,let-767,pod-2

i-6

-116,vps-35,vps-4

l-7A,rpl-9,rps-0,rps-1,rps-10,rps-11,rps-12,rps-13,rps-14,rps-15,rps-1
'686.3,ZK809.3,acd-1,acd-10,acd-12,acd-7,acd-9,acly-2,aco-2,ac
,sdha-2,ucr-2.1,ucr-2.2,ucr-2.3,vha-11,vha-12,vha-13,vha-14,vha-15.

6,rps-17,rps-18,rps-19,rps-2,rps-20,rps-22,rps-23,rps-24,rps-25,rps-2
acs-13,acs-4,ads-1,ahcy-1,alh-4,alh-8,ama-1,asb-1,asb-2,asg-1,asg-2,
,vha-16,vha-19,vha-5,vha-6,vha-8

!6,rps-27,rps-28,rps-3,rps-4,rps-5,rps-7,rps-8,rps-9,ubl-1,ubq-2
atp-2,atp-3,atp-5,ctps-1,cts-1,cyc-1,dad-1,dhs-28,dlat-1,dlst-1,dpm-1

.,ears-1,ech-6,epi-1,fbp-1,gdh-1,gpd-1,gpd-2,gpd-3,gsy-1,icl-1,idha-1

,idhb-1,idhg-1,isp-1,ivd-1,let-767,mdh-2,mev-1,mmcm-1,nduf-2.2,nc

duf-7, nuo-1, nuo-2, nuo-4, nuo-5, ostb-1, ostd-1, pck-1, pck-2, pdhb-1, po

d-2,pyc-1,pyk-1,pyr-1,qars-1,ribo-1,rnr-1,rnr-2,sdha-1,sdha-2,spl-1,s

tt-3,suca-1,sucl-1,tkl-1,tyr-4,ucr-2.1,ucr-2.2,ucr-2.3,ugt-62,vha-11,vh

ia-12,vha-13,vha-14,vha-15,vha-16,vha-19,vha-5,vha-6,vha-8