

*Cell-Specific Proteomic Analysis
in Caenorhabditis elegans*

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2.1 Abstract

Proteomic analysis of rare cells in heterogeneous environments presents difficult challenges. Systematic methods are needed to enrich, identify, and quantify proteins expressed in specific cells in complex biological systems including multicellular plants and animals. Here, we have engineered a *Caenorhabditis elegans* phenylalanyl-tRNA synthetase capable of tagging proteins with the reactive non-canonical amino acid *p*-azido-L-phenylalanine. We achieved spatiotemporal selectivity in the labeling of *C. elegans* proteins by controlling expression of the mutant synthetase using cell-selective (body wall muscles, intestinal epithelial cells, neurons, and pharyngeal muscle) or state-selective (heat-shock) promoters in several transgenic lines. Tagged proteins are distinguished from the rest of the protein pool through bio-orthogonal conjugation of the azide side chain to probes that permit visualization and isolation of labeled proteins. By coupling our methodology with stable-isotope labeling of amino acids in cell culture (SILAC), we successfully profiled proteins expressed in pharyngeal muscle cells, and in the process, identified proteins not previously known to be expressed in these cells. Our results show that tagging proteins with spatiotemporal selectivity can be achieved in *C. elegans* and illustrate a convenient and effective approach for unbiased discovery of proteins expressed in targeted subsets of cells.

2.2 Introduction

Caenorhabditis elegans is a small, free-living nematode (roundworm) (**Figure 2.1**) [97]. Since *C. elegans*'s introduction as a model organism to study development, neurobiology, and behavior by Sydney Brenner several decades ago [98], this multicellular organism has been used to study a variety of intricate biological and molecular processes such as ageing [99], apoptosis [100], cell cycle [101], cell polarity [102], cell signaling [103], disease pathogenesis [104], gene regulation [105], host-pathogen

interactions [106], and metabolism [107].

C. elegans biology has several attractive features that contribute to its increasing popularity in research. First, *C. elegans* is easy to maintain in the laboratory. Although the worm normally colonizes various microbe-rich habitats like decaying plant matter, it survives in liquid culture or on the surface of agar plates, feeding on a lawn of *Escherichia coli* [108,109]. Second, *C. elegans* has a short generation time and reproduces prolifically. Following hatching, it progresses through 4 larval stages (L1 to L4) in about 3 days on route to becoming a mature, egg-bearing adult capable of yielding up to 300 progeny (**Figure 2.2**) [110]. Third, *C. elegans* is small (about 1.3 mm in length and 80 microns in diameter in the adult stage), cell number invariant, and transparent. Hermaphrodites contain exactly 959 and males 1031 somatic cells whose lineage have been completely characterized by light microscopy [111,112]. Fourth, *C. elegans* is a sophisticated multi-cellular animal. Despite the small number of cells, these cells generate a diverse set of organs and tissues such as an excretory system, gonads, a hypodermis, intestine, muscle, and a nervous system comprising 302 neurons. Last, numerous *C. elegans* genetic and genomic resources are available [113], and *C. elegans* was the first multi-cellular organism whose genome was fully sequenced [114], permitting its genomic and proteomic interrogation.

In a complex eukaryote like *Caenorhabditis elegans*, cell heterogeneity restricts the usefulness of large-scale, mass spectrometry-based proteomic analysis. Enriching for specific cells is challenging, and researchers cannot systematically identify low-abundance proteins expressed in specific cells from whole-organism lysates. Cell-selective bio-orthogonal non-canonical amino acid tagging (cell-selective BONCAT) offers a way to overcome these limitations [115,116]. We have previously engineered a family of mutant *Escherichia coli* methionyl-tRNA synthetases (MetRSs) capable of appending the azide-bearing L-methionine (Met) analog L-azidonorleucine (Anl) to its cognate tRNA in competition with Met [87,88]. Because Anl is a poor substrate

for any of the natural aminoacyl-tRNA synthetases, it is excluded from proteins made in wild-type cells but is incorporated readily into proteins made in cells that express an appropriately engineered MetRS. Controlling expression of mutant MetRSs by expression only in specific cells restricts Anl labeling to proteins produced in those cells. Tagged proteins can be distinguished from the rest of the protein pool through bio-orthogonal conjugation of the azide side chain to alkynyl or cyclooctynyl probes that permit facile detection, isolation, and visualization of labeled proteins. This strategy has been used to selectively enrich microbial proteins from mixtures of bacterial and mammalian cells. For example, Ngo et al. [89] found that proteins made in an *E. coli* strain outfitted with a mutant MetRS could be labeled with Anl in coculture with murine alveolar macrophages, which were not labeled. Using similar approaches, Grammel et al. [90] identified virulence factors from *Salmonella typhimurium* that were expressed in the course of infection of murine macrophages, and Mahdavi et al. [117] profiled *Yersinia enterocolitica* proteins that were injected into HeLa cells. In a complementary approach, Chin and coworkers [118] recently reengineered orthogonal *Methanosarcina barkeri* and *Methanosarcina mazei* pyrrolysyl-tRNA synthetase/tRNA pairs for codon-selective incorporation of a cyclopropene lysine derivative into proteins made in *E. coli*, *Drosophila melanogaster* ovaries, and HEK293 cells; however, this technique requires the expression of both exogenous aminoacyl-tRNA synthetases and tRNAs. Here, we configure cell-selective BONCAT for cell-specific proteomic analysis in the nematode *C. elegans* (**Figure 2.3**). We first demonstrate that restricted expression of a mutant *C. elegans* phenylalanyl-tRNA synthetase (*CePheRS*) can label proteins with *p*-azido-L-phenylalanine (Azf; **Figure 2.4**) with spatiotemporal selectivity in the live worm. We then show that cell-selective BONCAT combined with stable-isotope labeling of amino acids in cell culture (SILAC) provides a convenient and effective approach for unbiased discovery of proteins uniquely expressed in a subset of cells.

2.3 Results and Discussion

2.3.1 Engineering a *C. elegans* PheRS Capable of Activating Azf

We focused our attention on the heterotetrameric *CePheRS* because we could not prepare healthy transgenic *C. elegans* strains that express mutant *E. coli* MetRSs. Furthermore, we found that *C. elegans* variants of the mutant *E. coli* MetRSs that we had used to activate Anl in our previous experiments showed no activity toward Anl (**Table 2.1**). *CePheRS* catalyzes esterification of L-phenylalanine (Phe; **Figure 2.4**) to its cognate tRNA (*CetRNA^{Phe}*) to form phenylalanyl-tRNA. A conserved “gatekeeper” threonine (Thr412 [the first methionine in the alpha subunit of *CePheRS*, isoform A, exon 3 is designated as residue 1 (“Met1”), *C. elegans* numbering; **Figures 2.5 and 2.6**) in the alpha subunit has been proposed to play a key role in determining substrate specificity in both prokaryotic and eukaryotic PheRSs [119, 120]. Therefore, we hypothesized that mutating this residue to smaller residues should enable *CePheRS* to activate and charge the larger azide-bearing Phe analog Azf to *CetRNA^{Phe}*. To screen for such an enzyme, we cultured KY14[pKPY93/pKPY1XX], a phenylalanine-auxotrophic strain of *E. coli* that expresses mutant forms of *CePheRS*, in M9 minimal medium supplemented with different concentrations of Phe and Azf (**Figure 2.7**). To assess *CePheRS* activity toward Azf, we detected Azf-labeled proteins by conjugation to dibenzocyclooctyne-functionalized tetramethylrhodamine (TAMRA-DBCO; **Figure 2.4**) and subsequent SDS/PAGE-in-gel fluorescence scanning. Although several mutants (Thr412Ser, Thr412Ala, Thr412Gly) showed evidence of labeling with Azf, only the Thr412Gly mutant (Thr412Gly-*CePheRS*) displayed robust labeling in cells treated with equimolar amounts of Phe and Azf (**Figure 2.8**). We confirmed by *in vitro* ATP-PP_i exchange assays that Thr412Gly-*CePheRS* is highly selective toward Azf: it activates Azf more than 20-fold faster than its canonical substrate Phe (**Table 2.2**). Thus, Azf labeling

does not require depletion of Phe from an animal’s diet, making cell-selective labeling feasible in live worms. Although Thr412Gly-*CePheRS* also activates tryptophan threefold faster than Phe, MALDI-TOF mass spectrometry measurements of tryptic GFP peptides did not detect misincorporation of tryptophan or any other canonical amino acid when GFP was expressed in media supplemented with either Phe or Azf (**Figure 2.9**). Collectively, these results suggest that Thr412Gly-*CePheRS* selectively activates Azf with catalytic efficiency similar to that observed in the activation of Phe by wild-type *CePheRS*. Introducing the Thr412Gly mutation into PheRSs of other eukaryotic cells including human also permits Azf activation (**Figure 2.10**). From these observations, we conclude that Thr412Gly-*CePheRS* is the best aminoacyl-tRNA synthetase candidate for cell-selective labeling in *C. elegans*. Although we generated transgenic *C. elegans* by DNA injection into the syncytial germ line in this work, inducible or cell-selective genome editing technologies could be used to quickly and efficiently generate transgenic animals because a single mutation in *CePheRS* is sufficient for Azf activity [121].

2.3.2 Characterizing Azf Labeling in *C. elegans*

To evaluate the performance of Thr412Gly-*CePheRS* in *C. elegans*, we first generated transgenic *C. elegans* lines that express both the mutant alpha subunit and GFP under control of the *hsp-16.2* promoter. *hsp-16.2* encodes a 16-kDa protein that is induced in multiple tissues in response to heat shock and other stresses [122]. Upon heat shock, we expected that the mutant alpha subunit would be expressed and form a hybrid heterotetramer with the endogenous beta subunit to produce fully active Thr412Gly-*CePheRS*. In our initial feeding experiments, we did not detect strong Azf labeling in transgenic animals when Azf was added exogenously to either liquid culture or solid agar plates. However, we found that these lines could be labeled by replacing their normal food source (e.g., *E. coli* OP50) with bacteria whose

proteins contain the non-canonical amino acid of choice in a fashion analogous to isotopic labeling [30, 35, 36]. We first labeled bacteria by culturing KY14[pKPY514], a phenylalanine-auxotrophic strain of *E. coli* that expresses the *E. coli* variant of Thr412Gly-*Ce*PheRS (Thr251Gly-*Ec*PheRS), in M9 minimal medium supplemented with Azf (**Figure 2.11**). The extent of replacement of Phe by Azf in total *E. coli* protein was determined by amino acid analysis to be 50-51% (**Figure 2.12**). We next fed labeled bacteria to *hsp-16.2::Thr412Gly-CePheRS* worms previously grown on OP50. We induced heat shock in worms grown at 20°C by 1-h exposure to 33°C and cleared external as well as ingested bacteria by washing worms with S medium over a period of 30 min. We could not detect *E. coli* protein (even the overexpressed Thr251Gly-*Ec*PheRS) in processed *C. elegans* lysate (**Figure 2.13**). Both in-gel fluorescence scanning of TAMRA-DBCO-treated lysates and fluorescence microscopy of TAMRA-DBCO-treated fixed animals revealed that only heat-shocked worms exhibited ubiquitous Azf labeling 24 h after heat shock (**Figure 2.14**). We detected labeled proteins as early as 1 h after heat shock (shorter times were not tested). Moreover, we observed no differences in behavior, development, or survival in worms fed with Azf-labeled *E. coli* versus worms fed with unlabeled *E. coli* for up to 72 h after heat shock (longer times were not tested).

2.3.3 Labeling Spatially Defined Protein Subpopulations

The core concept of cell-selective BONCAT is that restricting expression of mutant aminoacyl-tRNA synthetases by using promoters active only in specific cells restricts non-canonical amino acid labeling to those cells. Enrichment of labeled proteins permits examination of proteomic changes in those cells. Encouraged by the performance of *hsp-16.2::Thr412Gly-CePheRS* worms, we next tested cell-specific expression of Thr412Gly-*Ce*PheRS by generating transgenic *C. elegans* lines that express both the mutant alpha subunit and GFP under control of promoters shown previously to be

active specifically in the 95 body wall muscle cells (*myo-3*; [123]), the 20 intestinal cells (*ges-1*; [124]), neurons (*rab-3*; [125]), and the 20 pharyngeal muscle cells (*myo-2*; [126]) (**Figure 2.15**). We first fed Azf-labeled bacteria to each of these animals. We also observed no differences in behavior, development, or survival in worms fed with Azf-labeled *E. coli* versus worms fed with unlabeled *E. coli* for up to 72 h (longer times were not tested). We then treated fixed worms with TAMRA-DBCO to visualize sites of Azf incorporation. Fluorescence microscopy revealed that labeling in *myo-3::Thr412Gly-CePheRS*, *ges-1::Thr412Gly-CePheRS*, *rab-3::Thr412Gly-CePheRS*, and *myo-2::Thr412Gly-CePheRS* worms was confined to the body wall muscle, intestine, neurons, and pharyngeal muscle, respectively (**Figures 2.16 and 2.17**).

2.3.4 Identifying Pharyngeal Muscle-Specific Proteins

We next investigated whether proteins isolated from worms with cell-specific Thr412Gly-CePheRS fit characteristics of the targeted cell type. We were particularly interested in the *C. elegans* pharynx, a widely used model to study organ formation during embryogenesis [127]. The pharynx is a tube-like muscular pump that concentrates, grinds, and transports bacteria from the mouth to the intestine and comprises 68 cells: 9 epithelial, 4 gland, 9 marginal, 20 muscle, 20 neuronal, and 6 valve cells. We aimed to identify proteins expressed in pharyngeal muscle cells of *myo-2::Thr412Gly-CePheRS* worms by using a combined cell-selective BONCAT and SILAC approach. We first triply labeled food by culturing KY33[pKPY514], an arginine-, lysine-, and phenylalanine-auxotrophic strain of *E. coli*, in M9 minimal medium supplemented with “heavy” arginine ($^{13}\text{C}_6\text{ }^{14}\text{N}_4\text{ }^1\text{H}_{14}\text{ }^{16}\text{O}_2$), heavy lysine ($^{13}\text{C}_6\text{ }^{15}\text{N}_2\text{ }^1\text{H}_{14}\text{ }^{16}\text{O}_2$), and Azf. We next fed these bacteria to fourth larval stage *myo-2::Thr412Gly-CePheRS* animals previously grown on “light” OP50. According to our model of the cell-selective BONCAT method, all newly synthesized proteins in the animal should contain both

heavy arginine and heavy lysine, but only newly synthesized proteins made in pharyngeal muscle cells – the cells that express Thr412Gly-*Ce*PheRS – should contain Azf. This model gives rise to four classes of proteins: (i) light preexisting proteins synthesized in pharyngeal muscle cells before the shift in food source, (ii) light preexisting proteins synthesized in nontargeted cell types, (iii) heavy newly synthesized proteins – labeled with Azf – made in pharyngeal muscle cells after the shift in food source, and (iv) heavy newly synthesized proteins made in nontargeted cell types. The use of heavy labels enables relative quantitation of newly synthesized (heavy, H) and preexisting (light, L) peptides. Because enrichment concentrates heavy peptides derived from Azf-labeled proteins, cell selectivity can be quantified by comparing H/L ratios for “enriched” and “unenriched” proteins derived from the same worm sample. Proteins with the highest values of $(H/L)_E/(H/L)_U$ should have originated from pharyngeal muscle cells. Following 24 h of labeling through feeding, worm lysates were incubated with sodium dithionite-cleavable dibenzocyclooctyne-functionalized biotin (Diazo Biotin-DBCO; **Figure 2.4**), and Azf-containing proteins were isolated by streptavidin affinity chromatography (**Figures 2.18, 2.19, and 2.20**). Enriched and unenriched samples were resolved by SDS/PAGE and subjected to in-gel proteolytic digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

We identified and quantified 2,270 proteins across triplicate paired (enriched and unenriched) experiments (**Figures 2.21, 2.22, 2.23, and 2.24 and Table 2.3**). Of the quantified proteins, 1,607 (71%) had $(H/L)_E/(H/L)_U$ values greater than one, which indicates that the purification method successfully enriched newly synthesized, cell-specific proteins. Among the enriched proteins were 782 proteins that have expression patterns reported in the literature according to WormBase WS244 [128]; of these proteins, 409 are known to be expressed in the pharynx (**Table 2.4**). We expected that proteins expressed in pharyngeal muscle cells would be overrepresented

among proteins with high $(H/L)_E/(H/L)_U$ values and, indeed, found that of the top 12 proteins quantified, two [TNI-4 [129], a troponin I protein and TNC-2 [130], a troponin C protein] are expressed exclusively in pharyngeal muscle cells. Two proteins [SHL-1 [131], a voltage-gated potassium channel and NCX-2 [132], a sodium-calcium exchanger] are expressed in many muscle cells including pharyngeal muscle cells. A fifth protein F59F4.1 [133] is an acyl-CoA oxidase that is also expressed in the pharynx. Also, three well-known pharyngeal muscle-specific myosin heavy chains were among the top 3% of most highly enriched proteins: MYO-1 (top 1.0%, 22/2,270), MYO-2 (top 1.3%, 29/2,270), and MYO-5 (top 2.6%, 58/2,270). Although the right tail of the $(H/L)_E/(H/L)_U$ distribution contains relatively few members, they represent pharynx-specific proteins: of the 1,100 proteins in our dataset with known expression patterns, 7 of the 18 that are thought to be expressed exclusively in the pharynx have $(H/L)_E/(H/L)_U$ values greater than two ($P = 1.25 \times 10^8$; Fisher’s exact test).

Three of the remaining seven “top-12” proteins in our dataset (C53C9.2, K03E5.2, and CPN-4) share similarity with Calponin-1 [134], a human protein implicated in the regulation of smooth muscle contraction, but their expression patterns have not been reported. To determine whether they are expressed in pharyngeal muscle cells, we generated transgenic *C. elegans* lines that express GFP under control of each of the respective 5’ regulatory regions. We detected strong GFP fluorescence exclusively in pharyngeal muscle cells in *C53C9.2::gfp*, *K03E5.2::gfp*, and *cpn-4::gfp* animals (**Figure 2.25**). Calponin-1 has a single calponin homology (CH) and multiple calponin family repeat (CFR) domains. Notably, like its muscle-specific paralog CPN-3, CPN-4 has a CH domain, but no CFRs (**Figure 2.26**). In contrast, C53C9.2 and K03E5.2 have multiple CFRs but no CH domains. Only four *C. elegans* proteins contain multiple CFRs: C53C9.2, K03E5.2, T25F10.6, and UNC-87 [135], a protein required to maintain structure of myofilaments in muscle cells. T25F10.6 (top 5.3%,

121/2,270) and UNC-87 (top 7.5%, 170/2,270) were also among the top 10% of most highly enriched proteins. Although the characterization of new pharyngeal proteins was beyond the scope of this work, their placement among highly enriched proteins, localization, and similarity to other muscle-specific proteins suggest that C53C9.2, K03E5.2, and CPN-4 are excellent candidates for regulating aspects of pharyngeal muscle biology.

Finally, we asked whether our approach undesirably identified any off-target or “non-pharyngeal” proteins. Miller and coworkers used epitope-tagged (FLAG) polyA-binding protein (PAB-1) under the control of cell-specific promoters to isolate RNA from specific larval cells [136]. This work had larval data sets for body wall muscle, intestine, and neurons, but not pharyngeal muscle. For each body wall muscle, intestine, and neurons dataset, we examined the top 12 most enriched genes versus a reference sample of all cells that also has protein abundance information in PaxDb [137] and therefore had been identified in previous mass spectrometry experiments. SUR-5 appeared in all three lists because SUR-5 is highly expressed in body wall muscle, intestine, and neurons [138]. In this list of 34 “non-pharyngeal” proteins, only three appeared in our dataset: SUR-5 ($(H/L)_E/(H/L)_U = 1.21$), ELPC-3 ($(H/L)_E/(H/L)_U = 0.77$), and C24H12.4 ($(H/L)_E/(H/L)_U = 0.97$). Upon further examination, SUR-5 and ELPC-3 have been found to be also expressed in the pharynx in GFP fusion experiments [138, 139]. C24H12.4 has no expression data reported in the literature. Although the absence of a protein from a proteomic dataset cannot be taken as evidence that the protein is absent from the sample, this result is consistent with the hypothesis that the method described here provides an effective means of enriching pharyngeal proteins.

Together, these results demonstrate that the approach described here can be used to identify proteins (including proteins with previously unknown expression patterns) that are expressed in targeted subsets of cells. We note that in a cell-selective BONCAT experiment, proteins are labeled only after the shift in food source. Although long labeling times can be used to profile the majority of proteins in specific cells, short labeling times can be used to capture rapid changes in protein expression in those cells.

2.4 Conclusions

In summary, by using cell-specific promoters to drive expression of an engineered *CePheRS*, we demonstrated that cell-selective BONCAT coupled with SILAC can be used to identify proteins with spatiotemporal selectivity in living *C. elegans*. For future experiments, we suggest several avenues for improvement. First, we incubated worm lysates with Diazo Biotin-DBCO and isolated Azf-labeled proteins by streptavidin affinity chromatography. Because streptavidin affinity chromatography requires mild washing conditions to preserve streptavidin's bioactivity, enrichment quality might be affected by background proteins due to insufficient washing. Alternatively, Azf-labeled proteins can be selectively captured on commercially available alkynyl- or cyclooctynyl-functionalized resins that allow for highly stringent washing conditions to remove nonspecifically bound proteins. Second, we processed samples by SDS/PAGE and in-gel proteolytic digestion before LC-MS/MS analysis, but this approach can introduce contaminants and is time-consuming and laborious. Because chemical tagging of Azf-labeled proteins occurs immediately after animal lysis, our strategy is compatible with new advances in analytical proteomic workflows such as in StageTip-based filter-aided sample preparation (FASP) [140]. Third, in the LC-MS/MS analysis, we normalized H/L ratios of enriched proteins to total unenriched proteins derived from the same worm sample. Because the H/L ratio variability

depends on different intrinsic rates of protein synthesis in different cells, we advise investigators to additionally normalize H/L ratios of enriched proteins from one cell type to total proteins derived from mixed-stage worms or enriched proteins from another cell type for a more comprehensive analysis of cell-specific proteins.

Finally, our methodology should prove useful in multiple contexts. For example, one could easily build cell-specific proteomic atlases because (i) a catalog of cell-specific transcriptional enhancers is readily available and (ii) the creation of transgenic organisms is both rapid and routine. In addition, using regulatory elements to drive intersectional patterns of expression, one could restrict labeling to cells that express both elements and, thus, enhance spatiotemporal selectivity with either a FLP recombinase-based [141] or protein reconstitution-based [94] approach. Furthermore, this technique could be used to study protein-protein interactions in a cell-specific manner because aryl azides like Azf are activated upon UV light irradiation to form covalent adducts with proteins in close proximity [142]. In principle, the methodology described here could be applied to other organisms in which efficient delivery of non-canonical amino acids is feasible, alleviating the need for cell sorting or laser capture techniques to isolate protein from specific cells in intact organisms.

2.5 Figures

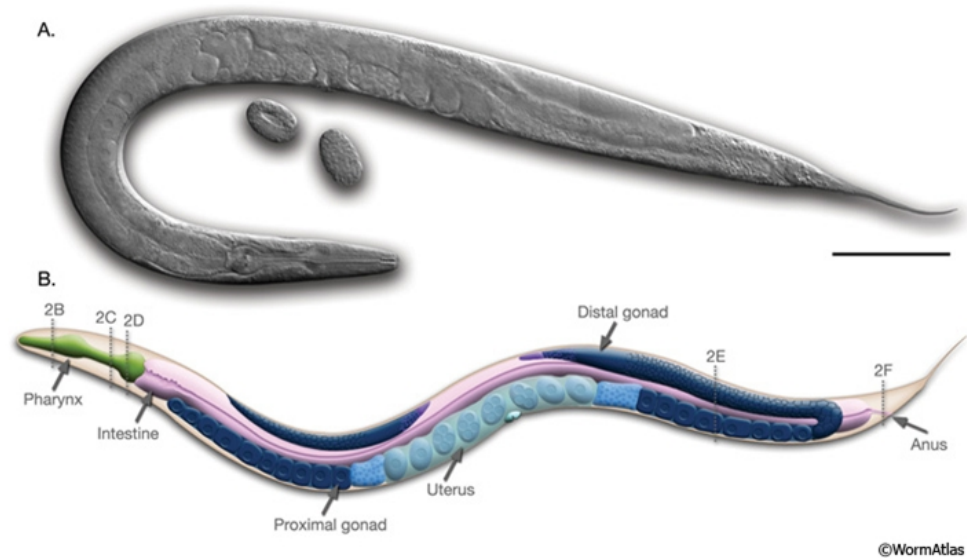
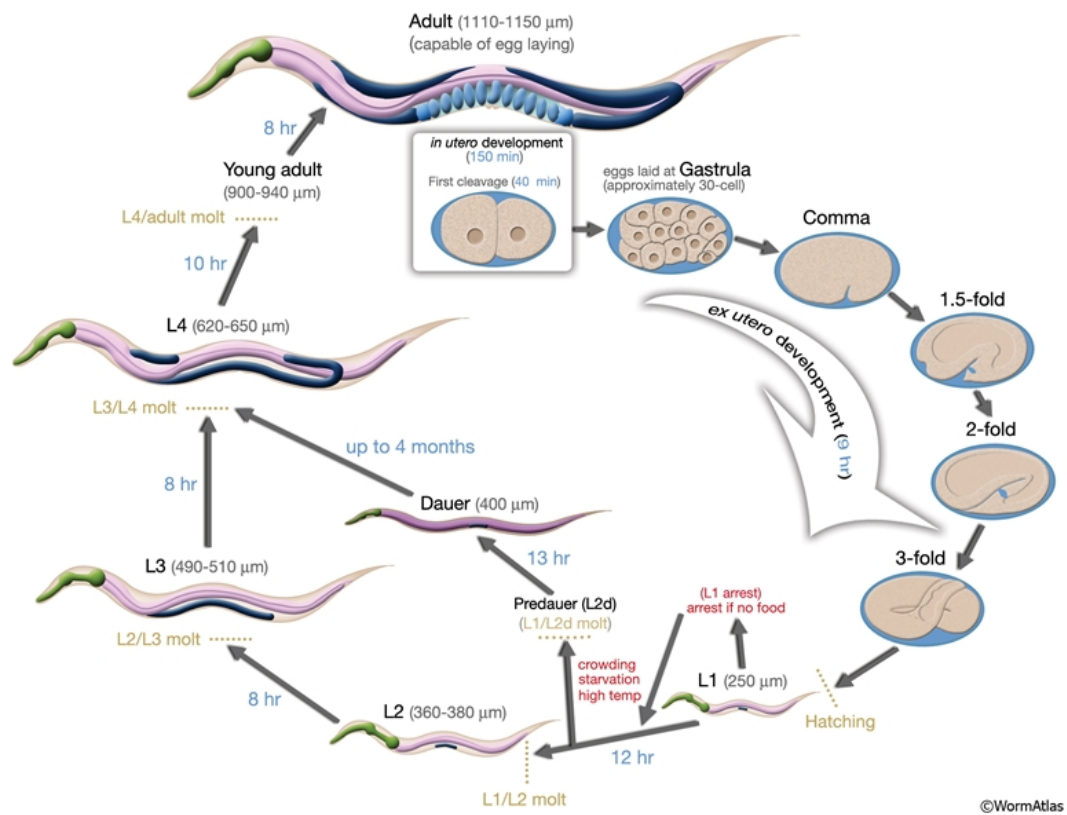


Figure 2.1: (Top) *Differential interference contrast (DIC) micrograph of an adult C. elegans hermaphrodite (Scale Bar: 0.1 mm).* (Bottom) *Schematic drawing of anatomical structures of an adult hermaphrodite (left lateral side).*

Figure 2.2: *Life cycle of C. elegans at 22°C*

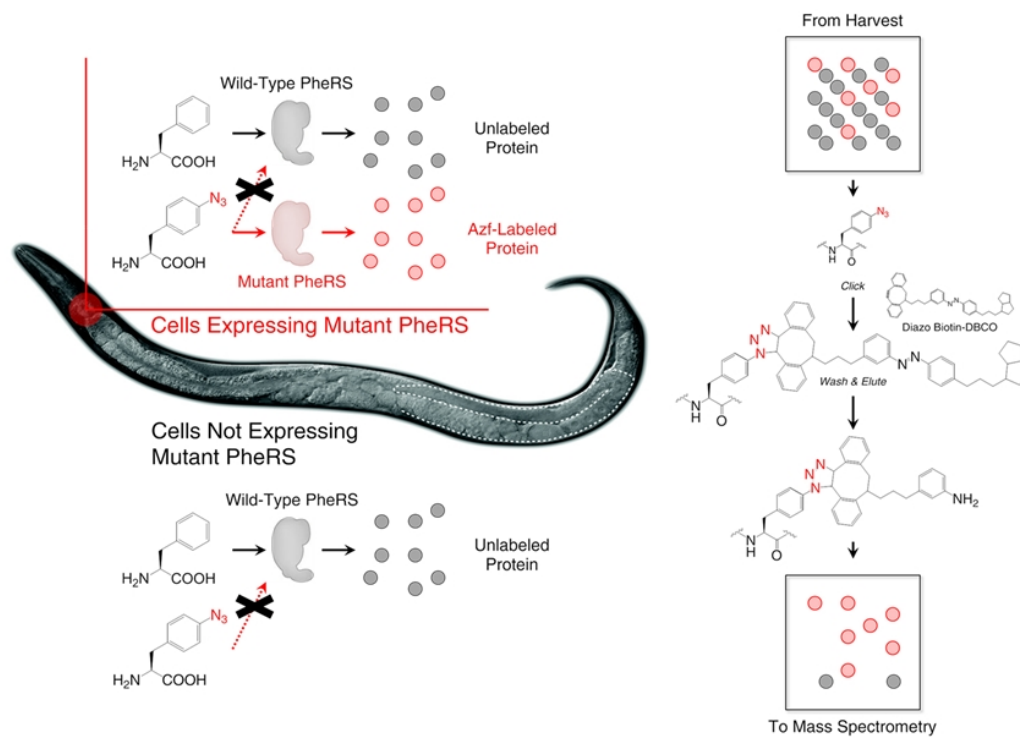


Figure 2.3: A mutant *C. elegans* PheRS is capable of tagging proteins with the reactive non-canonical amino acid Azf. Spatiotemporal selectivity is achieved by controlling expression of the mutant synthetase using cell-selective promoters in transgenic lines. Tagged proteins are distinguished from other proteins through conjugation of the azide side chain to probes that permit isolation of the labeled proteins. Worm lysates are reacted with sodium dithionite-cleavable Diazo Biotin-DBCO, and Azf-labeled proteins are isolated by streptavidin affinity chromatography. The structure of the biotin reagent has been simplified; the full structure is shown in **Figure 2.4**

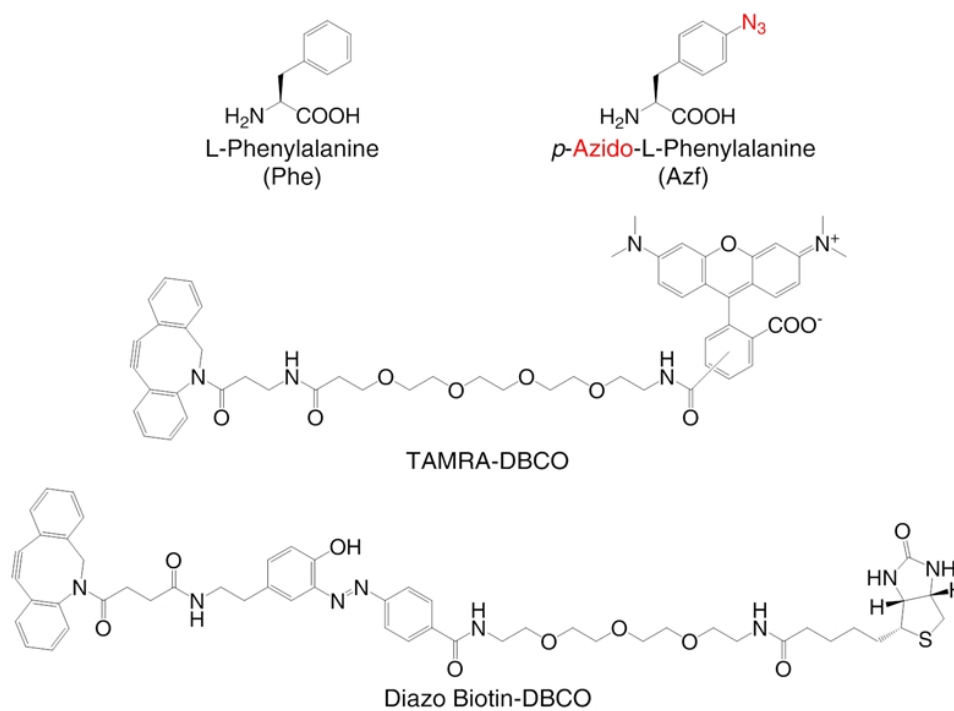


Figure 2.4: Structures of amino acids and probes used in Chapter 2: L-phenylalanine (Phe), p-azido-L-phenylalanine (Azf), dibenzocyclooctyne tetramethylrhodamine (TAMRA-DBCO), and sodium dithionite-cleavable dibenzocyclooctyne biotin (Diazo Biotin-DBCO).

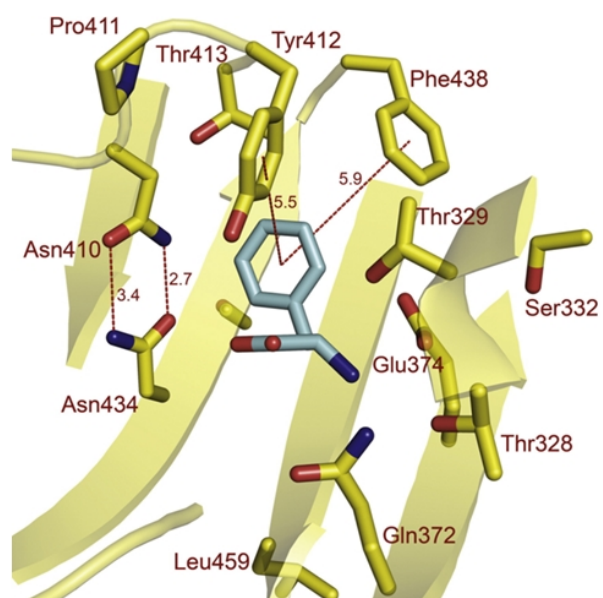


Figure 2.5: *Active site of H. sapiens PheRS with bound Phe (PDB ID code 3L4G).*

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Sc      HPLMKVREEFRQIFFSMGFTEMPNRYVETGFWNFDALYVPPQHPARDLQDTFFYIKDPLT
Ce      HPLMKVRSEFRQIFFSMGFSEMATNRYVSSFWNF DALFQPQHPARDAHDTFFVSDPAI
Dm      HPLLKVRTEFRQIFLEMGFSEMPNRYVSSFWNF DALYQPQHPARDAHDTFFVNHPAK
Dr      HPLMKVRTQFRQIFLEMGFTEMPNRFIESSFWNFDSLFPQHPARDQHDFFFISDPAL
Mm      HPLLKVRSQFRQIFLEMGFTEMPDNFIESSFWNF DALFQPQHPARDQHDFFF LRDPAE
Hs      HPLLKVRSQFRQIFLEMGFTEMPDNFIESSFWNF DALFQPQHPARDQHDFFF LRDPAE

Sc      ADLPDDKTYMDNIKAVHEQGRFGS IGYRYNWKPEECQKLVLRTHSTAI SARMLHDLAKD-
Ce      ST-KFPEDYLERVKTVHSKGGYSGAGYNDWKIEEAQKNVLRTHTTAVSARQLYQLAQ--
Dm      SH-KFPQDYLERVKKVHSVGGYSGKYGYDWKLEEAQKNLLRTHTTAVSARMLYKLANQE
Dr      AH-EFPRDYLERVKKVHSEGGYSGQGYKYDWKIEEAQKNLLRTHTTAVSARMLYKLAQQ-
Mm      AL-QLPMGYVQRVKRTHSQGGYSGQGYKYTWKLEEARKNLLRTHTTASARALYQLAQK-
Hs      AL-QLPMDYVQRVKRTHSQGGYSGQGYKYNWKLDEARKNLLRTHTTASARALYRLAQK-

Sc      --PKPTRLFSIDRVFRNEAVDATHLAEFHQVEGLADYINITLGDLIKFMEEFFERMVGTG
Ce      EGFRRPSKLFSDRVFRNETLDATHLAEFHQVEGVIAEKNLSLAHLIGIFTEFFKLGITN
Dm      GGFKAAKYFSDIKVFRNETLDATHLAEFHQVEGVIAADVGLTLDGLIGTLYEFFKLGITQ
Dr      EKFTPVKYFSDRVFRNETLDATHLAEFHQIEGVVADYGLTLGNLMGVLRHQFFTKLGITK
Mm      KPFTPAKYFSDRVFRNETLDATHLAEFHQIEGVIA DHGLTLGHLMGVLRHEFFTKLGITQ
Hs      KPFTPVKYFSDRVFRNETLDATHLAEFHQIEGVVADHGLTLGHLMGVLRHEFFTKLGITQ

Sc      LRFKPTYNPTYEPSMEIFSWHEGLQKWEI GNSGMFRPEMLES MGLPKDLRVLGWGLSLE
Ce      LRFKPTYNPTYEPSMEIFAYHQGLTKWVEI GNSGMFRPEMLLPMGLPADVNVAGYGLSLE
Dm      LEFKPAYNPTYEPSMEIFCYHPLAKWIEVGN S G VFRPEMLLPMGLPENVNVIAWGLSLE
Dr      LRFKPAYNPTYEPSMEVFSYHEGLKWEVGN S G VFRPEMLLPMGLPEGVSVIAWGLSLE
Mm      LRFKPAYNPTYEPSMEVFSYHQGLKWEVGN S G VFRPEMLLPMGLPENVSVIAWGLSLE
Hs      LRFKPAYNPTYEPSMEVFSYHQGLKWEVGN S G VFRPEMLLPMGLPENVSVIAWGLSLE

Sc      RPTMIKYQVNI REL LGHKVSLDFIETNPAARLDEDLYE-----
Ce      RPTMIKYGINNIRDLFGSKIDLNVVYNNPICRLDK-----
Dm      RPTMIKYGINNIRDLVGPVLDKQVVEEGPICRLDHA-----
Dr      RPTMIKYGINNIRELVGHKVNLMQVYDSPICRDLS-----
Mm      RPTMIKYGINNIRELVGHKVNLMQVYDSVPCRLDIEPRSSKTQEA
Hs      RPTMIKYGINNIRELVGHKVNLMQVYDSPICRLDAEPRPPPTQEA

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Ce = *C. elegans*, *Sc* = *S. cerevisiae*, *Dm* = *D. melanogaster*,
Dr = *D. rerio*, *Mm* = *M. musculus*, *Hs* = *H. sapiens*

Figure 2.6: Alignment of *S. cerevisiae* (*Sc*, *His228-Glu503*), *C. elegans* (*Ce*, *His225-Lys496*), *D. melanogaster* (*Dm*, *His224-Ala498*), *D. rerio* (*Dr*, *His225-Ser497*), *M. musculus* (*Mm*, *His225-Ala508*), and *H. sapiens* (*Hs*, *His225-Ala508*) *PheRS* catalytic cores. A conserved “gatekeeper” threonine (highlighted in yellow) in the alpha subunit has been proposed to play a key role in determining substrate specificity in *PheRS*s.

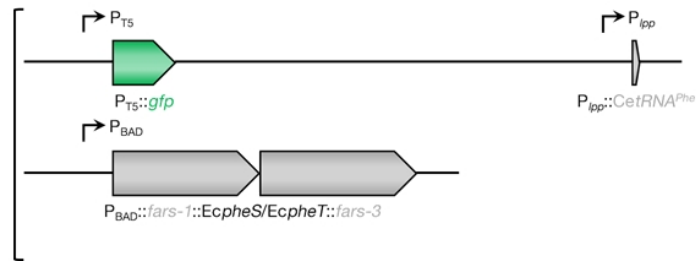


Figure 2.7: To screen for a mutant *CePheRS* that activates *Azf*, we used *KY14*[*pKPY93/pKPY1XX*], a phenylalanine-auxotrophic strain of *E. coli*. This strain houses two compatible plasmids: (i) *pKPY93* encodes *CetRNA*^{Phe} under constitutive *E. coli* murein lipoprotein (*lpp*) promoter control and IPTG-inducible (P_{T5}) 6xHis-tagged GFP and (ii) *pKPY1XX* encodes both *CePheRS* alpha and beta subunits under arabinose-inducible (P_{BAD}) control.

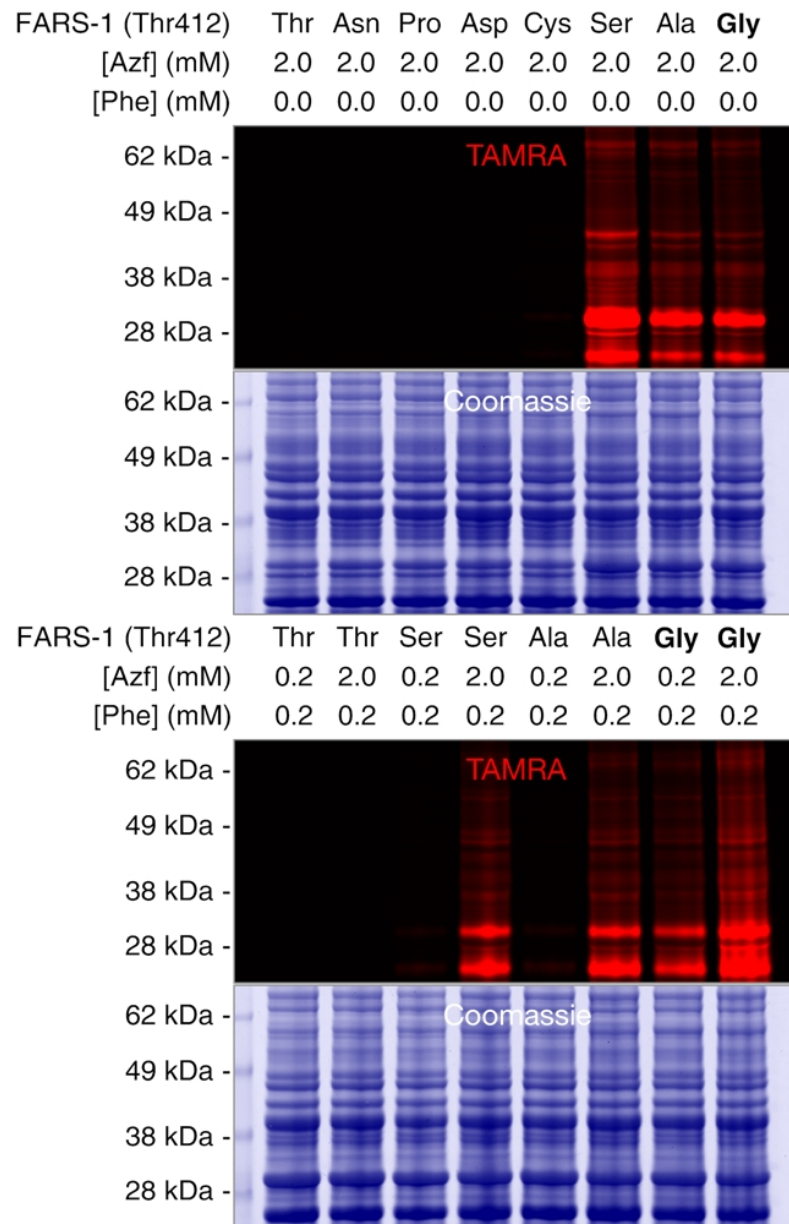


Figure 2.8: *SDS/PAGE and in-gel fluorescence scanning detection of conjugation of TAMRA-DBCO to Azf-labeled proteins derived from KY14[pKPY93/pKPY1XX] lysates.*

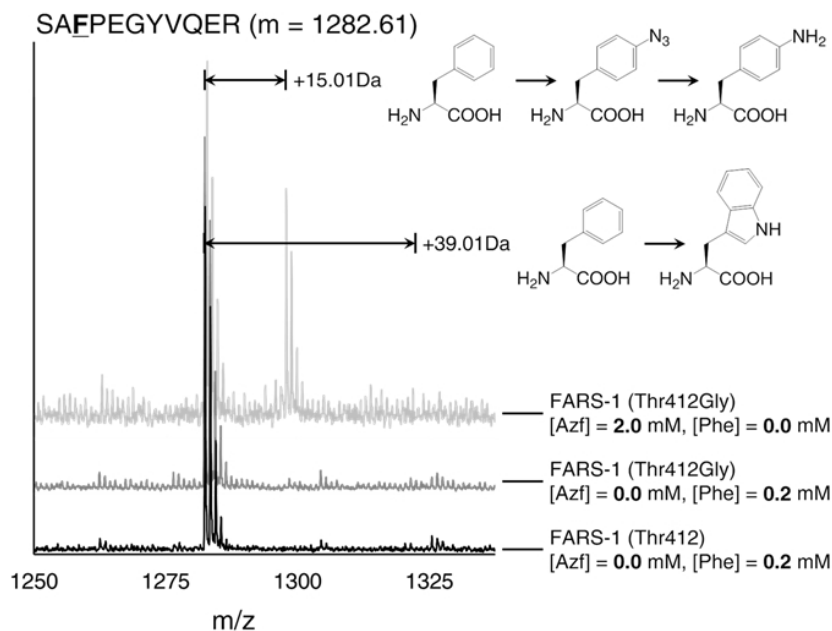
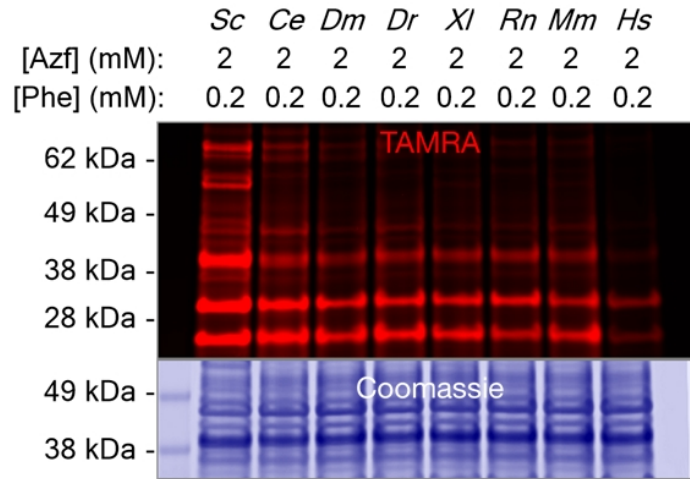


Figure 2.9: MALDI-TOF mass spectra of the tryptic GFP peptide SAFPEGYVQER. 6xHis-tagged GFP was purified from either KY14[pKPY93/pKPY100] or KY14[pKPY93/pKPY102]. GFP was expressed in cells bearing i) Thr412Gly-CePheRS and treated with 2.0 mM Azf and 0.0 mM Phe, ii) Thr412Gly-CePheRS, treated with 0.0 mM Azf and 0.2 mM Phe or iii) wild-type CePheRS with 0.0 mM Azf and 0.2 mM Phe. Azf was reduced to p-amino-L-phenylalanine during the experiment. Therefore, incorporation of Azf in this peptide results in a +15.01 Da m/z shift. Misincorporation of L-tryptophan in this peptide results in a +39.01 Da m/z shift.



Sc = *Saccharomyces cerevisiae*; *Ce* = *Caenorhabditis elegans*;
Dm = *Drosophila melanogaster*; *Dr* = *Danio rerio*; *Xl* = *Xenopus laevis*;
Rn = *Rattus norvegicus*; *Mm* = *Mus musculus*; *Hs* = *Homo sapiens*

Figure 2.10: SDS-PAGE and in-gel fluorescence scanning detection of conjugation of TAMRA-DBCO to Azf-labeled protein from KY14 containing *pKPY93* and *pKPY509*, *pKPY102*, *pKPY503*, *pKPY505*, *pKPY647*, *pKPY655*, *pKPY507*, or *pKPY137* lysates. These strains house two compatible plasmids: i) *pKPY93* encodes *CetRNA^{Phe}* under constitutive *E. coli* murein lipoprotein (*lpp*) promoter control as well as IPTG-inducible (P_{T5}) 6xHis-tagged GFP and ii) *pKPY509*, *pKPY102*, *pKPY503*, *pKPY505*, *pKPY647*, *pKPY655*, *pKPY507*, and *pKPY137* are plasmids that encode *Saccharomyces cerevisiae* (*Sc*), *Caenorhabditis elegans* (*Ce*), *Drosophila melanogaster* (*Dm*), *Danio rerio* (*Dr*), *Xenopus laevis* (*Xl*), *Rattus norvegicus* (*Rn*), *Mus musculus* (*Mm*), and *Homo sapiens* (*Hs*), respectively, *PheRS* alpha and beta subunits under arabinose-inducible (P_{BAD}) control.

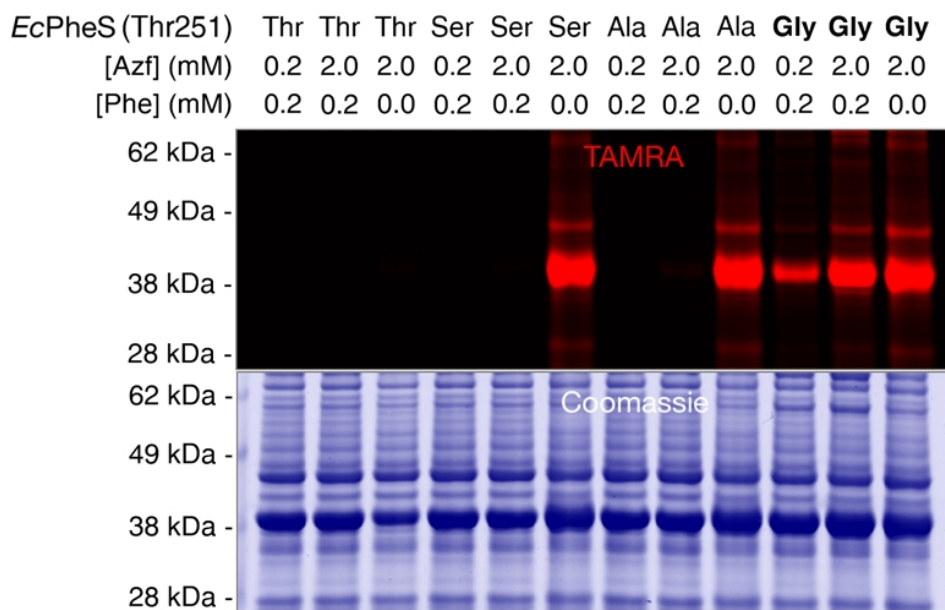


Figure 2.11: *SDS-PAGE and in-gel fluorescence scanning detection of conjugation of TAMRA-DBCO to Azf-labeled proteins from KY14 containing pKPY513, pKPY566, pKPY565, or pKPY514 lysates. pKPY513, pKPY566, pKPY565, and pKPY514 encode the IPTG-inducible (PT5) 6xHis-tagged E. coli variants of wild-type, Thr₄₁₂Ser, Thr₄₁₂Ala, or Thr₄₁₂Gly-CePheRS, respectively.*

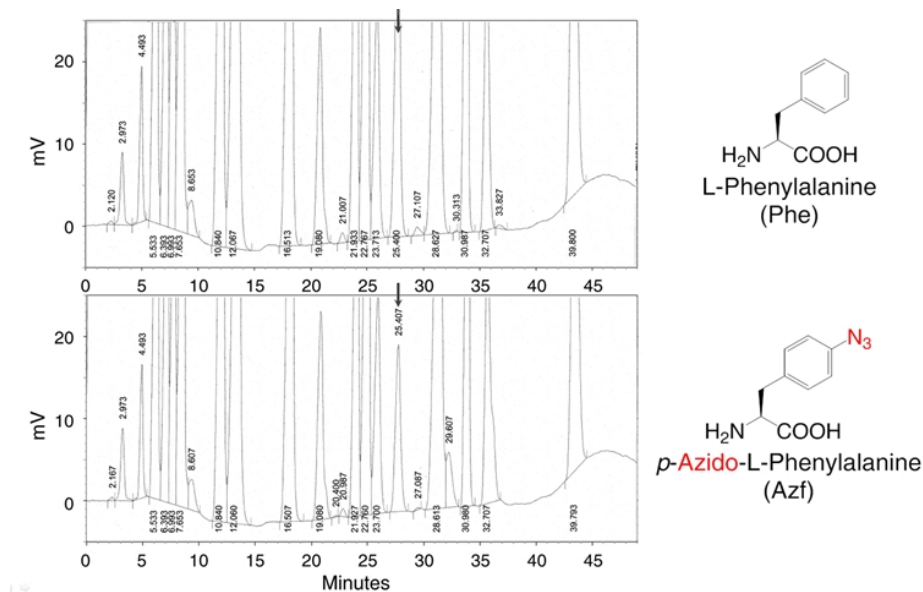


Figure 2.12: Amino acid analysis of the extent of replacement of Phe by Azf in total *E. coli* protein of KY14[pKPY514]. Cells bearing Thr251Gly-EcPheRS were treated with i) 0.0 mM Azf and 0.2 mM Phe or ii) 2.0 mM Azf and 0.0 mM Phe. The extent of replacement of Phe by Azf was calculated from the change in the ratio of Phe to other amino acids in cells treated with only Phe versus the ratio of Phe to other amino acids in cells treated with only Azf. Phe elutes around 25.4 minutes as indicated by the black arrow.

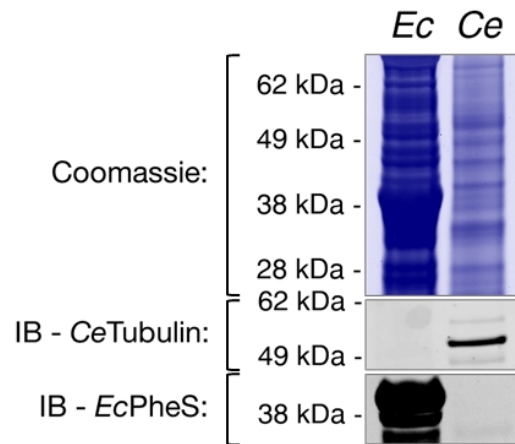


Figure 2.13: We fed *Azf*-labeled bacteria (left lane) to *hsp-16.2::Thr412Gly-CePheRS* worms previously grown on *OP50* and cleared digested bacteria by washing worms with *S* medium. We could not detect *E. coli* protein (even the overexpressed *Thr251Gly-EcPheRS*) in processed *C. elegans* lysate (right lane). Tubulin is a marker for *C. elegans* proteins.

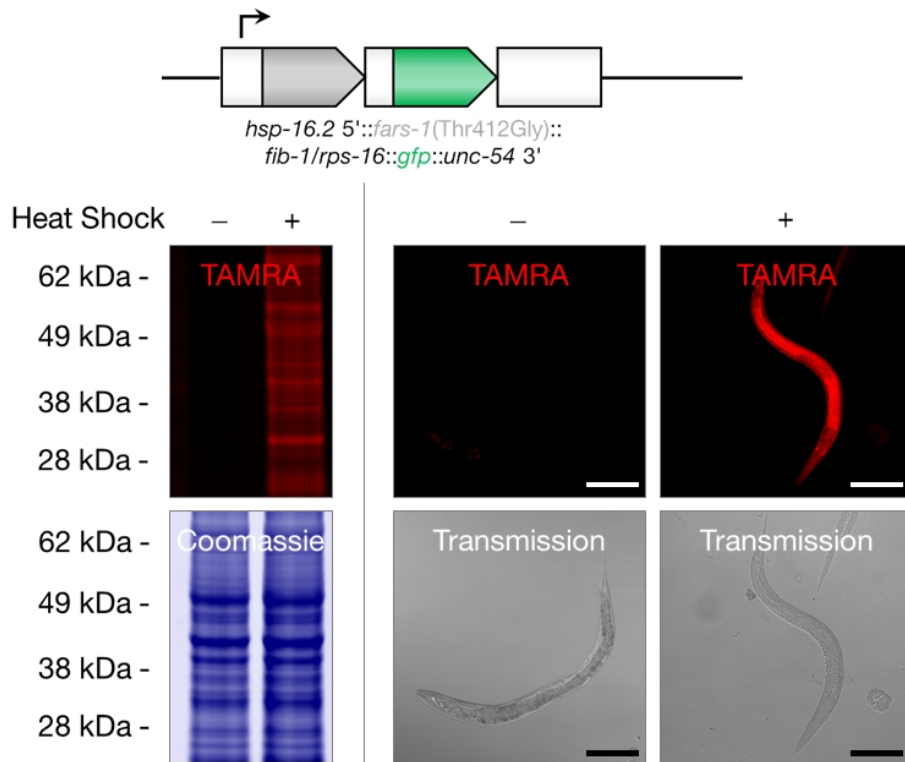


Figure 2.14: We fed *KY14*[*pKPY514*] to *hsp-16.2::Thr412Gly-CePheRS* animals. This *E. coli* strain harbors *pKPY514*, a plasmid encoding *Thr251Gly-EcPheRS* under IPTG-inducible (P_{T5}) control. *hsp-16.2::Thr412Gly-CePheRS C. elegans* express both the mutant alpha subunit and GFP under control of the *hsp-16.2* promoter. In-gel fluorescence scanning of TAMRA-DBCO-treated lysates (Left) and fluorescence microscopy of TAMRA-DBCO-treated fixed animals (Right) showed that only heat-shocked worms exhibited ubiquitous Azf labeling 24 h after heat shock. (Scale Bars: 100 μm .)

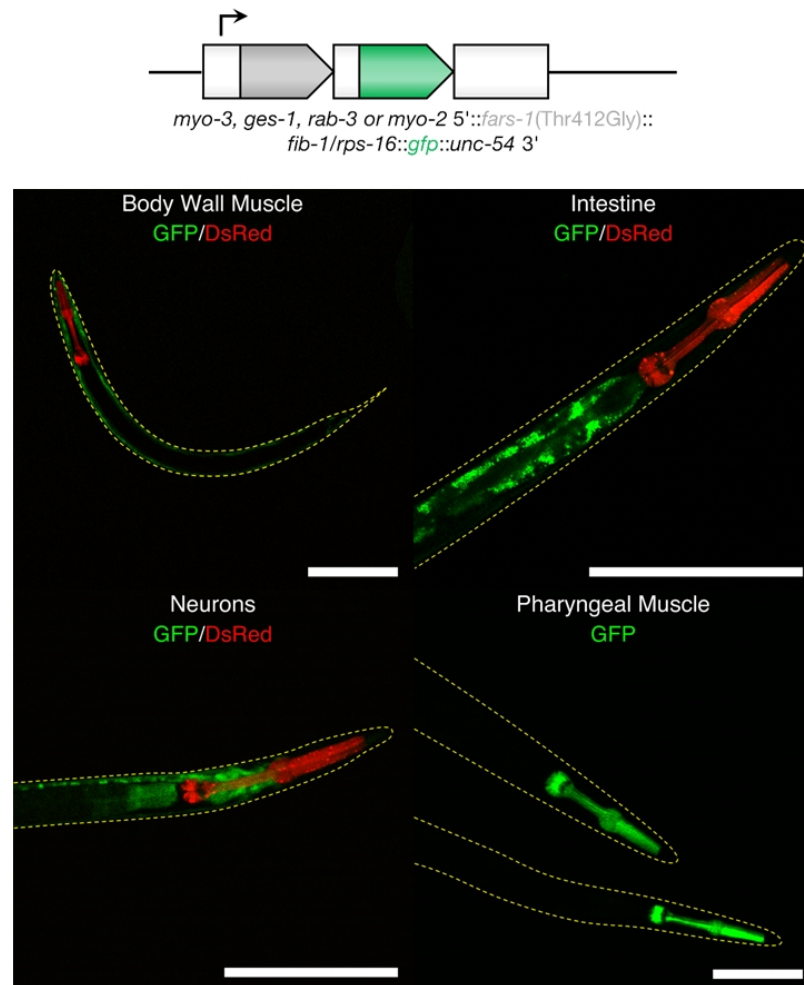


Figure 2.15: We fed *E. coli* strain KY14[pKPY514] to *myo-3::* (Top Left), *ges-1::* (Top Right), *rab-3::* (Bottom Left), and *myo-2::Thr412Gly-CePheRS* (Bottom Right) animals. These transgenic *C. elegans* strains express both the mutant PheRS alpha subunit and GFP (green) under control of the *myo-3*, *ges-1*, *rab-3*, or *myo-2* promoters, respectively. *myo-3*, *ges-1*, and *rab-3* worms also express the co-injection marker *myo-2::dsRed* which marks the pharyngeal muscle cells (red). (Scale Bar: 100 μm.)

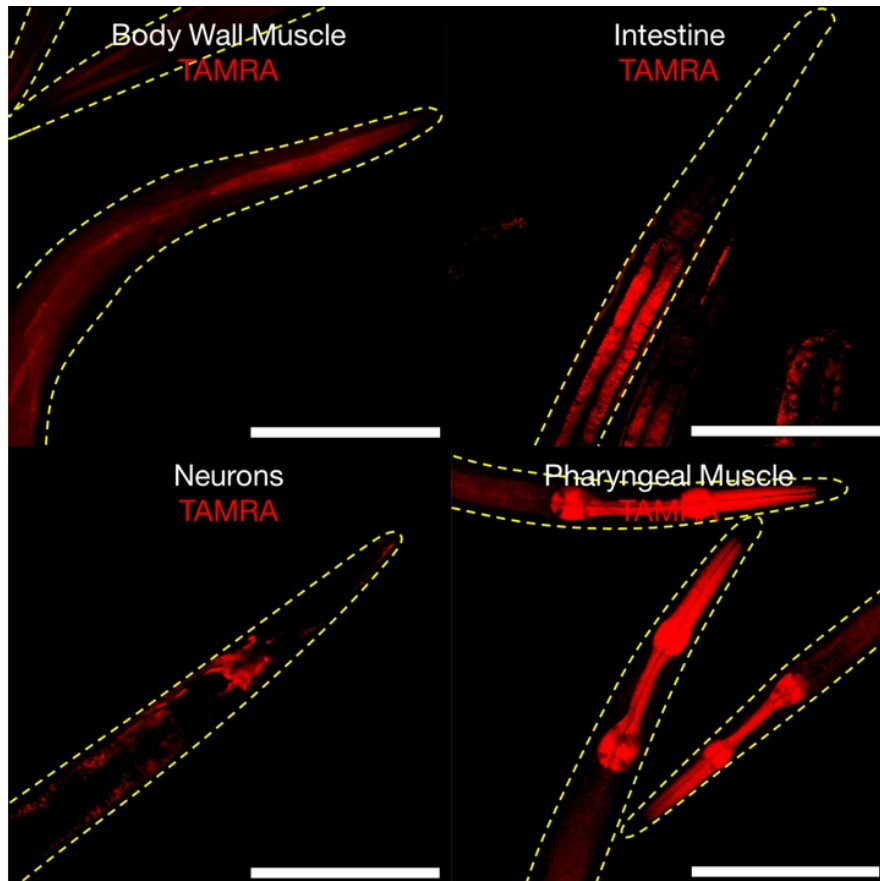


Figure 2.16: *Fluorescence microscopy revealed that labeling in myo-3 (Left), ges-1 (Center Left), rab-3 (Center Right), and myo-2::Thr412Gly-CePheRS (Right) worms was localized to body wall muscle, intestine, neurons, and pharyngeal muscle, respectively; the surrounding tissues were not labeled. (Scale Bar: 100 μm.)*

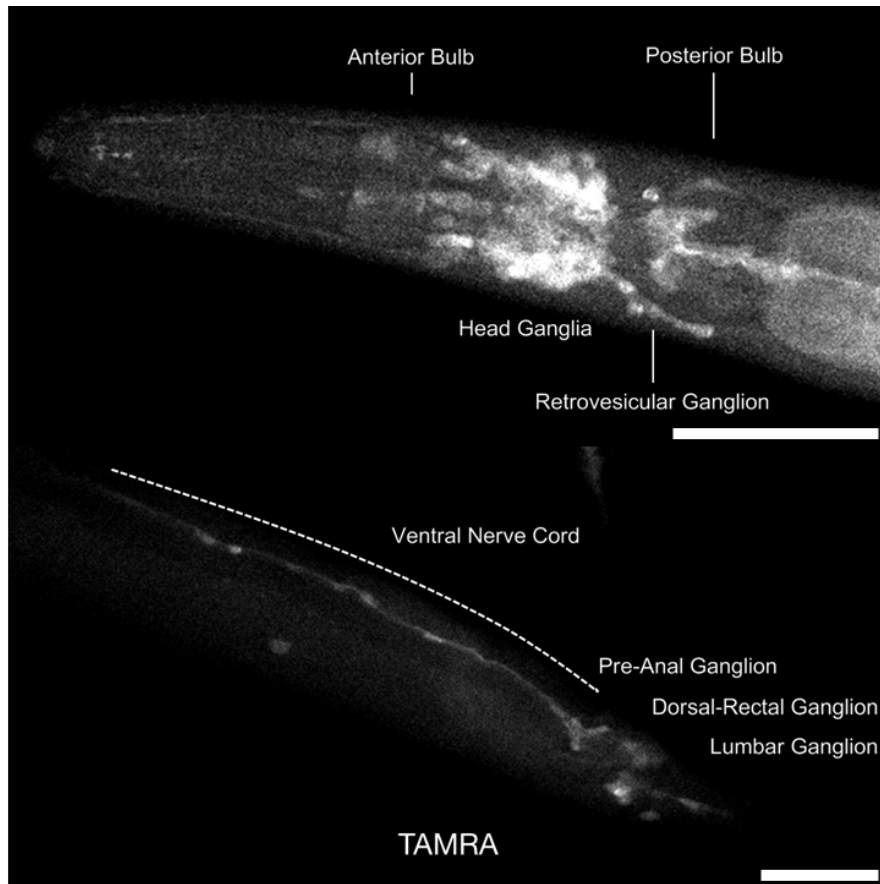


Figure 2.17: *Fluorescence microscopy revealed that worms with rab-3-driven expression of Thr412Gly-CePheRS exhibit selective Azf-labeling in neuronal cell-types. (Scale Bar: 50 μ m.)*

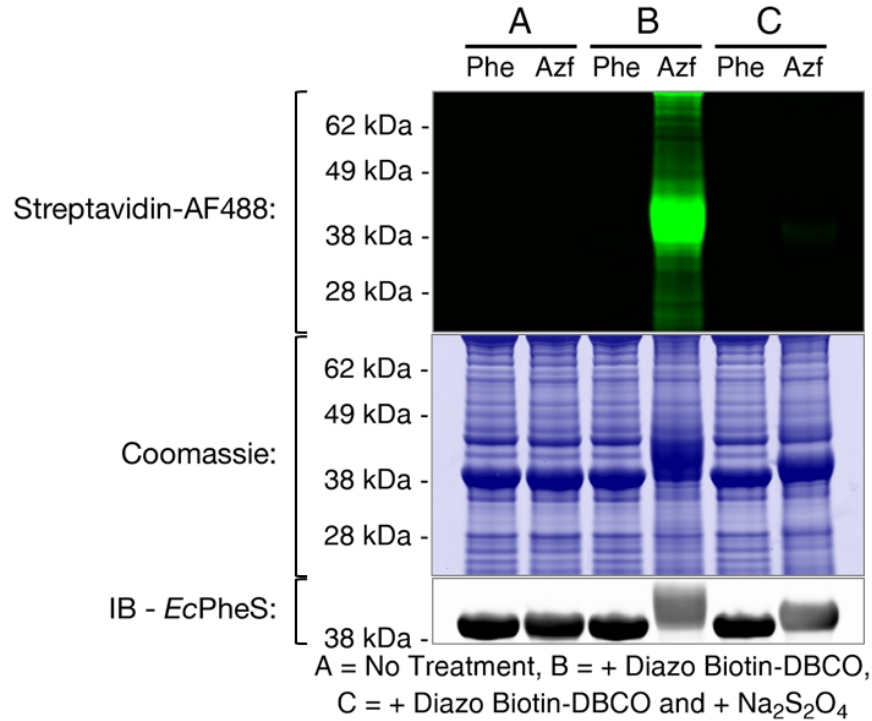


Figure 2.18: *Model labeling of KY14[pKPY514] lysates with Diazo Biotin-DBCO. First, cells bearing Thr251Gly-EcPheRS were treated with i) 0.0 mM Azf and 0.2 mM Phe (lanes marked with "Phe") or ii) 2.0 mM Azf and 0.0 mM Phe (lanes marked with "Azf"). Next, proteins were treated with water ("A" lanes) or Diazo Biotin-DBCO ("B" lanes). Diazo Biotin-DBCO-modified proteins were treated with sodium dithionite ("C" lanes). SDS-PAGE, Western blotting, and in-gel fluorescence scanning detection of Streptavidin-Alexa Fluor 488 (Streptavidin-AF488) revealed that only Azf-labeled proteins are biotinylated after treatment with Diazo Biotin-DBCO and that biotin is removed by treatment with sodium dithionite.*

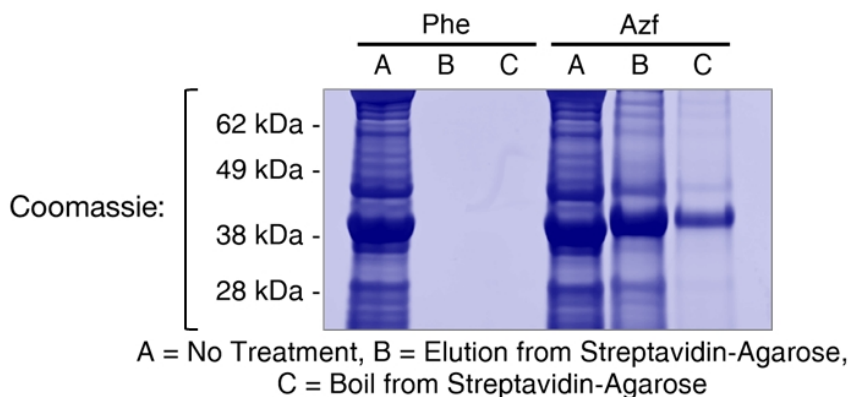


Figure 2.19: *Model enrichment of KY14[pKPY514] lysates with Diazo Biotin-DBCO. First, cells bearing Thr251Gly-EcPheRS were treated with i) 0.0 mM Azf and 0.2 mM Phe (lanes marked “Phe”) or ii) 2.0 mM Azf and 0.0 mM Phe (lanes marked “Azf”). Next, proteins were treated with Diazo Biotin-DBCO and incubated with Streptavidin agarose resin. Diazo Biotin-DBCO-modified proteins were eluted by treatment with sodium dithionite. Proteins that remained bound to resin were released after boiling in the presence of SDS. SDS-PAGE revealed that only Azf-labeled proteins are eluted after treatment with sodium dithionite. Unmodified lysates are in “A” lanes, proteins eluted after treatment with sodium dithionite are in “B” lanes, and proteins eluted after boiling are in “C” lanes.*

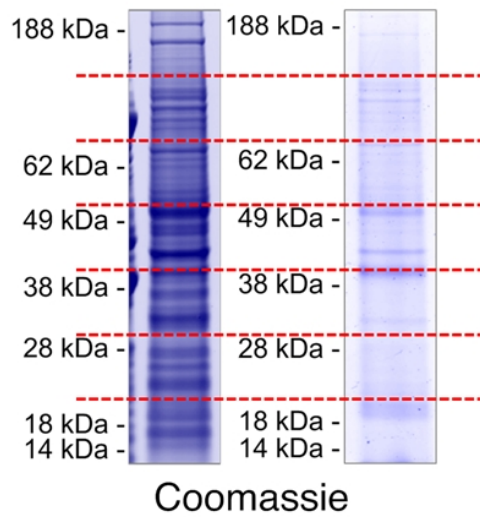


Figure 2.20: *Unenriched (Left) and enriched (Right) samples prepared from labeled myo-2::Thr₄₁₂Gly-CePheRS worms were subjected to in-gel proteolytic digestion and LC-MS/MS. After SDS-PAGE, gels were sliced as indicated by the dotted red lines.*

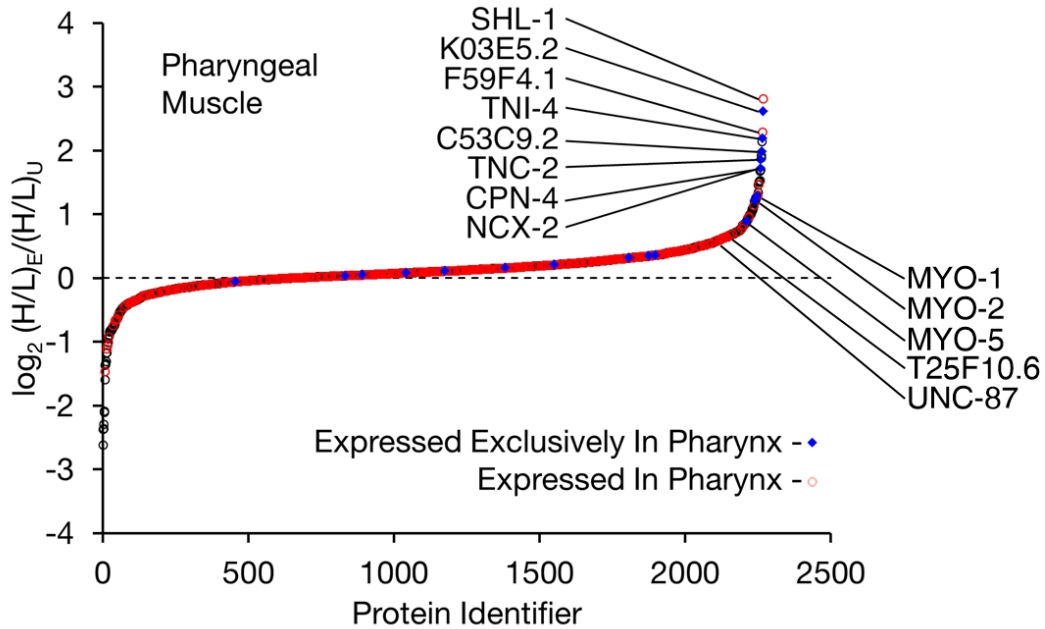


Figure 2.21: *LC-MS/MS analysis of myo-2::Thr412Gly-CePheRS animals fed with triply labeled E. coli (heavy arginine, heavy lysine, and Azf) for 24 h. Solid blue diamond markers represent proteins that are expressed exclusively in the pharynx. Open red circle markers represent proteins that are expressed in the pharynx. Open black circle markers represent proteins that have either unknown expression patterns or expression patterns not associated with the pharynx. The proteins discussed in this work are marked.*

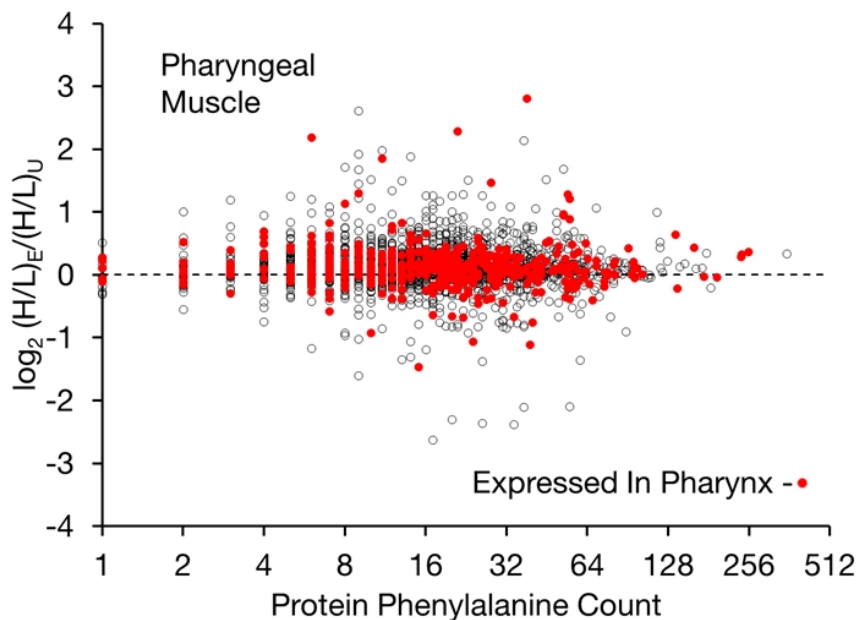


Figure 2.22: *LC-MS/MS analysis of myo-2::Thr412Gly-CePheRS animals fed with triply labeled E. coli (“heavy” arginine, “heavy” lysine, and Azf) for 24 hours: $(H/L)_E/(H/L)_U$ values versus phenylalanine counts. Solid red markers represent proteins that are expressed in the pharynx. Open black markers represent proteins that have either unknown expression patterns or expression patterns not associated with the pharynx. The number of phenylalanine residues in a protein does not influence the extent of enrichment.*

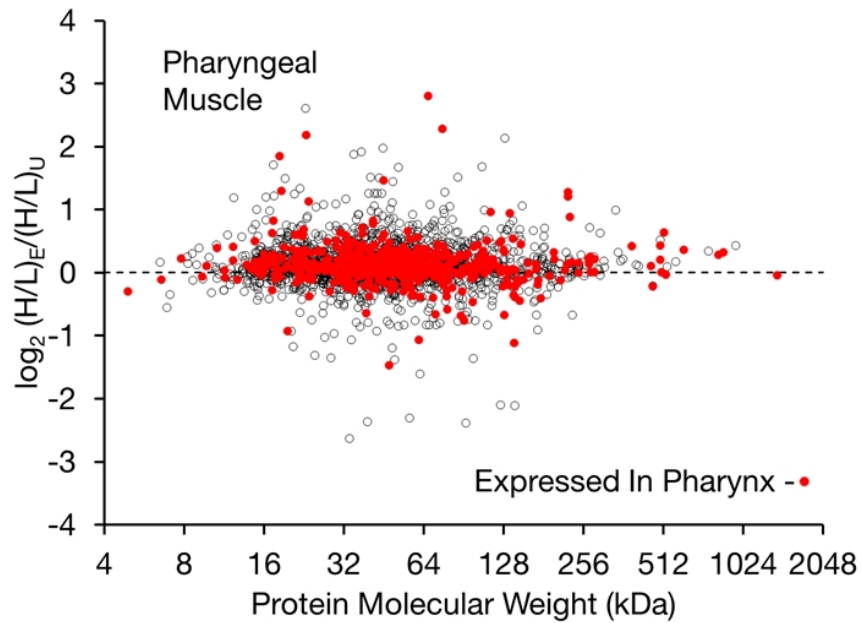


Figure 2.23: *LC-MS/MS analysis of myo-2::Thr412Gly-CePheRS animals fed with triply labeled E. coli ("heavy" arginine, "heavy" lysine, and Azf) for 24 hours: $(H/L)_E / (H/L)_U$ values versus molecular weights. Solid red markers represent proteins that are expressed in the pharynx. Open black markers represent proteins that have either unknown expression patterns or expression patterns not associated with the pharynx. The protein size does not influence the extent of enrichment.*

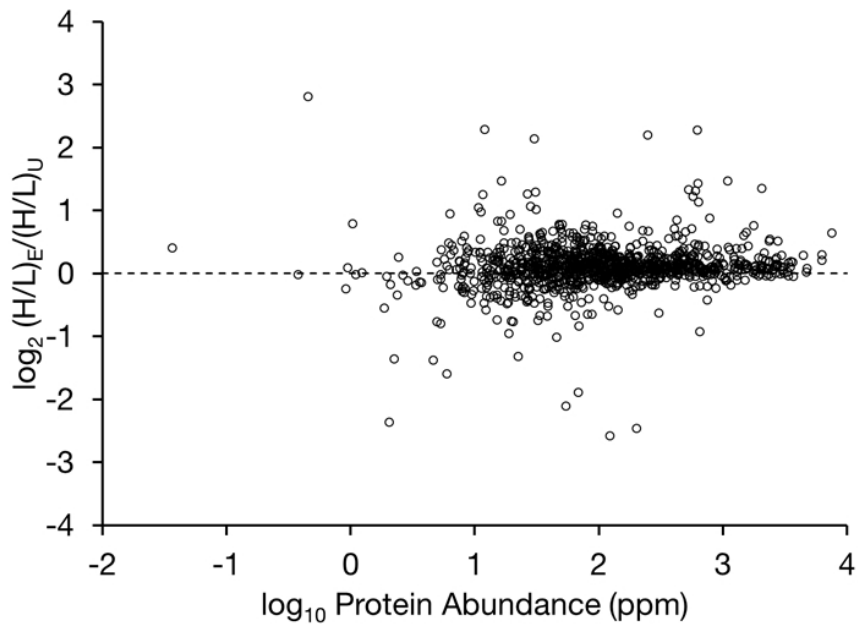


Figure 2.24: *LC-MS/MS analysis of myo-2::Thr412Gly-CePheRS animals fed with triply labeled E. coli (“heavy” arginine, “heavy” lysine, and Azf) for 24 hours: $(H/L)_E / (H/L)_U$ values versus protein abundance values obtained from the PaxDb database (Wang, M., et al., Mol. Cell. Proteomics **11**, 492–500 (2012)). Protein abundance does not influence the extent of enrichment.*

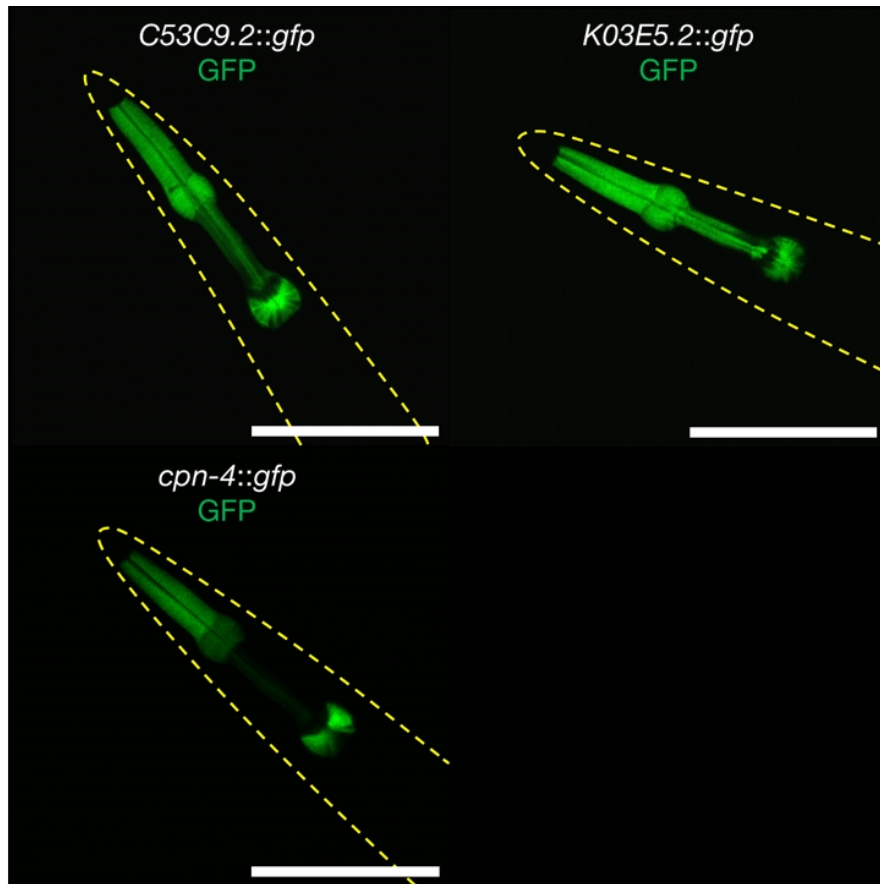


Figure 2.25: *Fluorescence microscopy confirmed GFP fluorescence was exclusively localized to pharyngeal muscle cells in C53C9.2::gfp (Top Left), K03E5.2::gfp (Top Right), and cpn-4::gfp (Bottom Left) animals. (Scale bars: 100 μ m.)*

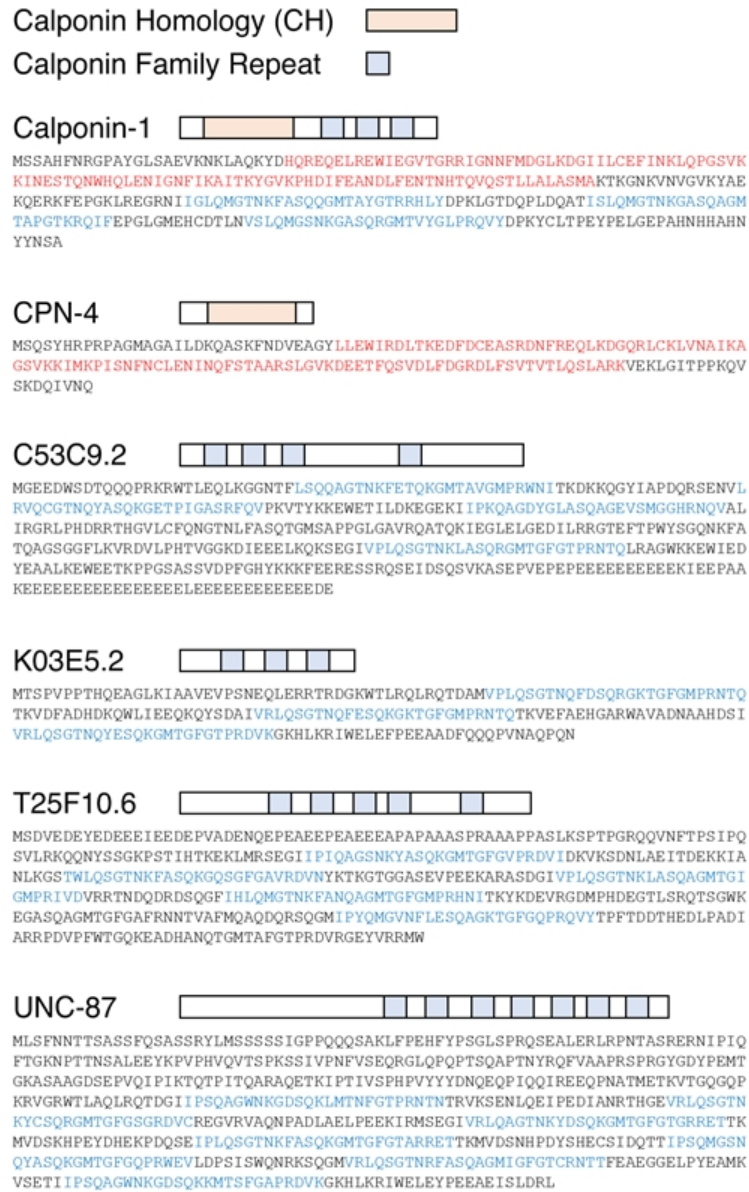


Figure 2.26: Schematic diagram of human Calponin-1 (Accession Number P51911), *C. elegans* proteins CPN-4, C53C9.2, K03E5.2 (Isoform A), T25F10.6 (Isoform A), and UNC-87 (Isoform A). Proteins and their domains are drawn in proportion to their length.

2.6 Tables

Substrate	K_m (10^{-6} M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
<i>C. elegans</i> Methionyl-tRNA Synthetase			
Met	27.9 ± 3.3	4.0 ± 0.5	140000 ± 24000
Mutant <i>C. elegans</i> Methionyl-tRNA Synthetases ¹			
Met	N.D.	N.D.	N.D.
Anl	N.D.	N.D.	N.D.

Table 2.1: *Kinetic parameters (determined by in vitro ATP-PP_i exchange) for activation of amino acids by wild-type and mutant C. elegans methionyl-tRNA synthetases (CeMetRS) (Met: L-Methionine, Anl: L-Azidonorleucine; N.D.: Not Detected). ¹Mutant CeMetRS: Leu42Gly; Leu42Cys, Tyr297Leu, His333Leu; Leu42Asn, Tyr297Leu, His333Leu; Leu42Pro, Tyr297Leu, His333Leu; Leu42Ser, Tyr297Leu, His333Leu.*

Substrate	K_m (10^{-6} M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	Selectivity ¹	Relative Activity ²
<i>C. elegans</i> Phenylalanyl-tRNA Synthetase					
Phe	0.972 ± 0.116	0.301 ± 0.027	312000 ± 46500	-	1
<i>C. elegans</i> Phenylalanyl-tRNA Synthetase (Thr412Gly)					
Phe	39.9 ± 4.1	0.327 ± 0.034	8280 ± 1220	1	1/37.7
Azf	1.95 ± 0.34	0.327 ± 0.026	172000 ± 33000	20.8	1/1.8
Trp	20.6 ± 1.6	0.507 ± 0.039	24600 ± 2690	3.0	1/12.7
Tyr	N.D.	N.D.	N.D.	-	-

Table 2.2: *Kinetic parameters (determined by in vitro ATP-PP_i exchange) for activation of amino acids by wild-type CePheRS and Thr412Gly-CePheRS (Phe: L-Phenylalanine, Azf: p-Azido-L-Phenylalanine, Trp: L-Tryptophan, Tyr: L-Tyrosine; N.D.: Not Detected). ¹Selectivity is defined as $(k_{cat}/K_m)_{xxx}$ of substrates of Thr412Gly-CePheRS divided by $(k_{cat}/K_m)_{Phe}$ of Thr412Gly-CePheRS. ²Relative Activity is defined as $(k_{cat}/K_m)_{xxx}$ of substrates of Thr412Gly-CePheRS divided by $(k_{cat}/K_m)_{Phe}$ of wild-type CePheRS.*

Protein	$\log_2 (H/L)_E/(H/L)_U$
Q95XD1 Q95XD1_CAEEL Protein SHL-1 OS=Caenorhabditis elegans GN=shl-1 PE=4 SV=2	2.81
O61848 O61848_CAEEL Protein K03E5.2, isoform a OS=Caenorhabditis elegans GN=CELE_K03E5.2 PE=2 SV=2; H2KZW0 H2KZW0_CAEEL Protein K03E5.2, isoform b OS=Caenorhabditis elegans GN=CELE_K03E5.2 PE=4 SV=1; H2KZW1 H2KZW1_CAEEL Protein K03E5.2, isoform d	2.61
Q93839 Q93839_CAEEL Acyl-coenzyme A oxidase OS=Caenorhabditis elegans GN=CELE_F59F4.1 PE=3 SV=2	2.29
O44572 TNNI4_CAEEL Troponin I 4 OS=Caenorhabditis elegans GN=tni-4 PE=2 SV=2	2.19
Q18529 Q18529_CAEEL Protein C39D10.7 OS=Caenorhabditis elegans GN=C39D10.7 PE=1 SV=3	2.14
Q09936 YSE2_CAEEL Uncharacterized protein C53C9.2 OS=Caenorhabditis elegans GN=C53C9.2 PE=3 SV=2	1.98
O45577 O45577_CAEEL Protein CPF-2 OS=Caenorhabditis elegans GN=cpf- 2 PE=4 SV=1	1.93
P90879 P90879_CAEEL Protein F49C12.9 OS=Caenorhabditis elegans GN=CELE_F49C12.9 PE=1 SV=1	1.88
Q09665 TNNC2_CAEEL Troponin C, isoform 2 OS=Caenorhabditis elegans GN=tni-2 PE=2 SV=1	1.85
O44727 O44727_CAEEL Protein CPN-4 OS=Caenorhabditis elegans GN=cpn-4 PE=4 SV=3	1.72

Table 2.3: *Proteins identified and quantified from LC-MS/MS analysis of myo-2::Thr412Gly-CePheRS animals fed with triply labeled E. coli (“heavy” arginine, “heavy” lysine, and Azf) for 24 hours. The full table is available as a PDF in the online version of this thesis.*

Protein	$\log_2 (H/L)_E/(H/L)_U$
Q95XD1 Q95XD1_CAEEL Protein SHL-1 OS=Caenorhabditis elegans GN=shl-1 PE=4 SV=2	2.81
Q93839 Q93839_CAEEL Acyl-coenzyme A oxidase OS=Caenorhabditis elegans GN=CELE_F59F4.1 PE=3 SV=2	2.29
O44572 TNNI4_CAEEL Troponin I 4 OS=Caenorhabditis elegans GN=tmi-4 PE=2 SV=2	2.19
Q09665 TNNC2_CAEEL Troponin C, isoform 2 OS=Caenorhabditis elegans GN=tnc-2 PE=2 SV=1	1.85
H2KYP4 H2KYP4_CAEEL Protein NCX-2, isoform d OS=Caenorhabditis elegans GN=ncx-2 PE=4 SV=1; H2KYP2 H2KYP2_CAEEL Protein NCX-2, isoform a OS=Caenorhabditis elegans GN=ncx-2 PE=4 SV=1	1.69
Q9U3N4 INX6_CAEEL Innexin-6 OS=Caenorhabditis elegans GN=inx-6 PE=2 SV=1	1.47
P19626 MLR2_CAEEL Myosin regulatory light chain 2 OS=Caenorhabditis elegans GN=mlc-2 PE=1 SV=1; P19625 MLR1_CAEEL Myosin regulatory light chain 1 OS=Caenorhabditis elegans GN=mlc-1 PE=4 SV=1	1.30
P02567 MYO1_CAEEL Myosin-1 OS=Caenorhabditis elegans GN=let-75 PE=2 SV=3	1.28
P12845 MYO2_CAEEL Myosin-2 OS=Caenorhabditis elegans GN=myo-2 PE=2 SV=2	1.21
Q86GU1 Q86GU1_CAEEL Protein HSP-25, isoform b OS=Caenorhabditis elegans GN=hsp-25 PE=2 SV=1; Q17849 Q17849_CAEEL Protein HSP-25, isoform a OS=Caenorhabditis elegans GN=hsp-25 PE=2 SV=1; Q5H9M9 Q5H9M9_CAEEL Protein HSP-25, isoform c OS=Caenorhabdi	1.14

Table 2.4: *Proteins previously known to be expressed in the pharynx from LC-MS/MS analysis of myo-2::Thr412Gly-CePheRS animals fed with triply labeled E. coli (“heavy” arginine, “heavy” lysine, and Azf) for 24 hours. The full table is available as a PDF in the online version of this thesis.*

Body Wall Muscle	Intestine	Neurons
<i>pgp-8</i>	<i>T08B6.2</i>	<i>Y48G10A.6</i>
<i>C37H5.5</i>	<i>K10D11.6</i>	<i>C09B9.1</i>
<i>sur-5</i>	<i>flr-4</i>	<i>sur-5</i>
<i>elpc-3</i>	<i>fbxa-51</i>	<i>daf-11</i>
<i>sdz-3</i>	<i>sur-5</i>	<i>nlp-5</i>
<i>unc-93</i>	<i>inx-17</i>	<i>K07C11.10</i>
<i>T28D9.7</i>	<i>F01D5.3</i>	<i>ZC13.2</i>
<i>F46C5.6</i>	<i>clec-166</i>	<i>Y42A5A.1</i>
<i>ugt-39</i>	<i>Y22D7AL.15</i>	<i>nhr-167</i>
<i>Y48G1A.4</i>	<i>R05D8.9</i>	<i>gpa-2</i>
<i>sul-1</i>	<i>clec-66</i>	<i>pde-5</i>
<i>C24H12.4</i>	<i>ZC196.5</i>	<i>fkh-8</i>

Table 2.5: We examined a test set of 34 proteins whose genes are highly expressed in body wall muscle, intestinal epithelia, and neuronal cells (Spencer, W.C., et al., *Genome Res.* **21**, 325–341 (2011)).

2.7 Materials and Methods

2.7.1 *ATP-PP_i Exchange Assay*

Aminoacylating Solution: In double-distilled water, 50 mM HEPES (Sigma-Aldrich Corporation), pH 7.6, 20 mM magnesium chloride (VWR International), 1 mM DL-dithiothreitol (Sigma-Aldrich Corporation), 2 mM adenosine 5'-triphosphate disodium salt hydrate (Sigma-Aldrich Corporation), 2 mM sodium pyrophosphate (VWR International), 0.1 mg/mL bovine serum albumin (Sigma-Aldrich Corporation), 0.14% (v/v) 2-mercaptoethanol (Sigma-Aldrich Corporation), 3 $\mu\text{Ci}/200 \mu\text{L}$ [³²P]-sodium pyrophosphate (PerkinElmer)

Quenching Solution: In double-distilled water, 7% (v/v) perchloric acid (EMD Millipore), 3% (w/v) activated charcoal (Sigma-Aldrich Corporation), 200 mM sodium pyrophosphate (VWR International)

Washing Solution: In double-distilled water, 0.5% (v/v) perchloric acid (EMD Millipore), 10 mM sodium pyrophosphate (VWR International)

Wild-type *CePheRS* and Thr412Gly-*CePheRS* were expressed as described in the **Labeling in *E. coli*** procedure and purified according to the **Isolating 6xHis-Tagged Proteins** procedure. Protein concentrations of purified proteins were determined by absorbance at 280 nm. To assay activation of L-phenylalanine (Sigma-Aldrich Corporation) by wild-type *CePheRS*, wild-type *CePheRS* (500 nM) and varying concentrations of L-phenylalanine (0.1 to 50 μM) were mixed together in Aminoacylating Solution and incubated for 16 minutes at room temperature. To assay activation of L-phenylalanine by Thr412Gly-*CePheRS*, Thr412Gly-*CePheRS* (500 nM) and varying concentrations of L-phenylalanine (1 to 500 μM) were mixed together in Aminoacylating Solution and incubated for 16 minutes at room temperature. To assay activation of *p*-azido-L-phenylalanine (Chem-Impex International) by Thr412Gly-*CePheRS*, Thr412Gly-*CePheRS* (500 nM) and varying concentrations

of *p*-azido-L-phenylalanine (0.1 to 50 μM) were mixed together in Aminoacylating Solution and incubated for 16 minutes at room temperature. To assay activation of L-tryptophan (Sigma-Aldrich Corporation) by Thr412Gly-*Ce*PheRS, Thr412Gly-*Ce*PheRS (500 nM) and varying concentrations of L-tryptophan (1 to 500 μM) were mixed together in Aminoacylating Solution and incubated for 16 minutes at room temperature. To assay activation of L-tyrosine (Sigma-Aldrich Corporation) by Thr412Gly-*Ce*PheRS, Thr412Gly-*Ce*PheRS (500 nM) and varying concentrations of L-tyrosine (1 to 500 μM) were mixed together in Aminoacylating Solution and incubated for 16 minutes at room temperature. Every four minutes, 0.20 volume was removed from the mixtures and blended with charcoal-containing Quenching Solution (2.50 volumes). The charcoal was centrifuged at 4,000 $\times g$ for five minutes at room temperature, and the top aqueous layer was discarded. Washing Solution (2.50 volumes) was added and mixed. The charcoal was centrifuged at 4,000 $\times g$ for five minutes at room temperature. The last three steps were repeated twice. The charcoal was transferred to scintillation vials containing Safety-Solve Complete Counting Cocktail (50.00 volumes, Research Products International) and counted with a Liquid Scintillation Analyzer Tri-Carb B2910TR (PerkinElmer) at the Caltech Environmental Health and Safety (EH&S) Office.

2.7.2 Chloroform/Methanol Precipitation

Methanol (EMD Millipore, 2.40 volumes) was added to 1.00 volume of protein solution and vortexed. Chloroform (EMD Millipore, 0.80 volume) was added and vortexed. Double-distilled water (3.20 volumes) was added and vortexed. The mixture was centrifuged at 20,000 $\times g$ for 20 minutes at room temperature, and the top layer was discarded. Methanol (5.00 volumes) was added and vortexed, and the mixture was centrifuged at 20,000 $\times g$ for five minutes at room temperature. The last three steps were repeated for a total of five times. The top layer was discarded, and the protein

pellet was air-dried.

2.7.3 Enrichment of Azf-Labeled Proteins

Diazo Biotin-DBCO Solution: 1 mM Diazo Biotin-DBCO (Click Chemistry Tools) in dimethyl sulfoxide, anhydrous (Life Technologies)

Binding/Washing Solution: In double-distilled water, 200 mM Trizma base (Sigma-Aldrich Corporation), 150 mM sodium chloride (VWR International), 0.8% (w/v) sodium dodecyl sulfate (Sigma-Aldrich Corporation), pH 8.0

Eluting Solution: In double-distilled water, 200 mM Trizma base (Sigma-Aldrich Corporation), 150 mM sodium chloride (VWR International), 0.8% (w/v) sodium dodecyl sulfate (Sigma-Aldrich Corporation), pH 8.0, 50 mM sodium dithionite (Sigma-Aldrich Corporation)

Lysing Solution A: In double-distilled water, 200 mM Trizma base (Sigma-Aldrich Corporation), 4% (w/v) sodium dodecyl sulfate (Sigma-Aldrich Corporation), pH 8.0

Lysates (1.00 volume) were incubated with Diazo Biotin-DBCO Solution (0.11 volume) for 20 minutes at room temperature. Excess Diazo Biotin-DBCO was removed by the **Chloroform/Methanol Precipitation** procedure. Protein pellets were re-suspended in Lysing Solution A (0.55 volume). To the protein solutions, Pierce Streptavidin UltraLink Resin (8.33 volumes, 10 μ L re-suspended resin/mg protein in Binding/Wash Solution, Thermo Fisher Scientific) was added. The mixtures were mildly agitated for five hours at room temperature, and centrifuged at 1,000 x *g* for five minutes at room temperature. The top aqueous layer was discarded. To deplete unlabeled proteins, Binding/Wash Solution (75.00 volumes) was added and vortexed. The mixtures were centrifuged at 1,000 x *g* for five minutes at room temperature. The last three steps were repeated for a total of ten times. To elute bound labeled proteins, the top aqueous layer was discarded, and freshly prepared Eluting Solution (5.55 volumes) was added and mildly agitated for 20 minutes at room temperature.

The mixtures were centrifuged at 1,000 x *g* for five minutes at room temperature, and the top aqueous layer was removed and saved. Freshly prepared Eluting Solution (5.55 volumes) was added and mildly agitated for 20 minutes at room temperature. The last three steps were repeated for a total of five times. Pooled protein samples were concentrated and buffer exchanged with Lysing Solution A using an Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 Membrane (EMD Millipore) according to the manufacturer's instructions. Samples were protected from light at all times.

2.7.4 Fluorescence Microscopy of Live C. elegans

Anesthetizing Solution: In double-distilled water, 6.8 g/L sodium phosphate dibasic anhydrous (VWR International), 3 g/L potassium phosphate monobasic (VWR International), 0.5 g/L sodium chloride (VWR International), 1 g/L ammonium chloride (VWR International), 2 mM magnesium sulfate heptahydrate (VWR International), 0.1 mM calcium chloride dihydrate (VWR International), 20 mM D-glucose (VWR International), 25 mM sodium azide (Sigma-Aldrich Corporation)

C. elegans were re-suspended in Anesthetizing Solution. Anesthetized worms were mounted on 3% (w/v) UltraPure Low Melting Point Agarose (Life Technologies) pads and imaged with a LSM 510 META microscope (Carl Zeiss AG) at the Biological Imaging Center in the Beckman Institute at Caltech. Samples were protected from light at all times.

2.7.5 Fluorescence Microscopy of Fixed C. elegans

Alkylating Solution: In double-distilled water, 100 mM Trizma base (Sigma-Aldrich Corporation), pH 8.0, 100 mM 2-chloroacetamide (Sigma-Aldrich Corporation)

C. elegans Fixing Solution: 160 mM potassium chloride (VWR International), 40 mM sodium chloride (VWR International), 20 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid tetrasodium salt (Sigma-Aldrich Corporation), 10 mM spermidine trihydrochloride (Sigma-Aldrich Corporation), 30 mM PIPES, pH 7.4, 50% (v/v) methanol (EMD Millipore), 1.6% (w/v) paraformaldehyde (Electron Microscopy Sciences)

Permeabilizing Solution A: In 1X Dulbecco's phosphate buffered saline, no calcium chloride, no magnesium chloride (Life Technologies), 1% (v/v) Triton X-100 (Sigma-Aldrich Corporation), 0.1% (w/v) bovine serum albumin (Sigma-Aldrich Corporation), 0.05% (w/v) sodium azide (Sigma-Aldrich Corporation)

Permeabilizing Solution B: In 1X Dulbecco's phosphate buffered saline, no calcium chloride, no magnesium chloride (Life Technologies), 10% (v/v) dimethyl sulfoxide (VWR International), 1% (v/v) Triton X-100 (Sigma-Aldrich Corporation), 0.1% (w/v) bovine serum albumin (Sigma-Aldrich Corporation), 0.05% (w/v) sodium azide (Sigma-Aldrich Corporation)

Reducing Solution: In double-distilled water, 100 mM Trizma base (Sigma-Aldrich Corporation), pH 8.0, 10 mM tris(2-carboxyethyl)phosphine hydrochloride (Thermo Fisher Scientific)

TAMRA-DBCO Solution: 1 mM TAMRA-DBCO (Click Chemistry Tools) in dimethyl sulfoxide, anhydrous (Life Technologies)

Washing Solution A: In double-distilled water, 100 mM Trizma base (Sigma-Aldrich Corporation), pH 8.0

Washing Solution B: In 1X Dulbecco's phosphate buffered saline, no calcium chloride, no magnesium chloride (Life Technologies), 20% (v/v) dimethyl sulfoxide (VWR In-

ternational), 1% (v/v) Triton X-100 (Sigma-Aldrich Corporation), 0.1% (w/v) bovine serum albumin (Sigma-Aldrich Corporation), 0.05% (w/v) sodium azide (Sigma-Aldrich Corporation)

Washing Solution C: In 1X Dulbecco's phosphate buffered saline, no calcium chloride, no magnesium chloride (Life Technologies), 20% (v/v) methanol (EMD Millipore), 1% (v/v) Triton X-100 (Sigma-Aldrich Corporation), 0.1% (w/v) bovine serum albumin (Sigma-Aldrich Corporation), 0.05% (w/v) sodium azide (Sigma-Aldrich Corporation)

Cell or worm pellets were re-suspended in double-distilled water (1.00 volume) and incubated for five minutes in ice. To fix animals and crack their cuticles, ice-cold *C. elegans* Fixing Solution (1.00 volume) was added to the mixtures of worms and incubated for five minutes in liquid nitrogen. Frozen solutions were thawed for five minutes in room temperature water. The last two freeze-thaw steps were repeated for a total of three times. Thawed mixtures were incubated for an additional hour in ice. The mixtures were centrifuged at 1,000 x *g* for one minute at room temperature, and the top aqueous layer was discarded. Washing Solution A (2.00 volumes) was added and mixed, and the mixtures were centrifuged at 1,000 x *g* for one minute at room temperature. The last three steps were repeated for a total of five times. The top aqueous layer was discarded. To reduce animals' cuticles, Reducing Solution (2.00 volumes) was added and mildly agitated for 30 minutes at room temperature. The mixtures were centrifuged at 1,000 x *g* for one minute at room temperature, and the top aqueous layer was discarded. To alkylate animals' cuticles, Alkylating Solution (2.00 volumes) was added and mildly agitated for 30 minutes at room temperature. The mixtures were centrifuged at 1,000 x *g* for one minute at room temperature, and the top aqueous layer was discarded. 1X Dulbecco's phosphate buffered saline, no calcium chloride, no magnesium chloride (2.00 volumes, Life Technologies) was added and mixed. The mixtures were centrifuged at 1,000 x *g* for one minute at

room temperature. The last three steps were repeated for a total of five times. The top aqueous layer was discarded. Permeabilizing Solution A (2.00 volumes) was added and mildly agitated for one hour at room temperature. The mixtures were centrifuged at $1,000 \times g$ for one minute at room temperature, and the top aqueous layer was discarded. Permeabilizing Solution B (1.98 volumes) was added and mixed. TAMRA-DBCO Solution (0.02 volume) was added and mildly agitated for 20 minutes at room temperature. The mixtures were centrifuged at $1,000 \times g$ for one minute at room temperature, and the top aqueous layer was discarded. Washing Solution B (2.00 volumes) was added and mixed. The mixtures were centrifuged at $1,000 \times g$ for one minute at room temperature. The last three steps were repeated for a total of ten times. The top aqueous layer was discarded. Washing Solution C (2.00 volumes) was added and mixed. The mixtures were centrifuged at $1,000 \times g$ for one minute at room temperature. The last three steps were repeated for a total of ten times. The top aqueous layer was discarded. Permeabilizing Solution A (2.00 volumes) was added and mixed. The mixtures were centrifuged at $1,000 \times g$ for one minute at room temperature. The last three steps were repeated for a total of ten times. Processed worms were mounted on 3% (w/v) UltraPure Low Melting Point Agarose (Life Technologies) pads and imaged with a LSM 510 META microscope (Carl Zeiss AG) at the Biological Imaging Center in the Beckman Institute at Caltech. Samples were protected from light at all times.

2.7.6 In-Gel Fluorescence Scanning of Azf-Labeled Proteins

Lysing Solution A: In double-distilled water, 200 mM Trizma base (Sigma-Aldrich Corporation), 4% (w/v) sodium dodecyl sulfate (Sigma-Aldrich Corporation), pH 8.0

Lysing Solution B: 1X Lysing Solution A, 100 mM 2-chloroacetamide (Sigma-Aldrich Corporation), protease inhibitor cocktail (1 tablet/10 mL cOmplete, Mini, EDTA-free, Roche Diagnostics)

Lysing Solution C: 75% (v/v) Lysing Solution A, 18.75% (v/v) NuPAGE LDS Sample Buffer (Life Technologies), 6.25% (v/v) 2-mercaptoethanol (Sigma-Aldrich Corporation)

Protein Gel Fixing Solution: 40% (v/v) double-distilled water, 50% (v/v) methanol (EMD Millipore), 10% acetic acid, glacial (EMD Millipore)

TAMRA-DBCO Solution: 1 mM TAMRA-DBCO (Click Chemistry Tools) in dimethyl sulfoxide, anhydrous (Life Technologies)

Cell or worm pellets were re-suspended in freshly prepared Lysing Solution B and lysed by sonication. Lysates were clarified by centrifugation at 20,000 x *g* for 20 minutes at room temperature. Protein concentrations of lysates were determined by bicinchoninic acid assay (BCA Protein Assay Kit, Thermo Fisher Scientific) according to the manufacturer's instructions and normalized to 1.11 mg/mL with additional Lysing Solution B. Lysates (1.00 volumes) were incubated with TAMRA-DBCO Solution (0.11 volume) for 20 minutes at room temperature. Excess TAMRA-DBCO was removed according to the **Chloroform/Methanol Precipitation** procedure. Protein pellets were re-suspended in freshly prepared Lysing Solution C (1.11 volumes) and incubated for 20 minutes at 100°C. The samples were electrophoresed on NuPAGE Novex 4-12% Bis-Tris Protein Gels (Life Technologies) according to the manufacturer's instructions. Protein gels were incubated with Protein Gel Fixing Solution for 20 minutes at room temperature and imaged with a Typhoon Trio+ instrument (GE Healthcare Life Sciences). Protein gels were stained using the Colloidal Blue Staining Kit (Life Technologies) according to the manufacturer's instructions. Samples were protected from light at all times.

2.7.7 In-Gel Proteolytic Digestion of Azf-Labeled Proteins

Lysing Solution A: In double-distilled water, 200 mM Trizma base (Sigma-Aldrich Corporation), 4% (w/v) sodium dodecyl sulfate (Sigma-Aldrich Corporation), pH 8.0

Lysing Solution B: 1X Lysing Solution A, 100 mM 2-chloroacetamide (Sigma-Aldrich Corporation), protease inhibitor cocktail (1 tablet/10 mL cOmplete, Mini, EDTA-free, Roche Diagnostics)

Lysing Solution C: 75% (v/v) Lysing Solution A, 18.75% (v/v) NuPAGE LDS Sample Buffer (Life Technologies), 6.25% (v/v) 2-mercaptoethanol (Sigma-Aldrich Corporation)

Cell or worm pellets were re-suspended in freshly prepared Lysing Solution B and lysed by sonication. Lysates were clarified by centrifugation at 20,000 x *g* for 20 minutes at room temperature. Protein concentrations of lysates were determined by bicinchoninic acid assay (BCA Protein Assay Kit, Thermo Fisher Scientific) according to the manufacturer's instructions and normalized to 5.55 mg/mL with additional Lysing Solution B. The samples are equally divided into two groups, one designated as "unenriched" and the other as "enriched".

"Unenriched" samples were re-normalized to 1.11 mg/mL with additional Lysing Solution B. Lysates (1.11 volumes) were subjected to the **Chloroform/Methanol Precipitation** procedure. Protein pellets were re-suspended in freshly prepared Lysing Solution C (1.11 volumes) and incubated for 20 minutes at 100°C. The samples were electrophoresed on NuPAGE Novex 4-12% Bis-Tris Protein Gels (Life Technologies) according to the manufacturer's instructions. Protein gels were stained using the Colloidal Blue Staining Kit (Life Technologies) according to the manufacturer's instructions.

“Enriched” samples were subjected to the **Enrichment of Azf-Labeled Proteins** procedure and dried by centrifugal evaporation. Protein pellets were re-normalized to 1.11 mg/mL with freshly prepared Lysing Solution C and incubated for 20 minutes at 100°C. The samples were electrophoresed on NuPAGE Novex 4-12% Bis-Tris Protein Gels (Life Technologies) according to the manufacturer’s instructions. Protein gels were stained using the Colloidal Blue Staining Kit (Life Technologies) according to the manufacturer’s instructions. Peptides were extracted from protein gels according to the in-gel proteolytic digestion procedures described by Shevchenko et al. (Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V., and Mann, M. *Nat. Protoc.* **1**, 2856–2860 (2007)) and dried by centrifugal evaporation. Peptides were desalted according to the micro-purification procedures described by Rappsilber et al. (Rappsilber, J., Mann, M., and Ishihama, Y. *Nat. Protoc.* **2**, 1896–1906 (2007)) and dried by centrifugal evaporation.

2.7.8 Isolation of 6xHis-Tagged Proteins

Native Lysing Solution: In double-distilled water, 50 mM sodium phosphate, monobasic monohydrate (VWR International), 300 mM sodium chloride (VWR International), 10 mM imidazole (Sigma-Aldrich Corporation), pH 8.0, 4 mg/mL lysozyme from chicken egg white (Sigma-Aldrich Corporation)

Cell pellets were re-suspended in freshly prepared Native Lysing Solution, incubated for one hour in ice and lysed by sonication. Lysates were clarified by centrifugation at 20,000 x *g* for 20 minutes at 4°C. 6xHis-tagged proteins were extracted from lysates using Ni-NTA Agarose (Qiagen) according to the manufacturers instructions. Proteins were desalted using PD-10 Desalting Columns (GE Healthcare Life Sciences) according to the manufacturers instructions and stored in 100 mM Trizma base (Sigma-Aldrich Corporation), pH 7.4 at 4°C.

2.7.9 Labeling in *C. elegans*

Potassium Citrate Solution: In double-distilled water, 20 g/L citric acid monohydrate (VWR International), 293.5 g/L potassium citrate monohydrate (VWR International), pH 6.0

S Medium: In double-distilled water, 5.85 g/L sodium chloride (VWR International), 1 g/L potassium phosphate dibasic (VWR International), 6 g/L potassium phosphate monobasic (VWR International), 5 mg/L cholesterol (Sigma-Aldrich Corporation), 1% (v/v) potassium citrate solution, 1% (v/v) Trace Metals Solution, 3 mM calcium chloride dihydrate (VWR International), 3 mM magnesium chloride hexahydrate (VWR International)

Trace Metals Solution: In double-distilled water, 1.86 g/L ethylenediaminetetraacetic acid disodium salt dihydrate (Sigma-Aldrich Corporation), 0.69 g/L iron(II) sulfate heptahydrate (Sigma-Aldrich Corporation), 0.2 g/L manganese(II) chloride tetrahydrate, 0.29 g/L zinc sulfate heptahydrate (Sigma-Aldrich Corporation), 0.025 g/L copper(II) sulfate pentahydrate (Sigma-Aldrich Corporation)

hsp-16.2::Thr412Gly-CePheRS, *myo-3::Thr412Gly-CePheRS*, *ges-1::Thr412Gly-CePheRS*, *rab-3::Thr412Gly-CePheRS*, and *myo-2::Thr412Gly-CePheRS* worms: *C. elegans* strains previously maintained in S Medium supplemented with 25 mg/mL *E. coli* OP50 (*Caenorhabditis* Genetics Center) at 20°C with agitation were pelleted by centrifugation at 1000 x *g* for five minutes at room temperature, washed three times with S Medium, and re-suspended in S Medium supplemented with 25 mg/mL *E. coli* KY14[pKPY514] or KY33[pKPY514]. KY14[pKPY514] or KY33[pKPY514] was prepared according to the **Labeling in *E. coli*** procedure. For *hsp-16.2::Thr412Gly-CePheRS* worms, we induced heat shock of worms grown at 20°C by one hour exposure to 33°C. After 24 hours of agitation at 20°C, worms were harvested by centrifugation at 1000 x *g* for five minutes at room temperature and cleaned according to the sucrose flotation procedures described by Portman (Portman, D.S., *WormBook*,

ed. The *C. elegans* Research Community, *WormBook*, doi/10.1895/wormbook.1.104.1 (2006)). Digested bacterial material was cleared by washing worms with S Medium over a period of 30 minutes. Worms were pelleted by centrifugation at 1000 x *g* for five minutes at room temperature and frozen in liquid nitrogen. Samples were protected from light at all times.

2.7.10 Labeling in *E. coli*

M9 Minimal Medium A: In double-distilled water, 6.8 g/L sodium phosphate dibasic Anhydrous (VWR International), 3 g/L potassium phosphate monobasic (VWR International), 0.5 g/L sodium chloride (VWR International), 1 g/L ammonium chloride (VWR International), 2 mM magnesium sulfate heptahydrate (VWR International), 0.1 mM calcium chloride dihydrate (VWR International), 35 mg/L thiamine hydrochloride (Sigma-Aldrich Corporation), 40 mg/L L-alanine (Sigma-Aldrich Corporation), 40 mg/L L-cysteine (Sigma-Aldrich Corporation), 40 mg/L L-aspartic Acid (Sigma-Aldrich Corporation), 40 mg/L L-glutamic Acid (Sigma-Aldrich Corporation), 40 mg/L glycine (Sigma-Aldrich Corporation), 40 mg/L L-histidine (Sigma-Aldrich Corporation), 40 mg/L L-isoleucine (Sigma-Aldrich Corporation), 40 mg/L L-leucine, 40 mg/L L-methionine (Sigma-Aldrich Corporation), 40 mg/L L-asparagine (Sigma-Aldrich Corporation), 40 mg/L L-proline (Sigma-Aldrich Corporation), 40 mg/L L-glutamine (Sigma-Aldrich Corporation), 40 mg/L L-serine (Sigma-Aldrich Corporation), 40 mg/L L-threonine (Sigma-Aldrich Corporation), 40 mg/L L-valine (Sigma-Aldrich Corporation), 40 mg/L L-tryptophan (Sigma-Aldrich Corporation), 40 mg/L L-tyrosine (Sigma-Aldrich Corporation)

M9 Minimal Medium B: In M9 Minimal Medium A, 0.5% (v/v) glycerol (VWR International), 40 mg/L L-phenylalanine (Sigma-Aldrich Corporation), 40 mg/L L-lysine (Sigma-Aldrich Corporation), 40 mg/L L-arginine (Sigma-Aldrich Corporation), 200 mg/L ampicillin sodium salt (Sigma-Aldrich Corporation), 35 mg/L chloramphenicol

(Sigma-Aldrich Corporation)

M9 Minimal Medium C: M9 Minimal Medium A, 0.5% (v/v) glycerol (VWR International), 40 mg/L L-lysine (Sigma-Aldrich Corporation), 40 mg/L L-arginine (Sigma-Aldrich Corporation), 200 mg/L ampicillin sodium salt (Sigma-Aldrich Corporation), 35 mg/L chloramphenicol (Sigma-Aldrich Corporation)

M9 Minimal Medium D: In M9 Minimal Medium A, 20 mM D-glucose (VWR International), 40 mg/L L-phenylalanine (Sigma-Aldrich Corporation), 40 mg/L L-lysine (Sigma-Aldrich Corporation), 40 mg/L L-arginine (Sigma-Aldrich Corporation), 35 mg/L kanamycin sulfate (Sigma-Aldrich Corporation)

M9 Minimal Medium E: In M9 Minimal Medium A, 20 mM D-glucose (VWR International), 40 mg/L L-lysine (Sigma-Aldrich Corporation), 40 mg/L L-arginine (Sigma-Aldrich Corporation), 35 mg/L kanamycin sulfate (Sigma-Aldrich Corporation)

M9 Minimal Medium F: In M9 Minimal Medium A, 20 mM D-glucose (VWR International), 40 mg/L L-phenylalanine (Sigma-Aldrich Corporation), 40 mg/L L-lysine ($^{13}\text{C}_6\text{ }^{15}\text{N}_2\text{ }^1\text{H}_{14}\text{ }^{16}\text{O}_2$, Cambridge Isotope Laboratories), 40 mg/L L-arginine ($^{13}\text{C}_6\text{ }^{14}\text{N}_4\text{ }^1\text{H}_{14}\text{ }^{16}\text{O}_2$, Cambridge Isotope Laboratories), 35 mg/L kanamycin sulfate (Sigma-Aldrich Corporation)

M9 Minimal Medium G: In M9 Minimal Medium A, 20 mM D-glucose (VWR International), 40 mg/L L-lysine ($^{13}\text{C}_6\text{ }^{15}\text{N}_2\text{ }^1\text{H}_{14}\text{ }^{16}\text{O}_2$, Cambridge Isotope Laboratories), 40 mg/L L-arginine ($^{13}\text{C}_6\text{ }^{14}\text{N}_4\text{ }^1\text{H}_{14}\text{ }^{16}\text{O}_2$, Cambridge Isotope Laboratories), 35 mg/L kanamycin sulfate (Sigma-Aldrich Corporation)

Terrific Broth: In double-distilled water, 12 g/L Bacto tryptone (BD Biosciences), 24 g/L Bacto yeast extract (BD Biosciences), 0.4% (v/v) glycerol (EMD Millipore), 2.31 g/L potassium phosphate monobasic (VWR International), 12.54 g/L potassium phosphate dibasic (VWR International), 200 mg/L ampicillin sodium salt (Sigma-Aldrich Corporation)

KY14[pKPY66] and KY14[pKPY68]: A single colony was used to inoculate an overnight culture of freshly prepared Terrific Broth (5.0 mL). Overnight cultures were diluted into freshly prepared Terrific Broth (50 mL) and agitated at 37°C until reaching an OD₆₀₀ of 0.5. After another 30 minutes of agitation at 20°C, expression of 6xHis-tagged wild-type and mutant *CePheRSs* was induced by addition of 1 M isopropyl β -D-1-thiogalactopyranoside (50 μ L, Sigma-Aldrich Corporation). After 16 hours of agitation at 20°C, cells were harvested by centrifugation at 5000 x *g* for five minutes at 4°C and frozen in liquid nitrogen.

KY14[pKPY93 and pKPY100 or pKPY133 or pKPY127 or pKPY131 or pKPY129 or pKPY125 or pKPY101 or pKPY102 or pKPY509 or pKPY503 or pKPY505 or pKPY647 or pKPY655 or pKPY507 or pKPY137]: A single colony was used to inoculate an overnight culture of freshly prepared M9 Minimal Medium B (5.0 mL). Overnight cultures were diluted into freshly prepared M9 Minimal Medium B (50 mL) and agitated at 37°C until reaching an OD₆₀₀ of 0.5. Expression of wild-type and mutant *CePheRSs* was induced by addition of 1 M L-(+)-arabinose (500 μ L, Sigma-Aldrich Corporation). After 30 minutes, cells were pelleted by centrifugation at 5000 x *g* for five minutes at 4°C, washed three times with ice-cold 0.9% (w/v) sodium chloride (VWR International) and re-suspended in freshly prepared M9 Minimal Medium C supplemented with L-phenylalanine (0.0 mM or 0.2 mM final concentration, Sigma-Aldrich Corporation) or *p*-azido-L-phenylalanine (0.0 mM, 0.2 mM or 2.0 mM final concentration, Chem-Impex International). After another 30 minutes of agitation at 37°C, expression of 6xHis-tagged GFPs was induced by addition of 1 M isopropyl β -D-1-thiogalactopyranoside (50 μ L, Sigma-Aldrich Corporation). After four hours of agitation at 37°C, cells were harvested by centrifugation at 5000 x *g* for five minutes at 4°C and frozen in liquid nitrogen. Samples were protected from light at all times.

KY14[pKPY513], KY14[pKPY566], KY14[pKPY565], and KY14[pKPY514]: A single colony was used to inoculate an overnight culture of freshly prepared M9 Minimal Medium D (5.0 mL). Overnight cultures were diluted into freshly prepared M9 Minimal Medium D (50 mL) and agitated at 37°C until reaching an OD₆₀₀ of 0.5. Cells were pelleted by centrifugation at 5000 x *g* for five minutes at 4°C, washed three times with ice-cold 0.9% (w/v) sodium chloride (VWR International) and re-suspended in freshly prepared M9 Minimal Medium E supplemented with L-phenylalanine (0.0 mM or 0.2 mM final concentration, Sigma-Aldrich Corporation) or *p*-azido-L-phenylalanine (0.2 mM or 2.0 mM final concentration, Chem-Impex International). After another 30 minutes of agitation at 37°C, expression of wild-type or mutant *EcPheRSs* was induced by addition of 1 M isopropyl β -D-1-thiogalactopyranoside (50 μ L, Sigma-Aldrich Corporation). After four hours of agitation at 37°C, cells were harvested by centrifugation at 5000 x *g* for five minutes at 4°C and frozen in liquid nitrogen. Extent of replacement of L-phenylalanine by *p*-azido-L-phenylalanine was determined by amino acid analysis at the Protein Sequencing and Amino Acid Analysis Resources of the W.M. Keck Foundation Biotechnology Resource Laboratory at the Yale School of Medicine. Samples were protected from light at all times.

KY14[pKPY514]: A single colony was used to inoculate an overnight culture of freshly prepared M9 Minimal Medium D (5.0 mL). Overnight cultures were diluted into freshly prepared M9 Minimal Medium D (500 mL) and agitated at 37°C until reaching an OD₆₀₀ of 0.5. Cells were pelleted by centrifugation at 5000 x *g* for 15 minutes at 4°C, washed three times with ice-cold 0.9% (w/v) sodium chloride (VWR International) and re-suspended in freshly prepared M9 Minimal Medium E supplemented with *p*-azido-L-phenylalanine (2.0 mM final concentration, Chem-Impex International). After another 30 minutes of agitation at 37°C, expression of Thr251Gly-*EcPheRS* was induced by addition of 1 M isopropyl β -D-1-thiogalactopyranoside (500

μL , Sigma-Aldrich Corporation). After four hours of agitation at 37°C , cells were harvested by centrifugation at $5000 \times g$ for 15 minutes at 4°C , re-suspended with freshly prepared M9 Minimal Medium E supplemented with *p*-azido-L-phenylalanine (2.0 mM final concentration, Chem-Impex International) at a concentration of 250 mg wet cell mass/mL medium and stored at 4°C . Samples were protected from light at all times.

KY33[pKPY514]: A single colony was used to inoculate an overnight culture of freshly prepared M9 Minimal Medium F (5.0 mL). Overnight cultures were diluted into freshly prepared M9 Medium F (500 mL) and agitated at 37°C until reaching an OD_{600} of 0.5. Cells were pelleted by centrifugation at $5000 \times g$ for 15 minutes at 4°C , washed three times with ice-cold 0.9% (w/v) sodium chloride (VWR International) and re-suspended in freshly prepared M9 Minimal Medium G supplemented with *p*-azido-L-phenylalanine (2.0 mM final concentration, Chem-Impex International). After another 30 minutes of agitation at 37°C , expression of Thr251Gly-*Ec*PheRS was induced by addition of 1 M isopropyl β -D-1-thiogalactopyranoside (500 μL , Sigma-Aldrich Corporation). After four hours of agitation at 37°C , cells were harvested by centrifugation at $5000 \times g$ for 15 minutes at 4°C , re-suspended with freshly prepared M9 Minimal Medium G supplemented with *p*-azido-L-phenylalanine (2.0 mM final concentration, Chem-Impex International) at a concentration of 250 mg wet cell mass/mL medium and stored at 4°C . Samples were protected from light at all times.

2.7.11 LC-MS/MS of Azf-Labeled Proteins

Solvent A: 0.2% (v/v) formic acid (Sigma-Aldrich Corporation), 2.0% (v/v) LC-MS CHROMASOLV acetonitrile (Sigma-Aldrich Corporation), 97.8% (v/v) LC-MS CHROMASOLV water (Sigma-Aldrich Corporation)

Solvent B: 0.2% (v/v) formic acid (Sigma-Aldrich Corporation), 80.0% (v/v) LC-MS CHROMASOLV acetonitrile (Sigma-Aldrich Corporation), 19.8% (v/v) LC-MS

CHROMASOLV water (Sigma-Aldrich Corporation)

LC-MS/MS experiments were performed on an EASY-nLC1000 (Proxeon Biosystems, now Thermo Fisher Scientific) coupled to a hybrid LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) equipped with a nano-electrospray ion source (Thermo Fisher Scientific). Peptides were re-suspended in 0.1% (v/v) formic acid (Sigma-Aldrich Corporation) and loaded on a 15-cm reversed phase analytical column (75 μm ID) packed in-house with 3 μm C18AQ beads (ReproSil-Pur C18AQ, Dr. Maisch-GmbH). The chromatographic separation was achieved using a 90 minute elution gradient from 0% (100% Solvent A) to 30% Solvent B (70% Solvent A) at a flow rate of 350 nL/minute. The mass spectrometer was operated in data-dependent mode to switch automatically between MS and MS/MS scans as described by Kalli and Hess (Kalli, A. and Hess, S. *Proteomics* **12**, 21–31 (2012)). Survey full scan mass spectra were acquired in the Orbitrap (400–1600 m/z) with a resolution of 60,000 at 400 m/z. The top ten most intense ions from the survey scan were isolated and fragmented in the linear ion trap by collisionally induced dissociation (collisional energy 35% and isolation width 2 Da). Precursor ion charge state screening was enabled, and all singly charged and unassigned charge states were rejected. The dynamic exclusion list was set with a maximum retention time of 90 seconds and a relative mass window of 10 ppm. Thermo raw data files were analyzed by MaxQuant (v 1.5.0.0) (Cox, J. and Mann, M. *Nat. Biotechnol.* **26**, 1367–1372 (2008); Cox, J., et al. *J. Proteome Res.* **10**, 1794–1805 (2011)) and were searched against the UniProt *C. elegans* database (26900 sequences) and an in-house contaminant database (259 sequences) including human keratins and proteases. All default options were used except as follows: match between runs was enabled, re-quantify was enabled, and multiplicity of two with heavy labels “Arg6” (+6.0201) and “Lys8” (+8.0142). Variable modifications included oxidation of L-methionine (+15.9949), N-terminal protein acetylation (+42.0106), L-phenylalanine to *p*-azido-L-phenylalanine (+41.0014), L-phenylalanine

to *p*-amino-L-phenylalanine (+15.0109), and cleaved Diazo Biotin-DBCO-modified *p*-azido-L-phenylalanine (+482.2066). Tryptic digest was specified with up to two missed cleavages. Initial precursor mass tolerance was 7 ppm; however, MaxQuant calculates tighter individual precursor tolerances after recalibration. Fragment ion tolerance was 0.5 Da. Peptide, protein and site false discovery rates were fixed at 1% using the target-decoy approach with a reversed database. To account for conversion of heavy labeled L-Lysine and heavy labeled L-Arginine to other amino acids (Park, S.K., Liao, L., Kim, J.Y., and Yates III, J.R. *Nat. Methods* **6**, 184–185 (2009)), a linear regression model was constructed to calculate the effects each amino acid has on the peptide ratio. These coefficients were used to back-calculate peptide ratios without biases from the amino acid content. Overall enrichment factor is the mean of the log₂ biological replicate enrichment factors.

2.7.12 MALDI TOF-MS of 6xHis-Tagged Proteins

α-Cyano-4-Hydroxycinnamic Acid Solution: 10 mg/mL *α*-cyano-4-hydroxycinnamic Acid (Sigma-Aldrich Corporation) in 10% (v/v) trifluoroacetic acid (Sigma-Aldrich Corporation), 40% (v/v) LC-MS CHROMASOLV water (Sigma-Aldrich Corporation), 50% (v/v) LC-MS CHROMASOLV acetonitrile (Sigma-Aldrich Corporation)

Purified proteins were obtained from the **Isolation of 6xHis-Tagged Proteins** procedure. Protein concentrations of purified proteins were determined by absorbance at 280 nm. NuPAGE LDS Sample Buffer (0.25 volume, Life Technologies) and 2-Mercaptoethanol (0.08 volume, Sigma-Aldrich Corporation) were added to protein solutions (1.00 volume) and incubated for 20 minutes at 100°C. The samples were electrophoresed on NuPAGE Novex 4-12% Bis-Tris Protein Gels (Life Technologies) according to the manufacturers instructions. Protein gels were stained using the Colloidal Blue Staining Kit (Life Technologies) according to the manufacturers instructions. Peptides were extracted from protein gels according to the in-gel proteolytic

digestion procedures described by Shevchenko et al. (Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V., and Mann, M. *Nat. Protoc.* **1**, 2856–2860 (2007)) and dried by centrifugal evaporation. Peptides were desalted according to the micro-purification procedures described by Rappsilber et al. (Rappsilber, J., Mann, M., and Ishihama, Y. *Nat. Protoc.* **2**, 1896–1906 (2007)). The peptides were mixed with α -Cyano-4-Hydroxycinnamic Acid Solution (1-10 pmol peptide/ μ L solution) and assayed on a Voyager DE Pro instrument (Applied Biosystems) at the Caltech Division of Chemistry and Chemical Engineering Mass Spectrometry Facility.

2.7.13 Plasmids and Strains

pKPY66: The genes encoding wild-type *C. elegans* PheRS were isolated by RNA extraction (RNeasy Mini Kit, Qiagen) from wild-type (N2) *C. elegans*, reverse transcription (SuperScript III Reverse Transcriptase, Life Technologies) and PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies). The PCR product encodes the alpha (isoform A) and beta subunits of *C. elegans* PheRS as well as the intergenic region from *E. coli pheST*. The purified fragments were ligated into pQE-80L (Qiagen) to generate pKPY66. We have designated the first methionine in the alpha subunit of *CePheRS*, isoform A, exon 3 as residue 1 (“Met1”).

pKPY68: pKPY66 was subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the Thr412Gly mutation.

pKPY93: The *lpp* promoter region was isolated by genomic DNA extraction (DNeasy Blood and Tissue Kit, Qiagen) from *E. coli* DH10B (Life Technologies) and PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies). *C. elegans* tRNA^{Phe} was synthesized by primer annealing and extension with Klenow Fragment (3 to 5' exo-, New England Biolabs). The *rrnB* terminator region was isolated by PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent

Technologies) of pBAD33 (Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. *J. Bacteriol.* **177**, 4121–4130 (1995)). The purified fragments were ligated into the NdeI site of pJTN4 (Ngo, J.T., et al. *Nat. Chem. Biol.* **5**, 715–717 (2009)) to generate pKPY93.

pKPY100: The genes encoding wild-type *C. elegans* PheRS were isolated by PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies) of pKPY66. The purified fragments were ligated into pBAD33.

pKPY133: pKPY100 was subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the Thr412Asn mutation.

pKPY127: pKPY100 was subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the Thr412Pro mutation.

pKPY131: pKPY100 was subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the Thr412Asp mutation.

pKPY129: pKPY100 was subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the Thr412Cys mutation.

pKPY125: pKPY100 was subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the Thr412Ser mutation.

pKPY101: pKPY100 was subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the Thr412Ala mutation.

pKPY102: pKPY100 was subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to

generate the Thr412Gly mutation.

pKPY509: The genes encoding wild-type *Saccharomyces cerevisiae* PheRS were isolated by PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies) of plasmids YSC3867-202326582 from Thermo Fisher Scientific (Accession Number YFL022C) and YSC3867-202328360 from Thermo Fisher Scientific (Accession Number YLR060W). The purified fragments were ligated into pBAD33 and subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the equivalent Thr412Gly mutation.

pKPY503: The genes encoding wild-type *Drosophila melanogaster* PheRS were isolated by PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies) of genomic DNA from *Drosophila melanogaster* (0000-1012.01 from *Drosophila* Species Stock Center). The purified fragments were ligated into pBAD33 and subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the equivalent Thr412Gly mutation.

pKPY505: The genes encoding wild-type *Danio rerio* PheRS were isolated by PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies) of plasmids 6806236 from Thermo Fisher Scientific (Accession Numbers BC066611, CA975688) and 7046061 from Thermo Fisher Scientific (Accession Numbers CK126922, BC085625). The purified fragments were ligated into pBAD33 and subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the equivalent Thr412Gly mutation.

pKPY647: The genes encoding wild-type *Xenopus laevis* PheRS were isolated by PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies) of plasmids 5156818 from GE Healthcare Dharmacon (Accession Numbers CA790449 BC078035) and 5048633 from GE Healthcare Dharmacon (Accession Numbers BM262817 BM261881 BC056121). The purified fragments were ligated into pBAD33 and subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the equivalent Thr412 Gly mutation.

pKPY655: The genes encoding wild-type *Rattus norvegicus* PheRS were isolated by PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies) of plasmids 7314191 from GE Healthcare Dharmacon and 7109296 from GE Healthcare Dharmacon. The purified fragments were ligated into pBAD33 and subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the equivalent Thr412Gly mutation.

pKPY507: The genes encoding wild-type *Mus musculus* PheRS were isolated by PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies) of plasmids 3595108 from Thermo Fisher Scientific (Accession Number BC006862) and 4239906 from Thermo Fisher Scientific (Accession Number BC016428). The purified fragments were ligated into pBAD33 and subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the equivalent Thr412Gly mutation.

pKPY137: The genes encoding wild-type *Homo sapiens* PheRS were isolated by PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies) of plasmids 2823551 from Open Biosystems (Accession Numbers AW732158, AW732158.1, BC006495, BC006495.2, BE208518, BE208518.1) and 4664949 from Open Biosystems (Accession Numbers BC017783, BC017783.1, BG504513,

BG504513.1). The purified fragments were ligated into pBAD33 and subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the equivalent Thr412Gly mutation.

pKPY513: The genes encoding wild-type *E. coli* PheRS were isolated by genomic DNA extraction (DNeasy Blood and Tissue Kit, Qiagen) from *E. coli* DH10B (Life Technologies) and PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies). The purified fragments were ligated into pQE-80L-Kan (Qiagen) to generate pKPY513.

pKPY566: pKPY513 was subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the Thr251Ser mutation.

pKPY565: pKPY513 was subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the Thr251Ala mutation.

pKPY514: pKPY513 was subjected to site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies; DpnI, New England Biolabs) to generate the Thr251Gly mutation.

pKPY197 (*frs-1*(Thr412Gly)::*fib-1/rps-16*::*gfp*(S65C, SynIVS)::*unc-54*): The gene encoding the mutant alpha subunit of *C. elegans* PheRS was isolated by PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies) of pKPY102. The intercistronic region of the *C. elegans* operon CEOP5428 as described by Lee et al. (Lee, L.W., Lo, H.W., and Lo, S.J. *Gene* **455**, 16–21 (2010)) was isolated by genomic DNA extraction (DNeasy Blood and Tissue Kit, Qiagen) from wild-type (N2) *C. elegans* and PCR amplification (PfuUltra II Fusion HS DNA Polymerase, Agilent Technologies) The purified fragments were ligated into pPD95.75 (Fire Lab Vector Kit, Addgene) to generate pKPY197.

KY14: The lysine- and phenylalanine-auxotrophic strain of *E. coli* KY14 was made in-house using the red recombinase gene knockout method described by Datsenko and Wanner (Datsenko, K.A. and Wanner, B.L. *Proc. Natl. Acad. Sci. USA* **97**, 6640–6645 (2000)) to eliminate the gene *pheA* from the *E. coli* strain KY2. The lysine-auxotrophic strain of KY2 was made in-house using the red recombinase gene knockout method described by Datsenko and Wanner to eliminate the gene *lysA* from the *E. coli* strain TYJV2 (Van Deventer, J.A., Yuet, K.P., Yoo, T.H., and Tirrell, D.A. *ChemBioChem* **15**, 1777–1781 (2014)).

KY33: The arginine-, lysine- and phenylalanine-auxotrophic strain of *E. coli* KY33 was made in-house using the red recombinase gene knockout method described by Datsenko and Wanner (Datsenko, K.A. and Wanner, B.L. *Proc. Natl. Acad. Sci. USA* **97**, 6640–6645 (2000)) to eliminate the gene *argA* from the *E. coli* strain KY14.

Worm strains were cultured as described by Brenner (Brenner, S. *Genetics* **77**, 71–94 (1974)). The strains used in this study were as follows:

PS6741 (*ges-1::Thr412Gly-CePheRS*): *pha-1(e2123ts) III*, *him-5(e1490) V*; *syEx1341[ges-1::frs-1(Thr412Gly)::fib-1/rps-16::gfp(S65C, SynIVS)::unc-54* (1 ng/ μ L) + *myo-2::dsRed* (10 ng/ μ L) + *pha-1(+)* (90 ng/ μ L) + pBluescript (180 ng/ μ L)]

PS6742 (*myo-2::Thr412Gly-CePheRS*): *pha-1(e2123ts) III*, *him-5(e1490) V*; *syEx1342[myo-2::frs-1(Thr412Gly)::fib-1/rps-16::gfp(S65C, SynIVS)::unc-54* (1 ng/ μ L) + *unc-122::mRFP* (10 ng/ μ L) + *pha-1(+)* (90 ng/ μ L) + pBluescript (180 ng/ μ L)]

PS6743 (*myo-3::Thr412Gly-CePheRS*): *pha-1(e2123ts) III*, *him-5(e1490) V*; *syEx1343[myo-3::frs-1(Thr412Gly)::fib-1/rps-16::gfp(S65C, SynIVS)::unc-54* (1 ng/ μ L) + *myo-2::dsRed* (10 ng/ μ L) + *pha-1(+)* (90 ng/ μ L) + pBluescript (180 ng/ μ L)]

PS6744 (*rab-3::Thr412Gly-CePheRS*): *pha-1(e2123ts) III, him-5(e1490) V*;
syEx1344[rab-3::frs-1(Thr412Gly)::fib-1/rps-16::gfp(S65C, SynIVS)::unc-54
 (1 ng/ μ L) + *myo-2::dsRed* (10 ng/ μ L) + *pha-1(+)* (90 ng/ μ L) + pBluescript
 (180 ng/ μ L)]

PS6745 (*hsp-16.2::Thr412Gly-CePheRS*): *pha-1(e2123ts) III, him-5(e1490) V*;
syIs266[hsp-16.2::frs-1(Thr412Gly)::fib-1/rps-16::gfp(S65C, SynIVS)::unc-54
 (1 ng/ μ L) + *myo-2::dsRed* (10 ng/ μ L) + *pha-1(+)* (90 ng/ μ L) + pBluescript
 (180 ng/ μ L)]

2.7.14 Western Blotting

Western blots were performed using standard procedures. Antibodies were used at the concentrations suggested by the manufacturers. Alexa Fluor 488-Goat Anti-Rat IgG (H+L) antibody, Alexa Fluor 647-Goat Anti-Rat IgG (H+L) antibody, Streptavidin-Alexa Fluor 488 conjugate and Streptavidin-Alexa Fluor 647 conjugate were from Life Technologies. Penta-His-Alexa Fluor 488 conjugate and Penta-His-Alexa Fluor 647 conjugate were from Qiagen. Rat Monoclonal Antibody to Tubulin [YL1/2] was from Abcam.

