Stable-isotope labeling with amino acids in nematodes

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We describe an approach for accurate quantitation of global protein dynamics in *Caenorhabditis elegans*. We adapted stable-isotope labeling with amino acids in cell culture (SILAC) for nematodes by feeding worms a heavy lysine– and heavy arginine–labeled *Escherichia coli* strain and report a genetic solution to elminate the problem of arginine-to-proline conversion. Combining our approach with quantitative proteomics methods, we characterized the heat-shock response in worms.
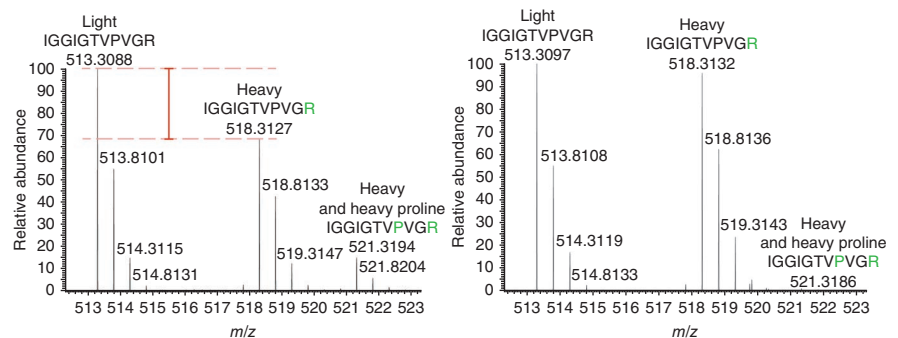
The stable-isotope labeling with amino acids in cell culture (SILAC) mass spectrometry approach facilitates the accurate and reproducible quantification of large numbers of proteins[1]. In contrast, ^{15}N-labeling, which has been used previously for proteomic analysis in *C. elegans*[2], has drawbacks for data analysis, including the unpredictable mass shifts induced by total ^{15}N labeling[3]. SILAC is typically used with tissue culture cells, and SILAC-based methods have not been reported previously for *C. elegans* to our knowledge. Whereas SILAC has been used to analyze several multicellular organisms, problems associated with the conversion of isotope-labeled arginine to proline and other amino

acids have complicated such studies[4]. Arginine-to-proline conversion reduces the signal from all 'heavy' SILAC-labeled peptides containing proline. The increased number of tandem mass spectrometry (MS/MS) target peptides resulting from arginine-to-proline conversion reduces the overall sensitivity of global MS/MS analysis. These additional peptide peaks also increase the likelihood of overlapping isotopic envelopes reducing peak discrimination.

We aimed to adapt SILAC methodology for *C. elegans* and to eliminate the arginine-to-proline conversion problem. Initially, we used an arginine and lysine auxotrophic version of *E. coli* BL21 (DE3), but *C. elegans* did not survive well on this strain (data not shown). We therefore modified *E. coli* HT115, a strain commonly used for the 'RNAi via feeding' procedure[5], by generating an arginine and lysine auxotroph termed SLE1 (**Supplementary Fig. 1a**). Analysis of the egg-laying rate and embryonic development and survival of *C. elegans* grown on the *E. coli* SLE1 strain indicated no difference (**Supplementary Fig. 1b**) compared to *E. coli* OP50, which is generally used as a food source in *C. elegans* studies. For *C. elegans* labeling, we grew the SLE1 strain in minimal medium containing the $^{15}N_4$-$^{13}C_6$-arginine ('heavy' arginine) and $^{15}N_2$-$^{13}C_6$-lysine ('heavy' lysine), and plated these bacteria on agarose Petri dishes. We placed *C. elegans* eggs onto a lawn of SLE1 bacteria and analyzed the incorporation of heavy arginine and lysine into protein in the F1 generation by mass spectrometry.

We observed ~93% heavy isotope incorporation as illustrated for a representative peptide (**Supplementary Fig. 2a**). However, arginine-to-proline conversion led to ~20% of the heavy peptide signal being diverted to a larger mass-to-charge ratio (*m/z*) signal. We saw this diversion of signal intensity more clearly when equal portions of heavy and light protein were mixed

Figure 1 | Elimination of arginine-to-proline conversion using *orn-1*–targeted RNAi via feeding facilitates stable-isotope labeling with amino acids. Lysates from light isotope– or heavy isotope–labeled *C. elegans* were mixed in equal proportions, fractionated by denaturing size-exclusion chromatography and fraction 12 was analyzed by trypsin digestion and LC-MS/MS. A representative proline-containing peptide derived from EF-1α was examined. Shown are mass spectra for the indicated peptide sequences. The peak intensity of the depicted 'light' model peptide approximately equals the combined intensities of the corresponding heavy peptide and the peptide that contains heavy proline (3 replicates).

Figure 2 | SILAC in nematodes, taking the analysis of the heat-shock response as an example. *E. coli* SLE1 strain expressing the *orn-1* RNAi construct are grown in 'light' medium (M9 minimal medium with arginine and lysine) or 'heavy' medium (M9 minimal medium and $^{15}N_4$-$^{13}C_6$-arginine and $^{15}N_2$-$^{13}C_6$-lysine). Bacteria are concentrated by centrifugation and plated on Petri dishes, onto which adult worms are transferred. Upon ingesting bacteria, worms incorporate 'light' or 'heavy' amino acids and *orn-1* knockdown occurs. Single worm progeny are transferred onto a new plate, which is then processed as shown.
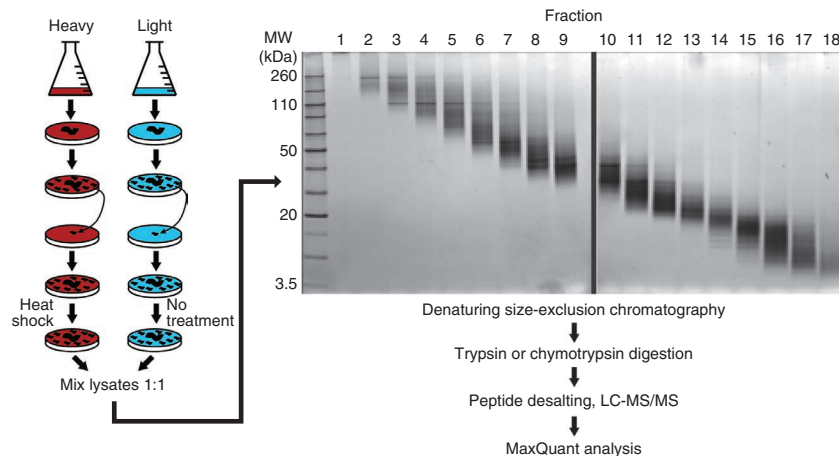

(**Fig. 1**). Analysis of each peak shown in **Supplementary Figure 2a** by MS/MS confirmed that the heavier peak contained ^{15}N-$^{13}C_5$-proline ('heavy' proline) and no labeled amino acids other than arginine (**Supplementary Fig. 2b**). The conversion of heavy arginine to heavy proline occurs in the nematode and not in the SLE1 strain of bacteria because *E. coli* did not generate heavy proline–containing peptides (**Supplementary Table 1**). Arginine-to-proline conversion occurs through the urea cycle[6] (**Supplementary Fig. 3**).

To eliminate the problem of arginine-to-proline conversion, we used the RNAi via feeding procedure, targeting the ornithine transaminase enzyme gene, *orn-1*. This enzyme converts ornithine to L-glutamate-5-semialdehyde (**Supplementary Fig. 3**) and is required for arginine-to-proline conversion in *Schizosaccharomyces pombe*[6]. We performed RNAi via feeding of *C. elegans* according to schemes in **Figure 2** and **Supplementary Figure 4a**, and evaluated the extent of *orn-1* transcript depletion by quantitative PCR (qPCR) (**Supplementary Fig. 4b**). Worms subjected to either control (*GFP*) or *orn-1* knockdown by RNAi had similar egg laying rates and embryonic development, which indicated that *orn-1* knockdown by RNAi did not have a major effect on viability (**Supplementary Fig. 4c**). We labeled worms subjected to *orn-1* knockdown by RNAi with both heavy arginine and heavy lysine or with light arginine and light lysine, for one generation, to determine the effect of RNAi via feeding on arginine-to-proline conversion. Mass spectrometry analysis again showed ~93% heavy arginine and lysine isotope incorporation (**Fig. 1** and **Supplementary Fig. 2c**), and that greater than 98% of total proline was ^{14}N-$^{12}C_5$-proline (light proline), indicative of a near-complete elimination of arginine-to-proline conversion (**Fig. 1** and **Supplementary Fig. 2a**). When we mixed

equal portions of untreated heavy and light protein and analyzed the sample by mass spectrometry, more than 95% of the proteins had $\log_2$ ratios of approximately zero (**Supplementary Table 1**). These data also indicate that fold-changes greater than ±50% would differentiate proteins whose expression has been altered from that of unaffected proteins.

Many temperature-sensitive mutants have been generated in *C. elegans*, allowing for a transient disruption of often essential proteins. One problem associated with this experimental procedure is background perturbations generated by the heat-shock response. We therefore used the SILAC in nematodes approach to examine the extreme end of the heat-shock response by shifting worms to 30 °C, as illustrated in **Figure 2**. We fractionated the *C. elegans* proteome using a detergent-free denaturing size exclusion chromatography method (Online Methods), which proved effective (**Fig. 2**).

Liquid chromatography–MS/MS (LC-MS/MS) analysis of each fraction yielded a dataset of ~19,000 peptides corresponding to > 1,400 proteins, each identified with at least two peptides (**Supplementary Tables 1** and **2**). Four small heat-shock proteins were among the nine proteins upregulated more than fourfold and had MaxQuant significance *B* values[1] < 0.05 (**Fig. 3** and **Supplementary Tables 1** and **2**). These data validate our technique and provide a global overview describing changes in protein abundance upon heat-shock treatment. Three cathepsin-like aspartic acid proteases of both lysozomal and non-lysozomal origin (ASP-1, ASP-2 and ASP-6) were downregulated more than threefold after heat shock and had MaxQuant significance *B* values[1] less than 0.05 (**Fig. 3** and **Supplementary Tables 1** and **2**). ASP-1 is a lysozomal aspartic acid protease of the cathepsin-D family that is mainly expressed in intestinal cells and has been observed previously to be downregulated in response to heat shock[7]. Little is known about the role of ASP-1,
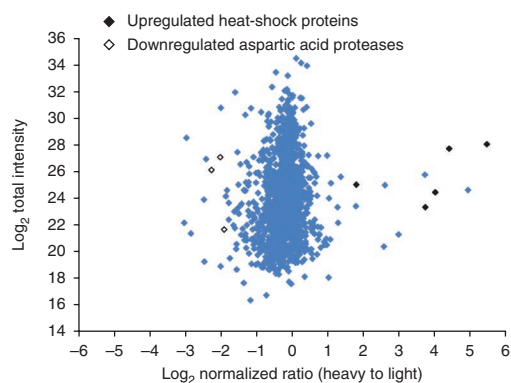
Figure 3 | Analysis of *C. elegans* heat-shock response using SILAC in nematodes. The abundance of ~1,400 proteins is indicated on the *y* axis using a $\log_2$ scale. The abundance of each protein indicated by the position of the dot on the *y* axis was determined by summing all individual light and heavy peptide intensities detected for each protein. The relative fold decrease or increase upon heat-shock treatment is indicated on the *x* axis. Heat shock–treated worms were grown on heavy isotope–labeled SLE1 bacteria, and untreated worms were grown on light bacteria (data from the average of two replicates are shown).

ASP-2 and ASP-6 in nematode biology, and future studies will be needed to reveal their role in the heat stress response.

Our subsequent studies using subcellular fractionation of unlabeled worms allowed approximately three times the number of proteins to be identified by mass spectrometry compared to our heat-shock dataset and provided valuable information about the subcellular distribution of proteins in untreated cells (**Supplementary Table 1**). Subcellular fractionation, in conjunction with the SILAC-based method presented here, will allow future studies to examine the dynamic re-localization of the *C. elegans* proteome in response to various conditions, as has been demonstrated recently in human cells[8].

Using the SILAC in nematodes approach we also characterized changes in the *C. elegans* proteome in response to *orn-1* knockdown by RNAi (**Supplementary Table 1**). These experiments compared the proteome of worms labeled with light amino acids and subjected to a control RNAi knockdown (targeting *GFP*) with worms labeled with heavy istotope–labeled amino acids and *orn-1*–targeted RNAi (replicate 1). These data showed the reduction of total ORN-1 protein by ~60% (**Supplementary Table 1**). The reduced knockdown could be the result of incomplete RNAi in *C. elegans* neurons, which has been observed previously[9]. However, the residual ORN-1 activity in RNAi-resistant cells appears to be minimal because arginine-to-proline conversion was mostly abolished. *orn-1*–targeted RNAi also generated some changes in protein expression compared with cells subjected to a control RNAi treatment, as expected. However, as *orn-1*–targeted RNAi should be used in both heavy isotope– and light isotope–labeled worms in the SILAC in nematodes method, these differential effects will be negated. One caveat may be that urea-cycle enzymes could be altered by *orn-1*–targeted RNAi, and this should be taken into account for any experiments targeting that pathway. Members of our laboratory have requested the generation of a null mutant for the *orn-1* gene by the *C. elegans* Knockout Consortium, and this mutant will facilitate future studies without the need for RNAi-mediated *orn-1* knockdown.

Our SILAC in nematodes methodology opens many opportunities for research in *C. elegans* using quantitative mass spectrometry–based proteomic strategies. Without arginine-to-proline conversion SILAC experiments can be performed more efficiently. SILAC in nematodes will also allow the application of methods previously used in tissue culture cells, for example, the relative quantification of protein-protein interactions and the elimination of contaminants in pulldown experiments, in worms[10]. The generation of the *E. coli* SLE1 strain for simultaneous stable-isotope labeling with amino acids and RNAi via feeding could also be used for double RNAi experiments where possible. For example, *orn-1* can be knocked down to eliminate arginine-to-proline conversion, and another *C. elegans* gene could be targeted to determine the effects on the proteome. This will be especially useful for the analysis of genes for which null mutations are embryonic lethal but RNAi leaves sufficient protein product for survival. The SILAC in nematodes technique will also help to determine global proteome changes during development, aging and stress responses.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

AUTHOR CONTRIBUTIONS

A.P.B. and E.P. cloned, passaged and treated all *C. elegans* samples. M.L. performed all protein analysis. M.L., A.P.B., A.G. and A.I.L. wrote the paper. A.P.B., R.T.H., G.B. and S.C. generated the *E. coli* auxotroph strains. D.P.X., A.G. and A.I.L. provided mentorship and financed the project.

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ONLINE METHODS

Materials. Coomassie Plus (Bradford) reagent and Tris-carboxy-ethylphosphine (TCEP) (Bond-breaker neutral pH solution) was from Pierce. Trypsin was from Promega. Oasis HLB 96-well μ-elution plates were from Waters. The Pepmap C18 columns and trapping cartridges were from Dionex. Complete protease inhibitor cocktail tablets and PhosStop phosphatase inhibitor tablets were from Roche. CBQCA assay kit was from Invitrogen. All other materials were obtained from Sigma.

***C. elegans* strains and maintenance.** *C. elegans* N2 Bristol strain[11] was used and maintained at 20 °C unless otherwise indicated. For iso-topic labeling *C. elegans* were grown on nematode growth medium, nitrogen free (NGM-N) plates. Larval stage 1 (L1) worms were separated from an unsynchronized population by filtering through a nylon filter (11 μm, NY11, Millipore) and 5 L1 stage *C. elegans* were incubated on a fresh NGM-N plate (plate 1). *C. elegans* were monitored every day and L4 larvae stage *C. elegans* of the following generation (F1) were picked onto a fresh plate (plate 2). This proce-dure was followed unless indicated otherwise.

SDS-PAGE. Size fractionation was confirmed by SDS-PAGE analysis on 4–12% (wt/vol) Bis-Tris NuPage gels using 2-(*N*-morpholino)ethanesulfonic acid (MES) running buffer (Invitrogen) according to manufacturer's instructions but with the addition of 25 mM TCEP, in the LDS sample buffer (Invitrogen). Equal amounts of protein were loaded with a maximum of 10 μg per lane. SYPRO Ruby staining was performed according to the manufacturer's instructions (Invitrogen).

Generation of *E. coli* mutant strains. The SLE1 auxotrophic derivative of *E. coli* HT115 was generated using bacteriophage P1 transduction[12] to introduce in-frame deletions in *argA* and *lysA* using the the *E. coli* Keio Collection single gene mutant library[13]. Briefly, the *argA* and *lysA* kanamycin restistance cassette replace-ment mutants were retrieved from the library and used to pre-pare P1 lysates. Generalized P1 transduction was then performed according to standard methods[12] to sequentially introduce each mutation into the recipient HT115 strain. Positive transductants were selected using kanamycin resistance and confirmed by testing auxotrophy for the corresponding amino acid (arginine or lysine). After the successful introduction of each mutation, the kanamycin resistance cassette was excised to leave an unmarked deletion, via the transient expression of the FLP recombinase as described pre-viously[14]. Auxotrophy was confirmed by assessing growth in the presence or absence of the relevant amino acid(s) at 40 μg ml^{-1} in M9 minimal medium (Na$_2$HPO$_4$ 5.8 g l^{-1}, KH$_2$PO$_4$ 3 g l^{-1}, NaCl 0.5 g l^{-1}, NH$_4$Cl$_2$ 1 g l^{-1}, glucose 0.2% (wt/vol), MgSO$_4$ 1 mM, thiamine 0.01% (wt/vol)). The full genotype of SLE1 is *argA, lysA,* F$^-$, *mcrA, mcrB, IN(rrnD-rrnE)1, lambda$^-$, rnc14∷Tn10* (DE3 lysogen: *lacUV5* promoter, T7 polymerase). The SLE1 strain will be available to the community via the Caenorhabditis Genetics Center at the University of Minnesota.

Bacterial growth conditions. *E. coli* HT115 cells knocked out for *lysA* and *argA* (SLE1) were grown in M9 minimal medium. M9 minimal medium was prepared by mixing 100 ml of 10 × M9 salts (420 mM disodium phosphate, 240 mM monopotassium phos-phate, 90 mM sodium chloride, 190 mM ammonium chloride)

and 893 ml of deionized water and autoclaved (120 °C for 20 min). After cooling to 55 °C, 5 ml of 40% (wt/vol) glucose, 1 ml of 1 M MgSO$_4$ and 1 ml of 1% (wt/vol) thiamine were added. Lysine and arginine were added to 40 μg ml^{-1} final concentrations from a 73 mg ml^{-1} or 42 mg ml^{-1} stock (in PBS), respectively. A single bacterial colony freshly streaked on an LB plate from a frozen stock was used to start a 200-ml culture. Bacteria were incubated at 37 °C under agitation (220 r.p.m.) until OD$_{600 nm}$ = 1 was reached. Bacteria were concentrated by centrifugation (8,000*g*, 20 min) to OD$_{600 nm}$ = 50, and 5 ml was plated onto NGM-N plates. Plates were then stored at 20 °C and used within 7 d.

***orn-1* RNAi via feeding construct.** For the ornithine transami-nase *orn-1* (Wormbase gene identifier C16A3.10), plasmid pAG608 was generated using the following primers and Not1 restriction: forward 5′-TATATATAGCGGCCGCACTTCTCGC ACACTACCACG-3′, reverse 5′-TATATATAGCGGCCGCTTA GTTTTGCTTTGCAAAATCG-3′.

E. coli SLE1 *argA, lysA* were made chemically competent by incubating 5 ml of freshly grown bacteria in LB (OD$_{600 nm}$ = 0.4) with ice-cold CaCl$_2$ (100 μM) for 2 h.

RNAi via feeding. RNAi experiments were performed according to the feeding method[5] with the following modifications: cells carrying the corresponding feeding vector were grown in 2 ml of M9 minimum medium supplemented with either heavy or light arginine and lysine (40 μg ml^{-1}) and carbenicillin (1 μg ml^{-1}) at 37 °C until OD$_{600 nm}$ = 1. Double-stranded RNA expression was induced by the addition of IPTG (1 mM final) for 3 h. Bacteria were pelleted and resuspended in 1 ml of worm M9 buffer sup-plemented with 1 mM IPTG and 1 μg ml^{-1} carbenicillin to which ~500 L1 larvae stage worms were added. This mixture was trans-ferred into a 50 ml falcon tube to allow oxygenation and incubated at 25 °C overnight under gentle agitation (150 r.p.m.). Worms and bacteria were spun down the following day, plated on 9 cm NGM-N plates and incubated at 20 °C. The following worm generation (F1) was analyzed for the corresponding phenotype.

Quantitative PCR. RNA was extracted by resuspending worms in Qiazol (Qiagen) disruption with 0.7 mm zirconia beads (Biospec Products). Five hundred nanograms of RNA was reverse-transcribed using a Quantitect kit (Qiagen) in a final volume of 20 μl of which 0.5 μl was used for the quantitative PCR. MesaGreen mix (Eurogentec) was used following manufacturer instructions on an iCycler iQ5 (Biorad). Cycling conditions were: 1 × (5 min at 95 °C) and 50 × (15 s at 95 °C, 20 s at 60 °C and 40 s at 72 °C); fluorescence was measured after each 72 °C step. Relative expression levels were determined using *tbg-1* (γ-tubulin) transcript as standard. Experiments were done in triplicate.

Labeling *C. elegans* plates. *C. elegans* were grown on NGM plates that did not contain any nitrogen source (NGM-N plates). For 1 l of NGM-N medium, 3 g of NaCl and 12 g of agarose (Invitrogen, molecular grade) were mixed with 970 ml of deionized water and autoclaved (120 °C for 20 min). After cooling to 55 °C the following compounds were added: 1 ml of 1 M CaCl$_2$, 1 ml of 1 M MgSO$_4$, 25 ml of 1 M KPO$_4$, 1 ml of cholesterol at 5 mg ml^{-1} (in ethanol), 1 ml of Nystatin (10,000 units ml^{-1}); 9-cm plates were used.

Generation of *C. elegans* lysates. *C. elegans* were collected from plates by flushing with PBS and washed three times with PBS. *C. elegans* were pelleted and resuspended in ice-cold 100 µl of PBS containing Complete protease inhibitors, PhosStop and 5 mM N-ethyl maleimide before snap-freezing in liquid nitrogen. For lysis, *C. elegans* were thawed on ice and powdered guanidine-HCl (76 mg) was added to generate a ~6 M guanidine-HCl solution. TCEP (reducing agent) was added to a concentration of 25 mM, the solution was vortexed and then heated to 65 °C for 10 min. Zirconia beads (0.7 mm, Biospec Products) were added to make a ~50% (vol/vol) slurry and the sample was bead-beated (Mini Beadbeater 8, Biospec Products) for 1 min at room temperature (25 °C). The lysate was then centrifuged for 10 min at 17,000g at room temperature. A Bradford assay was performed on the supernatant and for SILAC mixing; equal proportions of protein were combined.

Subcellular fractionation of *C. elegans*. The QProteome Cell Compartment fractionation kit (Qiagen) was used to fractionate worms according to the manufacturer's tissue fractionation protocol. Briefly, *C. elegans* were collected by flushing with PBS and washed three times with PBS. Freshly collected *C. elegans* were pelleted and resuspended in ice-cold buffer 1 containing Complete protease inhibitors. Zirconia beads (0.7 mm, Biospec Products) were added to make a ~50% (vol/vol) slurry and the sample was bead-beated (Mini Beadbeater 8, Biospec Products) for 5 s at 4 °C and lysis was checked by microscopy. The lysate was then processed for the remaining steps according to the manufacturer's instructions. A bicinchoninic acid assay (BCA) assay was performed on each fraction before denaturing gel filtration chromatography of each.

Denaturing gel filtration chromatography, trypsin digestion and peptide cleanup. To identify and quantify as many proteins as possible we used two strategies to maximize protein fractionation (**Fig. 2**). First, we separated proteins by molecular weight using denaturing size exclusion chromatography with a combination of urea and thiourea as denaturants. This enabled fractionation of small amounts of protein with minimal sample loss. It also allowed us to perform protein digestion in solution, which overcomes the problem of incomplete peptide extraction associated with the in-gel digestion procedure. The reduction of handling steps reduces protein contamination problems, for instance, keratin. Second, we digested the size exclusion chromatography fractions with either trypsin or chymotrypsin to generate complementary digests of the fractionated proteins. Trypsin (which cleaves after arginine and lysine) or chymotrypsin (which cleaves after bulky hydrophobic residues) yield quite different peptide fragments. Combining MS/MS datasets obtained upon trypsin and chymotrypsin cleavage increases sequence coverage and the number of protein identifications.

Size fractionation and protein digestion were performed as described below. Using a Dionex Ultimate 3000 HPLC system, lysates in 6 M guanidine-HCl were injected (20 µl per injection equals 80 µg protein) onto a mAbPacSEC column (Dionex) equilibrated with 6 M urea, 2 M thiourea and 0.1 M Tris-HCl pH 7.0. The flow rate was 0.2 ml min^{-1} and sixteen 100-µl fractions

were collected using a low protein binding 96-deep well plate (Eppendorf). Trypsin or chymotrypsin digestions in separate reactions for each fraction and peptide desalting was performed according to the scheme shown in **Supplementary Figure 5**. Briefly, three volumes of 0.1 M Tris-HCl pH 8.0 and 1 mM CaCl$_2$ were added to each fraction to dilute the urea. Five hundred nanograms of trypsin or chymotrypsin were subsequently added to each well. The plate was sealed with a rubber mat, vortexed and incubated overnight at 37 °C. Trifluoroacetic acid was added to 1% (vol/vol) final concentration and peptides were purified using an Oasis HLB 96-well µ-elution plate. Peptides were eluted in 100 µl of 50% (vol/vol) acetonitrile and dried in a SpeedVac before resuspension in 5% (vol/vol) formic acid. Peptide concentrations were determined using the CBQCA assay (Invitrogen) after 100-fold dilution of peptide samples in water.

LC-MS/MS and MaxQuant analysis. Using a Dionex Ultimate 3000 nanoHPLC system, 1 µg of peptides in 5% (vol/vol) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Dionex). After washing with 2% (vol/vol) acetonitrile 0.1% (vol/vol) formic acid peptides were resolved on a 150 mm × 75 µm Acclaim PepMap C18 reverse phase analytical column over a 100 min organic gradient with a flow rate of 300 nl min^{-1}. Peptides were ionized by nano-electrospray ionization at 1.2 kV using a fused silica emitter with an internal diameter of 5 µm (New Objective). Tandem mass spectrometry analysis was carried out on a LTQ-Velos Orbitrap mass spectrometer (Thermo Scientific). The data-dependent acquisition method used was the FT10 protocol as described previously[15]. Data were processed, searched and quantified using the MaxQuant software package version 1.1.1.36 as described previously[1], using the default settings and employing a combined *C. elegans* and *E. coli* Uniprot databases. The settings used for the MaxQuant analysis were: 2 failed cleavages were allowed; enzymes were trypsin or chymotrypsin; variable modifications included in the analysis were methionine oxidation, deamidation of glutamine or asparagine, N-terminal pyro-glutamic acid formation, protein N-terminal acetylation. To identify heavy proline containing peptides ^{15}N-^{13}C$_5$-proline was added as a variable modification. A mass tolerance of 7 p.p.m. was used for precursor ions and a tolerance of 0.5 Da was used for fragment ions. Using the default MaxQuant settings a maximum false positive rate of 1% was allowed for both peptide and protein identification. This cutoff was used for accepting individual spectra as well as whole proteins in the MaxQuant output. This threshold has previously been shown to be a rigorous method for identifying true positive matches[1]. Protein quantitation data was always derived from a minimum of two or more peptides per protein, and MaxQuant significance *B* values are provided as described previously to identify significant fold changes[1]. All replicates indicated are biological replicates.

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