

Metabolic labeling of *C. elegans* and *D. melanogaster* for quantitative proteomics

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A crucial issue in comparative proteomics is the accurate quantification of differences in protein expression levels. To achieve this, several methods have been developed in which proteins are labeled with stable isotopes either *in vivo* via metabolic labeling or *in vitro* by protein derivatization. Although metabolic labeling is the only way to obtain labeling of all proteins, it has thus far only been applied to single-celled organisms^{1,2} and cells in culture^{2,3}. Here we describe quantitative ¹⁵N metabolic labeling of the multicellular organisms *Caenorhabditis elegans*, a nematode, and *Drosophila melanogaster*, the common fruit fly, achieved by feeding them on ¹⁵N-labeled *Escherichia coli* and yeast, respectively. The relative abundance of individual proteins obtained from different samples can then be determined by mass spectrometry (MS). The applicability of the method is exemplified by the comparison of protein expression levels in two *C. elegans* strains, one with and one without a germ line. The methodology described provides tools for accurate quantitative proteomic studies in these model organisms.

Metabolic labeling of proteins in living cells with stable isotopes requires that the metabolic pathway be accessible to the label. Here we explored whether we could label the multicellular organisms *C. elegans* and *D. melanogaster* to an extent and within a time frame suitable for quantitative proteomics. Because *C. elegans* feeds on bacteria, we used a two-step approach to label the organism with ¹⁵N (Fig. 1). First, *E. coli* was grown in medium enriched in ¹⁵N to achieve >98% labeling. To check whether the label was fully incorporated into the bacteria, 1 ml of labeled culture was pelleted, washed twice in PBS and lysed. The protein extract (100 µg) was separated by two-dimensional (2D) gel electrophoresis, and then spots were digested in-gel and analyzed by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) MS. The isotope distribution of the peptides was indicative of 98% enrichment in ¹⁵N as evidenced by isotope simulation, whereas no unlabeled form of the peptides were detected (data not shown).

Next, labeled *E. coli* was spread over agar plates otherwise devoid of nutrients and any potential nitrogen source. *C. elegans* L1 larvae were seeded and grown for 2–3 d until the early adult stage.

Animals were harvested, washed and processed for 2D gel electrophoresis. Twelve protein spots were digested for MALDI-TOF MS analysis (Fig. 2). The spectra obtained indicate that for each protein analyzed, 96% of the total amount of protein was labeled with ¹⁵N and therefore was synthesized during larval development. The unlabeled fraction (4%) probably is a remaining pool from the egg stage. Within the labeled fraction, ¹⁵N incorporation was approximately 95%, demonstrating that 95% of the amino acids incorporated during protein synthesis were labeled (Fig. 2b). Given the efficiency of the amino acid labeling, the isotope envelopes of labeled and unlabeled peptides are well separated (compare Figs. 2a and 2b) and would therefore, in principle, be sufficient for proper calculation of abundance ratios. However, although the 4% of unlabeled protein can be corrected for, it cannot be assumed that this percentage is identical for every protein. To circumvent this potential problem for later applications, we extended the duration of labeling for one more generation. Eggs of first-generation nematodes were harvested and seeded on fresh plates containing labeled bacteria. Nematodes were grown to early adult stage, 2D gels were prepared and the same spots were analyzed as before. MALDI-TOF analysis revealed that ¹⁵N content in labeled peptides was now 98% and, more importantly, that no unlabeled form of the peptide was detectable (Fig. 2c). For all proteins analyzed this way, we observed that labeling was quantitative.

We extended our studies to determine whether isolation and quantification of proteins via ¹⁵N metabolic labeling could also be achieved in another multicellular model organism, *D. melanogaster*. Because *D. melanogaster* feeds on yeast, we used a two-step approach similar to that used for labeling *C. elegans* (Fig. 1). First, ¹⁵N-labeled and unlabeled yeast were generated by growing them in minimal medium supplemented with ¹⁵N-labeled or unlabeled ammonium sulfate as a source of nitrogen. We spread 5 mg of 0–12-h *D. melanogaster* embryos in larva boxes containing the ¹⁵N-labeled or unlabeled yeast, and other ingredients lacking nitrogen. Larva boxes were kept at 25 °C throughout the larval and pupal developmental stages until hatching at 10 d. Hatched flies (5 mg) were then transferred to a fly cage and kept on ¹⁵N-labeled or unlabeled yeast for 48 h before embryo collection. Thus the embryos were collected from labeled flies raised for a full generation on ¹⁵N-labeled yeast,

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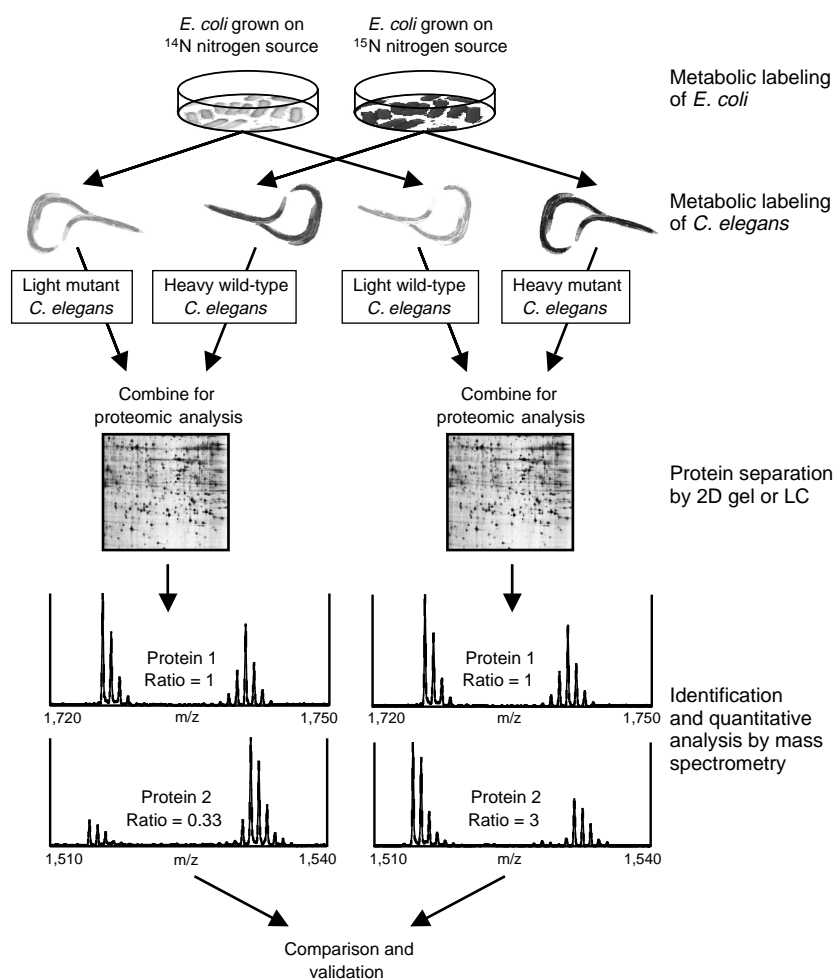


Figure 1 Schematic overview of the experimental approach for metabolic labeling. *E. coli* was grown in two separate batches on identical medium, except that one batch was 98% enriched in ^{15}N . Subsequently, wild-type and mutant nematodes were grown using the labeled and unlabeled batch of *E. coli*, respectively, as the only food source. Next, labeled wild-type and unlabeled mutant worms were pooled in a 1:1 ratio. Protein was extracted and separated by 2D-gel electrophoresis, protein spots were excised and tryptic digests were analyzed by MS. Protein identification and relative quantification of their expression levels were done using the labeled and unlabeled peptide doublets. As a validation of the quantification, this whole procedure was repeated in a reciprocal arrangement by labeling *glp-4* mutants instead of wild-type nematodes.

and the germline or maternal contribution to the second-generation embryos was therefore also labeled. We then collected 0–12 h embryos and subjected them to 2D gel electrophoresis. Analysis by MS of corresponding labeled and unlabeled protein spots showed that in all labeled proteins ^{15}N incorporation was 94–95% with no residual unlabeled protein left (Figs. 2d,e). These results establish that virtually complete labeling can be achieved within the lifespan of *D. melanogaster*.

The complete labeling of both multicellular organisms provides tools for quantitative proteomics studies without the need for chemical tagging, and with the important advantage that labeled and unlabeled animals can be combined before protein extraction, thereby minimizing differences due to sample handling. To illustrate the applicability of the approach, we compared protein expression profiles in the wild-type *C. elegans* strain N2 with those in the mutant strain *glp-4*, in which germline cells stop proliferating at an early stage⁴. As a result no sperm cells or oocytes are produced in

these mutants. *glp-4* and N2 nematodes were fed on normal and labeled bacteria, respectively, using the optimized protocol described above. All nematodes were harvested at the early adult stage, and equal volumes of labeled and unlabeled nematodes were mixed directly after harvesting. We extracted the protein from these combined batches and prepared 2D gels. We excised spots randomly and digested them for identification and relative quantification.

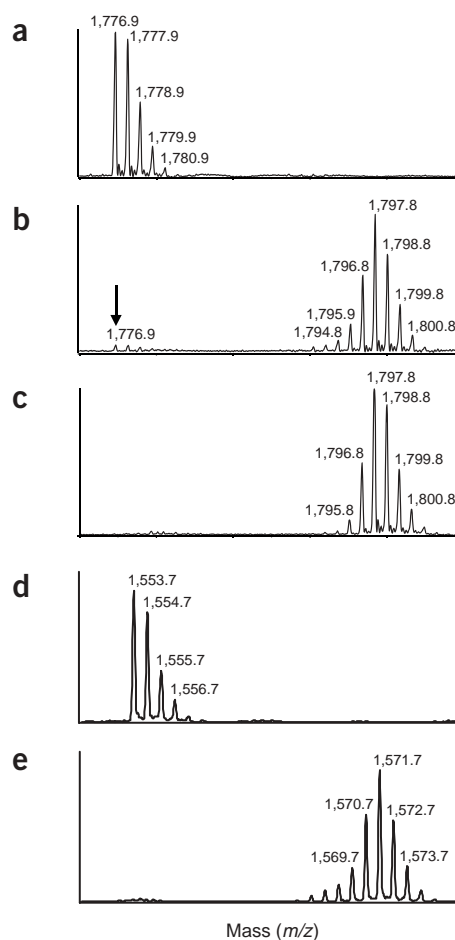
For relative quantification, ratios of peptide peak areas were calculated for multiple peak pairs per spectrum (minimum 3, average 6) and averaged. Because equal volumes of labeled and unlabeled nematodes were mixed before protein extraction in a ratio close to but not necessarily equal to 1, normalization was required to allow direct comparison of later independent data sets. We indeed observed a systematic deviation from 1 for several proteins in our data set, and therefore used the averaged abundance of each of six proteins (H28O16.1, T25C8.2, C07D8.6, F31C3.1, B0393.1 and F10C1.7a) as a normalization factor (0.87 ± 0.05), assuming that the abundance of these proteins (including structural proteins) do not change as a result of the *glp-4* mutation.

Figure 3a depicts the abundance of all identified proteins after normalization. One of the most prominent differences was that major sperm protein was found exclusively in N2 animals. The complete absence of this protein in *glp-4* was expected, as no sperm is formed in this strain. The absence of sperm may also have been reflected in the higher abundance of mitochondrial proteins in N2 animals. For instance, prohibitin (Y37E3.9) is a protein present primarily in mitochondria and was enriched by a factor of 1.4 in N2. Other proteins, such as acyl-CoA dehydrogenase (C55B7.4) and fructose-bisphosphate

aldolase (T05D4.1), are mitochondrial proteins but are also present in other compartments, and therefore displayed a less apparent upregulation. Another remarkable feature was the higher abundance (sevenfold upregulation) of F15E11.1 in *glp-4* mutants. This is a 17.4 kDa protein with unknown function. We analyzed the knockout phenotype of this gene by RNA interference⁵; however, this did not reveal any obvious phenotype.

We validated the results by repeating the entire experiment using reciprocally labeled samples where the *glp-4* strain was labeled instead of N2 (see Fig. 1). We identified and quantified the same set of proteins from 2D gels as represented in Figure 3a. The average abundance of the same six proteins as above (1.05 ± 0.05) was used as the correction factor for normalization. To illustrate the reproducibility of the experiment, we plotted the ratios of the abundance of proteins from both experiments (Fig. 3b). The average of these ratios is 0.98, with a median of 1.00 and s.d. of 0.16. Our results demonstrate that the experiment is highly reproducible for all pro-

Figure 2 Metabolic labeling of *C. elegans* and *D. melanogaster*. To obtain metabolically labeled *C. elegans*, we presented *E. coli*, grown on medium 98% enriched in ^{15}N , as a food source. (a,b) The mass spectra obtained after proteomic analysis of first-generation nematodes revealed that, as compared to the unlabeled control (a), labeling (b) was highly efficient but approximately 4% of the protein remained unlabeled (arrow). (c) Complete labeling was achieved in L4 larvae of second-generation worms. The difference in isotope pattern of the labeled peptide in b compared to c indicates an increase in ^{15}N content from 95% to 98%. The mass shift of 21 Da correlates with the number of nitrogens in this tryptic peptide derived from actin (SYELPDGQVITVGNER, ${}_{76}\text{H}_{122}\text{N}_{21}\text{O}_{28}$). *D. melanogaster* was labeled by culturing on ^{15}N -labeled yeast. The results of 2D gel electrophoresis and MALDI-TOF MS of extracted proteins indicated the incorporation of 94% ^{15}N . (d,e) An unlabeled (d) and a labeled (e) tryptic peptide of the ATP synthase alpha chain (EAYPGDVLYLHSR, $\text{C}_{72}\text{H}_{101}\text{N}_{18}\text{O}_{21}$) exemplify this.



teins analyzed, including the unknown protein F15E11.1 that was found to be sevenfold more abundant in the mutant strain. Apart from multifold differences in expression levels as shown for MSP, F15E11.1, minor differences like that for prohibitin (ratio 1.4), were also accurately reproduced. These data also demonstrate that the accuracy of quantification is irrespective of which of the two samples is labeled. Furthermore, by doing the experiments both ways, any isotope effects on nematode development, if expected at all, can be eliminated.

Researchers have compared the RNA expression profiles of *glp-4* mutants with those of wild-type nematodes in different developmental stages using DNA microarrays⁶. Although these arrays contained 11,917 genes, 11 out of the 25 proteins (44%) that we identified in our study were not represented on the DNA microarrays, including the novel F15E11.1 protein and Y37E3.9 (prohibitin). Of the remaining 14 genes, only those encoding C55B7.4, T05D4.1, ZK829.4 and T21C12.2 showed changes in expression levels, with ratios of 1.85, 1.21, 1.55 and 0.64, respectively⁶. We did not find these differences (Fig. 3), indicating that mRNA and protein expression levels do not necessarily correlate.

To further substantiate that differences in protein expression levels below a factor of 2 can be identified, we performed an orthogonal analysis to quantify prohibitin in N2 and *glp-4* strains. A quantitative western blot analysis of N2 and *glp-4* extracts was conducted using antibodies against actin and prohibitin to determine the abundance of these proteins relative to each other (Figs. 3c,d). Quantification of antibody signals revealed a twofold downregulation of prohibitin in *glp-4*. Considering the relatively large error in these experiments, the relative abundance of 1.4 that we determined falls well within this range.

To illustrate that quantification through metabolic labeling can be done equally well using liquid chromatography (LC)-MS, unlabeled N2 and labeled *glp-4* nematodes were mixed, boiled in Laemmli buffer and the resulting extract was run on an SDS-PAGE gel. The 30-kDa region (containing prohibitin) was excised and digested in-gel, and the peptide mixture was analyzed by LC coupled to tandem MS (LC-MS/MS). In this mixture, prohibitin was identified from two peptides, both of which were present in a ratio of 1.4 in N2 relative to *glp-4* (illustrated for one peak pair in Figure 3e). What also became readily apparent was that the unlabeled and labeled peptide coeluted perfectly (Figs. 3f,g), indicating that, in contrast to the conventional isotope coded affinity tag (ICAT) approach^{7,8}, there was no isotope effect on chromatographic behavior.

The approach presented here shows that *C. elegans* and *D. melanogaster* can be efficiently labeled with stable isotopes. We

have shown that using this approach both major and subtle differences in protein abundance can be determined accurately and reproducibly. We think that this results from three aspects of this method: (i) labeling is quantitative, (ii) variation in sample handling is eliminated because unlabeled and labeled organisms are processed simultaneously in the same vial and (iii) no derivatization steps are needed after protein extraction. All three aspects distinguish this approach from *in vitro* labeling methods such as ICAT⁸. Furthermore, we have shown that protein identification and quantification can be done using the two most frequently adopted approaches in proteomics, 2D-gel electrophoresis followed MALDI-TOF MS, as well as liquid-phase separations such as reversed-phase chromatography coupled to MS.

Taken together, our establishment of simple and efficient protocols for the virtually complete metabolic labeling of flies and worms offers a versatile approach for quantitative proteomics, which should now allow a fruitful integration of proteomics and developmental genomics studies in these two multicellular systems. We expect that metabolic labeling of these organisms will provide particularly useful tools for determining changes in protein expression levels in different developmental stages, in response to environmental changes and in specific mutants, and that it will provide a valuable complement to DNA array-based approaches in this field^{6,9}. Additionally, metabolic labeling can be readily integrated with already existing biochemical methods to elucidate biochemical pathways or functional protein-protein interactions as applied recently to cell cultures¹⁰.

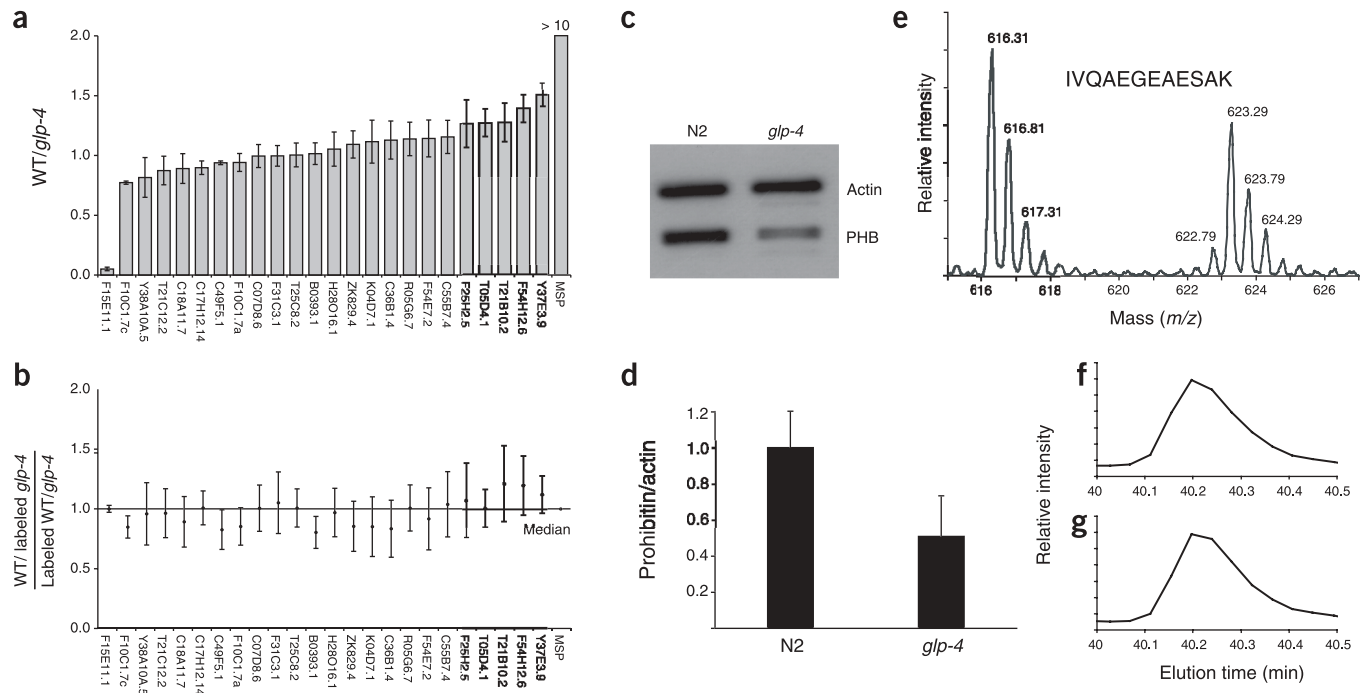


Figure 3 Relative quantification of expression levels of proteins in nematodes with and without a germ line. **(a)** Ratio of protein expression levels in wild-type (WT) *C. elegans* relative to the germline deficient *glp-4* mutant. The bars show the relative abundance of individual proteins in unlabeled WT nematodes relative to ^{15}N -labeled *glp-4* mutants. Error bars indicate the s.d. observed in these peptide-pair signals. **(b)** Validation of the quantification method in two independent experiments. The relative abundance of each protein in the first experiment (unlabeled N2 versus labeled *glp-4*) are divided by the abundance from the second experiment (labeled N2 versus unlabeled *glp-4*). The average value for all proteins is 0.98; the median is 1.00, with an s.d. of 0.16. Error bars indicate the s.d. of individual proteins over both experiments. For MSP no s.d. could be calculated because this protein was present only in strain N2. **(c)** Quantification of prohibitin Y37E3.9 expression levels in N2 and *glp-4* strains by western blotting and metabolic labeling. The western blot shows lower expression of prohibitin in *glp-4* mutants than in N2 (wild type) worms. **(d)** Quantification of antibody signals shows that the amount of prohibitin relative to actin drops by a factor of 2 in *glp-4* mutants. **(e)** Prohibitin was also quantified by LC-MS/MS. Unlabeled N2 and labeled *glp-4* worms were mixed, their extract was run on an SDS gel, and the 30-kD region was digested with trypsin. The peptide pair shown represents the unlabeled and labeled form of one of the tryptic peptides of prohibitin (IVQAEGEAESAK, $\text{C}_{51}\text{H}_{87}\text{N}_{14}\text{O}_{21}$) with an abundance ratio of 1.4. **(f,g)** Mass chromatograms show that the unlabeled **(f)** and labeled **(g)** peptides coelute, excluding any isotope effects on chromatographic behavior. F15E11.1, novel protein; F10C1.7c, intermediate filament protein; Y38A10A.5, calreticulin precursor; T21C12.2, 4-hydroxyphenylpyruvate dioxygenase; C18A11.7, Dim-1; C17H12.14, ATPase; C49F5.1, S-adenosylmethionine synthetase; F10C1.7a, intermediate filament protein; C07D8.6, aldehyde reductase; F31C3.1, cyclophilin; T25C8.2, actin-5; B0393.1, 40S ribosomal protein; H28O16.1, ATP synthase; ZK829.4, glutamate dehydrogenase; K04D7.1, guanine nucleotide-binding protein; C36B1.4, proteasome subunit; R05G6.7, channel protein; F54E7.2, 40S ribosomal protein; C55B7.4, acyl-CoA dehydrogenase; F25H2.5, nucleoside diphosphate kinase; T05D4.1, fructose-bisphosphate aldolase; T21B10.2, enolase; F54H12.6, elongation factor 1; Y37E3.9, prohibitin; ZK546.3, major sperm protein.

METHODS

Culturing of *C. elegans*. *C. elegans* was grown at 15 °C or 25 °C, on plates containing 12 g agarose, 3 g NaCl, 5 $\mu\text{g/ml}$ cholesterol, 1 mM CaCl_2 , 1 mM MgCl_2 and 25 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 6.0. These plates were seeded with bacteria (strain OP50) that were grown in either medium 98% enriched in ^{15}N (Martek 9-N) or the equivalent with natural isotope abundance (Martek 9-U, Spectra Stable Isotopes). To get a thick bacterial lawn, 1 liter of overnight culture was pelleted and resuspended in approximately 20 ml medium. Of this suspension, 400 μl was used to seed the plates. Eggs were isolated by standard bleach treatment and hatched overnight at 15 °C or 25 °C. The resulting L1 larvae were then distributed over the appropriate plates. The developmental stage of the animals was monitored visually. Worm strains used were Bristol N2 (wild type) and SS104 (*glp-4*(bn2)). The SS104 strain harbors a temperature-sensitive allele of *glp-4*, so that animals grown at 15 °C are wild type and animals grown at 25 °C are devoid of a germ line. For this reason, both N2 and SS104 were grown at 15 °C in the first generation of labeling, and at 25 °C in the second generation of labeling. After this second generation, the animals were washed off the plates and left in buffer for 30 min to digest the bacteria in their digestive system. Then they were washed with water and frozen at -80 °C.

Culturing and labeling of *D. melanogaster*. ^{15}N -labeled and unlabeled *Saccharomyces cerevisiae* type II (Sigma) was cultured by growing at 30 °C in minimal medium supplemented with Yeast Nitrogen Base (Difco), sucrose, and ^{15}N -labeled (Spectra Stable Isotopes) or unlabeled (Sigma) ammonium sulfate as a source of nitrogen. We spread 5 mg of 0–12 h *D. melanogaster* embryos on $9 \times 9 \times 6$ cm larva boxes containing 80 ml larva box mix (6 g sucrose, 24 μl propionic acid, 160 μl phosphoric acid, 0.08 % tegosept, 0.1 mg/ml ampicillin and 30 ml ^{15}N -labeled or unlabeled yeast) over a layer of cotton. Larva boxes were kept at 25 °C in 80% humidity until flies hatched at 10 d. We transferred 5 ml newly hatched flies to collection cages (radius 4.5 cm \times 10 cm) where they fed on ^{15}N -labeled or unlabeled yeast spread on plates with an agarose matrix for 48 h. We collected 20 mg 0–12 h embryos from agarose plates and pooled them. Embryos were washed, dechorionated by incubation in 2.5% sodium hypochlorite for 90 s followed by washing first with buffer containing 0.7% NaCl, 0.04% Triton X-100, and then thoroughly with H_2O , and kept at -80 °C.

Protein extraction. Labeled and unlabeled *C. elegans* were analyzed separately, or combined for the relative quantification of proteins in the N2 strain compared to the *glp-4* mutant. In the latter case, equal volumes of labeled and unla-

beled nematode suspensions were mixed before protein extraction to ensure identical treatment of each sample.

Washed *C. elegans* or *D. melanogaster* pellets were dried in a rotary evaporator and lysed in lysis solution (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM dithiothreitol (Sigma)) under vigorous shaking. Lysates were cleared by centrifugation (18,000g), and protein content was determined using a Bradford protein assay (Pierce).

2D electrophoresis. Approximately 200 µg of protein extract in 350 µl of sample buffer containing 7 M urea (Sigma), 2 M thiourea (Sigma), 4% CHAPS (Sigma), 100 mM dithiothreitol (Sigma) and 0.5% (vol/vol) carrier ampholytes, pH 3–10 nonlinear (NL), was applied to a 24-cm immobilized pH gradient (IPG) strip, pH 3–10 NL. Rehydration was carried out for 14 h at 30 V using an IPGphor, followed by isoelectric focusing (IEF) at a limiting current of 50 µA in consecutive steps at 500 V for 1 h, 1,000 V for 1 h and 8,000 V for 7.5 h. All IEF equipment and related reagents were from Amersham Biosciences. IPG strips were kept at –80 °C for later use, or equilibrated immediately for 15 min in a buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (vol/vol) glycerol, 2% (wt/vol) SDS and 2% (wt/vol) dithiothreitol (all from Sigma). For second-dimension analysis, we used a Dodeca Cell (Bio-Rad) with 12% acrylamide SDS-PAGE gels. Equilibrated IPG strips were put on top of 12% acrylamide SDS-PAGE gels, sealed in agarose containing a trace amount of bromophenol blue, and second-dimension separation was performed by electrophoresis at 200 mA until the front had reached the lower end of the gel. Proteins were detected by silver staining.

Mass spectrometry. Silver-stained spots were excised and destained for 10 min in a solution containing 30 mM potassium ferricyanide and 100 mM sodium thiosulfate (Sigma). After repeated washings with water and acetonitrile, proteins were digested in-gel with modified trypsin (Roche Diagnostics) in 50 mM ammonium bicarbonate (Sigma). After digestion, peptides were concentrated using µC18-ZipTips (Millipore) and eluted directly on the MALDI target in 1 µl of a saturated solution of α-cyanohydroxycinnamic acid in 50% acetonitrile (vol/vol). Peptides were analyzed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems) operated in reflectron mode at 20 kV accelerating voltage.

MS/MS measurements were performed on an ESI quadrupole time-of-flight instrument (Q-TOF; Micromass) operating in positive ion mode. For LC-MS, a nano-LC system was coupled to the Q-TOF essentially as described¹¹. Peptide mixtures were delivered to the system using a Famos autosampler (LC Packings) at 3 µl/min, and trapped on an AquaTM C18RP column (Phenomenex; column dimensions 1 cm × 100 µm internal diameter (i.d.), packed in-house). After flow splitting down to 150–200 nl/min, peptides were transferred to the analytical column (PepMap; LC Packings; column dimensions 25 cm × 50 µm i.d., packed in-house) in a gradient of acetonitrile (1% per min). Fragmentation of eluting peptides was performed in data-dependent mode, and mass spectra were acquired in full-scan mode.

Protein identification and quantification. MALDI-TOF mass spectra were calibrated internally with known trypsin peaks, and proteins were identified

by searching masses of measured peptides against *C. elegans* or *D. melanogaster* proteins in nonredundant protein databases using the peptide mass fingerprint tool in Mascot (Matrix Science) allowing a mass tolerance of 40 p.p.m. For relative protein quantification, peak areas of all isotopes of the unlabeled peptide were integrated and divided by the integrated peak area of the labeled peptide. This was done for several peak pairs in the same spectrum. The percentage of ¹⁵N incorporation was estimated by comparing the obtained isotope distribution of a labeled peptide with a predicted distribution using the isotope simulator IsoPro 3.0 (<http://members.aol.com/msmssoft>).

Western blot analysis. For western blots, N2 (wild type) and *glp-4* mutant worms were collected by centrifugation. Worm pellets were washed free of bacteria. Approximately 30 µl of pelleted worms were boiled in 5 volumes sample buffer for 5 min. Different aliquots of extracted proteins were resolved on 10% acrylamide SDS-PAGE gels¹². Proteins were electrophoretically transferred to nitrocellulose membranes and membranes were probed with anti-prohibitin-1 antibody¹³ and anti-actin antibody (ICN, clone C4). Immunoreactive material was visualized by enhanced chemiluminescence (Amersham) according to the manufacturer's instructions. Antibody signals were quantified using ImageQuant software (Molecular Dynamics).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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