CHAPTER 3

Split-Intein-Mediated Split-Aminoacyl-tRNA Synthetase System for Spatiotemporally Specific Proteomic Analysis

3.1 Introduction

Cell-selective metabolic labeling of proteins with functionalizable amino acids is a promising method to precisely interrogate protein synthesis in a living animal without dissection. Because labeled proteins – and only labeled proteins – are subject to conjugation to affinity probes, proteins from targeted cells can be separated from the rest of the organism and identified by mass spectrometry. This potentially powerful technique operates on the premise that restricting expression of engineered aminoacyl-tRNA synthetases with promoters active only in specific cells restricts labeling to those cells. For example, Chin and coworkers used the germline-specific driver GAL4::VP16-nos.UTR in combination with an engineered *Methanosarcina* prroylysyl-tRNA synthetase/tRNA pair to identify proteins synthesized in germ cells of the *Drosophila* ovary [118]. Dieterich and coworkers used the motor neuron-specific driver OK371-GAL4 in combination with an engineered *Drosophila* methionyl-tRNA synthetase to monitor differences in protein synthesis rates in motor neurons in a fly model for Charcot-Marie-Tooth neuropathy [143, 144]. We have used the pharyngeal muscle-specific driver *myo-2* 5 in combination with an engineered *Caenorhabditis* phenylalanyl-tRNA synthetase to discover previously unidentified pharyngeal-muscle-specific calponin-like proteins [145]. However in a multicellular organism, many cells cannot be targeted with a single promoter. Frequently, cell- and time-specific expression arises from the combinatorial action of multiple regulators. To overcome the limitations of single-component systems for cell-selective metabolic labeling of proteins, we have developed a two-component system based on split-intein-mediated reconstitution of a split-aminoacyl-tRNA synthetase (**Figure 3.1**).

Inteins (*in*tervening pro*teins*) are protein segments that excise themselves out from a larger precursor polypeptide and join the remaining portions – exteins (*ex*ternal pro*teins*) – together with a peptide bond in a spontaneous auto-processing event known as protein splicing [146]. A special class of inteins – split inteins – are tran-

scribed and translated as two separate polypeptide fragments: an N-terminal intein fused to an N-terminal extein and a C-terminal intein fused to a C-terminal extein. These intein fragments undergo non-convalent assembly into the canonical intein structure followed by protein splicing *in trans* [147]. Inteins exhibit high splicing efficiencies; consequently, protein chemists and engineers have exploited intein chemistry in a variety of applications including tagless protein purification [148], *in vitro* and *in vivo* protein semi-synthesis [149,150], segmental isotopic labeling [151], protein and peptide cyclization [152], as well as conditional protein splicing [153]. Here in this chapter, we also exploit intein chemistry by demonstrating the reconstitution of aminoacyl-tRNA synthetase activity from individually expressed fragments with the assistance of split inteins first in *E. coli* and then in *C. elegans*. Using a combination of a heat-shock promoter (*hsp-16.2*) and a cell-specific promoter (*myo-2*, pharyngeal-muscle-specific), we show that metabolic labeling of proteins with a functionalizable amino acid can be introduced in a targeted subset of cells at specific times in the live worm.

3.2 Results and Discussion

3.2.1 Engineering Split-Intein-Reconstituted Split-Thr412Gly-CePheRS

We have previously engineered a *C. elegans* phenylalanyl-tRNA synthetase (Thr412 Gly-*Ce*PheRS) capable of appending the azide-bearing L-phenylalanine (Phe; **Figure 3.2**) analog *p*-azido-L-phenylalanine (Azf; **Figure 3.2**) to its cognate tRNA in competition with Phe and achieved spatiotemporal selectivity in the labeling of *C. elegans* proteins by controlling expression of this mutant (Thr412Gly) synthetase using cell-selective promoters [145]. Here, we explore split-intein-mediated protein splicing as a means for efficient reconstitution of the alpha subunit of *Ce*PheRS,

FARS-1, from two separate polypeptides. We first identified the cyanobacteria *Nostoc punctiforme* (*Npu*) *DnaE* intein as a promising split intein candidate as it displays superior splicing kinetics: $t_{1/2}$ of 60 s for the splicing reaction at 37°C (k = 1.1 ± 0.2 x 10^{-2} s^{-1}) [154, 155]. The catalytic subunit of DNA polymerase III DnaE is encoded by two separate genes: *dnaE-n* and *dnaE-c*. *dnaE-n* contains the N-terminal segment (N extein, DnaE(N)) of the protein followed by a 102-amino acid N-intein (Int(N, DnaE)) (**Figure 3.3**), whereas the *dnaE-c* product consists of a 36-residue C-intein (Int(C, DnaE) followed by the C-terminal segment (C extein, DnaE(C)) of the protein (**Figure 3.4**). We exchanged the extein regions with the respective halves of FARS-1 (FARS-1(N) and FARS-1(C)). For the first version of the split-intein-reconstituted split-Thr412Gly-*Ce*PheRS system, we selected the split-sites of FARS-1 between Lys187 and Gln188 in a surface-exposed linker domain bridging the N-terminal tRNA-binding domains and the C-terminal catalytic domains (FARS-1(N, Met1-Lys187)-Int(N, DnaE) and Int(C, DnaE)-Cys-Phe-Asn-FARS-1(C, Gln188-Lys496); **Figures 3.5, 3.6, and 3.7**). We added the native DnaE C-extein tripeptide cysteine-phenylalanine-asparagine between Int(C, DnaE) and FARS-1(C, Gln188-Lys496) as the presence of non-native C-extein residues immediately next to the C-intein can lead to dramatic reductions in splicing efficiency [155].

To evaluate DnaE-intein-driven reconstitution of Thr412Gly-*Ce*PheRS activity in *E. coli*, we designed seven *E. coli* strains. *E. coli* **1** and **2** represent negative and positive controls, respectively, of Azf-labeling resulting from expression of full-length FARS-1. *E. coli* **3**, **4**, and **5** test whether splitting FARS-1 yields variants that require only one fragment that associates with the beta subunit to form an active enzyme. *E. coli* **6** and **7** test Azf-labeling resulting from expression of FARS-1(N, Met1-Lys187)-Int(N, DnaE) and Int(C, DnaE)-Cys-Phe-Asn-FARS-1(C, Gln188-Lys496) and subsequent reconstitution of full-length FARS-1.

- *E. coli* **1** (Lane 1 in **Figure 3.8**) houses the two compatible plasmids pKPY93 and pKPY100: (i) pKPY93 encodes *Ce*tRNA^{Phe} under constitutive *E. coli* murein lipoprotein (*lpp*) promoter control and (ii) pKPY100 encodes both *Ce*PheRS wild-type intact alpha and beta subunits under arabinose-inducible (P_{BAD}) control.

- *E. coli* 2 (Lane 2 in **Figure 3.8**) houses pKPY93 and pKPY102: pKPY102 encodes both *Ce*PheRS mutant intact alpha and beta subunits under P_{BAD} control.

- *E. coli* 3 (Lane 3 in **Figure 3.8**) houses pKPY386 and pKPY273: (i) pKPY386 encodes *Ce*tRNA^{Phe} under *lpp* promoter control as well as FARS-1(N, Met1-Lys187)-Int(N, DnaE) under P_{BAD} control and (ii) pKPY273 encodes just the *Ce*PheRS beta subunit under P_{BAD} control.

- *E. coli* 4 (Lane 4 in **Figure 3.8**) houses pKPY93 and pKPY387: pKPY387 encodes both wild-type Int(C, DnaE)-Cys-Phe-Asn-FARS-1(C, Gln188-Lys496) and the *Ce*PheRS beta subunit under P_{BAD} control.

- *E. coli* 5 (Lane 5 in **Figure 3.8**) houses pKPY93 and pKPY388: pKPY388 encodes both mutant Int(C, DnaE)-Cys-Phe-Asn-FARS-1(C, Gln188-Lys496) and the *Ce*PheRS beta subunit under P_{BAD} control.

- *E. coli* 6 (Lane 6 in **Figure 3.8**) houses pKPY386 and pKPY387.

- *E. coli* 7 (Lane 7 in **Figure 3.8**) houses pKPY386 and pKPY388.

We cultured these strains in M9 minimal medium supplemented with 2 mM Azf and 10 mM arabinose. To assess enzymatic activity toward Azf, we detected Azf-labeled proteins by conjugation to dibenzocyclooctyne-functionalized tetramethylrhodamine (TAMRA-DBCO; **Figure 3.2**) and SDS/PAGE-in-gel fluorescence scanning

(**Figure 3.8**). As expected, wild-type intact (Lane 1) and reconstituted *Ce*PheRS (Lane 6) does not activate Azf. Notably, the expression of the synthetase-intein fragments by themselves does not yield Azf-labeling (Lanes 3, 4, and 5). Only the mutant intact (Lane 2) and reconstituted *Ce*PheRS (Lane 7) displayed robust labeling cells treated with Azf.

These results demonstrate that FARS-1 can be split into two distinct fragments, which form a functional full-length synthetase subunit when brought back together by intein-assisted splicing in *E. coli*. Encouraged by the performance of DnaE-intein-reconstituted split-Thr412Gly-*Ce*PheRS (relative labeling of 50% compared to intact synthetase), we hypothesized that a more judicious placement of the native DnaE C-extein tripeptide Cys-Phe-Asn should enable higher labeling activity. As this tripeptide remains in the product after splicing, the post-splicing variant of FARS-1 may exhibit inferior amino acid activation in relation to its wild-type counterpart. While there is no natural occurrence of Cys-Phe-Asn in FARS-1, the tripeptide Glu26-Phe27-Asn28 exists in a surface-exposed section of the N-terminal tRNA-binding domains. Reassuringly, we mutated this glutamic acid residue to cysteine and observed that bacteria expressing Glu26Cys-Thr412Gly-*Ce*PheRS are Azf-labeled to the same extent as bacteria expressing Thr412Gly-*Ce*PheRS (**Figure 3.9**). Therefore, for the second version of the split-intein-reconstituted split-Thr412Gly-*Ce*PheRS system, we selected the split-sites of FARS-1 between Asn25 and Glu26 and introduced the Glu26Cys mutation (FARS-1(N, Met1-Asn25)-Int(N, DnaE) and Int(C, DnaE)-FARS-1(C, Glu26Cys-Lys496); **Figures 3.5, 3.10, and 3.11**). We designed five new *E. coli* strains in addition to *E. coli* **1** (Lane 1 in **Figure 3.12**) and *E. coli* **2** (Lane 2 in **Figure 3.12**).

- *E. coli* **8** (Lane 3 in **Figure 3.12**) houses pKPY393 and pKPY273: (i) pKPY393 encodes *Ce*tRNA^{Phe} under *lpp* promoter control as well as FARS-1(N, Met1-Asn25)-Int(N, DnaE) under P_{BAD} control.

- *E. coli* **9** (Lane 4 in **Figure 3.12**) houses pKPY93 and pKPY394: pKPY394 encodes both wild-type Int(C, DnaE)-FARS-1(C, Glu26Cys-Lys496) and the *Ce*PheRS beta subunit under P_{BAD} control.

- *E. coli* **10** (Lane 5 in **Figure 3.12**) houses pKPY93 and pKPY395: pKPY388 encodes both mutant Int(C, DnaE)-FARS-1(C, Glu26Cys-Lys496) and the *Ce* PheRS beta subunit under P_{BAD} control.

- *E. coli* **11** (Lane 6 in **Figure 3.12**) houses pKPY393 and pKPY394.

- *E. coli* **12** (Lane 7 in **Figure 3.12**) houses pKPY393 and pKPY395.

Again, only the mutant intact (Lane 2) and reconstituted *Ce*PheRS (Lane 7) displayed robust labeling cells treated with Azf; however, repositioning the split-site from Lys187/Gln188 to Asn25/Glu26Cys did not significantly improve Azf-labeling.

Next, we asked whether replacement of the DnaE intein with an intein that possess higher splicing rates and yields would enable higher labeling activity. To our knowledge, the *Prochlorococcus* cyanomyophage P-SSM2 Gp41-1 is the most active intein reported to date: it gives rise to completed reactions within 20-30 s at 37°C ($k = 1.4 \pm 0.2\ x\ 10^{-1}\ s^{-1}$) [156]. The DNA primase/helicase Gp41-1 is encoded by two separate genes: *gp41-1-n* and *gp41-1-c*. *gp41-1-n* contains the N-terminal segment (N extein, Gp41-1(N)) of the protein followed by an 88-amino acid N-intein (Int(N, Gp41-1)) (**Figure 3.13**), whereas the *gp41-1-c* product consists of a 38-residue C-intein (Int(C, Gp41-1)) followed by the C-terminal segment (C extein, Gp41-1(C)) of the protein (**Figure 3.14**). Fortuitously, the native Gp41-1 C-extein tripeptide serine-serine-aspartic acid exists in another surface-exposed section of the N-terminal

tRNA-binding domains: Ser148-Ser149-Asp150. Therefore, for the third version of the split-intein-reconstituted split-Thr412Gly-*Ce*PheRS system, we selected the split-sites of FARS-1 between Gly147 and Ser148 (FARS-1(N, Met1-Gly147)-Int(N, Gp41-1) and Int(C, Gp41-1)-FARS-1(C, Ser148-Lys496); **Figures 3.5, 3.15, and 3.16**). Again, we designed five new *E. coli* strains in addition to *E. coli* **1** (Lane 1 in **Figure 3.17**) and *E. coli* **2** (Lane 2 in **Figure 3.17**).

- *E. coli* **13** (Lane 3 in **Figure 3.17**) houses pKPY458 and pKPY273: (i) pKPY458 encodes *Ce*tRNA^{Phe} under *lpp* promoter control as well as FARS-1(N, Met1-Gly147)-Int(N, Gp41-1) under P_{BAD} control.

- *E. coli* **14** (Lane 4 in **Figure 3.17**) houses pKPY93 and pKPY459: pKPY459 encodes both wild-type Int(C, Gp41-1)-FARS-1(C, Ser148-Lys496) and the *Ce* PheRS beta subunit under P_{BAD} control.

- *E. coli* **15** (Lane 5 in **Figure 3.17**) houses pKPY93 and pKPY460: pKPY460 encodes both mutant Int(C, Gp41-1)-FARS-1(C, Ser148-Lys496) and the *Ce*Phe RS beta subunit under P_{BAD} control.

- *E. coli* **16** (Lane 6 in **Figure 3.17**) houses pKPY458 and pKPY459.

- *E. coli* **17** (Lane 7 in **Figure 3.17**) houses pKPY458 and pKPY460.

As in the cases of first and second version of the split-intein-reconstituted split-Thr412Gly-*Ce*PheRS system, only the mutant intact (Lane 2) and reconstituted *Ce*PheRS (Lane 7) displayed robust labeling cells treated with Azf. Encouragingly, replacing the DnaE intein with the Gp41-1 intein and repositioning the split-site from Asn25/Glu26Cys to Gly147/Ser148 significantly enhanced Azf-labeling (relative labeling of 80% compared to intact synthetase). From these observations, we conclude that the third version of the split-intein-reconstituted split-Thr412Gly-*Ce*PheRS system is the best candidate for precise spatiotemporal-selective Azf-labeling in a mul-

ticellular organism like *C. elegans*. For simplification, we refer to FARS-1(N, Met1-Gly147)-Int(N, Gp41-1) as FARS-1(N)-Int(N) and mutant Int(C, Gp41-1)-FARS-1(C, Ser148-Lys496) as Int(C)-FARS-1(C) for the remainder of this chapter.

3.2.2 Characterizing Split-Intein-Reconstituted Split-Thr412Gly-CePheRS in C. elegans

To evaluate the performance of the split-intein-reconstituted split-Thr412Gly-*Ce*Phe RS system in *C. elegans*, we generated transgenic *C. elegans* lines that (i) express both FARS-1(N)-Int(N) and GFP under control of the *hsp-16.2* promoter, a promoter shown previously to be active in multiple tissues in response to heat shock and other stresses [122], and (ii) express both Int(C)-FARS-1(C) and mCherry under control of the *myo-2* promoter, a promoter shown previously to be active in the 20 pharyngeal muscle cells [126] (**Figure 3.18**). We envision exploiting the dual component nature of the split-intein-reconstituted split-synthetase to metabolically label proteins in specific cells at specific times. Upon heat shock and only in pharyngeal muscle, we expect that both FARS-1(N)-Int(N) and Int(C)-FARS-1(C) would be expressed, resulting in the recovery of the full-length mutant alpha subunit after intein fragment association and protein splicing *in trans*. Fully active Thr412Gly-*Ce*PheRS is produced after the mutant alpha subunit forms a hybrid heterotetramer with the endogenous beta subunit.

Specifically, we designed four gene cassettes, each consisting of a promoter, a protein-coding segment, and a terminator: (i) *hsp-16.2* 5'::FARS-1(N)-Int(N)::*fib-1/rps-16*::*gfp*::*unc-54* 3', (ii) *myo-2* 5'::Int(C)-FARS-1(C)::*rpl-16/M01F1.8*::*mCherry*::*let-858* 3', (iii) *rps-27* 5'::*neoR*::*unc-54* 3', and (iv) *hsp-16.41* 5'::*peel-1*::*tbb-2* 3' (**Figure 3.19**). The *fib-1/rps-16* and *rpl-16/M01F1.8* intercistronic regions enable the control of FARS-1(N)-Int(N)/GFP and Int(C)-FARS-1(C)/mCherry, respectively, under the same promoter by instructing the genes encoding FARS-1(N)-Int(N)/GFP

and Int(C)-FARS-1(C)/mCherry to be first transcribed as a single polycistronic precursor mRNA and then trans-spliced into monocistronic mature mRNAs [157]. GFP and mCherry act as fluorescent reporters for the promoter activity of the two split-intein split-synthetase cassettes, confirming that the transgene is present and expressing in the expected cells. The *unc-54*, *let-858*, and *tbb-2* 3' regions act as putative transcriptional terminators that prevent misexpression of any downstream sequences. The positive-selection marker cassette *rps-27* 5'::*neoR*::*unc-54* 3' confers resistance to G-418 (Geneticin), a drug that inhibits protein synthesis in prokaryotes and eukaryotes, and therefore allows hands-off maintenance and enrichment of transgenic worms on G-418-containing plates [158]. The negative-selection marker cassette *hsp-16.41* 5'::*peel-1*::*tbb-2* 3' encodes the potent PEEL-1 toxin [159] under control of the *hsp-16.41* promoter, another promoter shown previously to be active in response to heat shock [122]. We assembled the four cassettes in a single plasmid pKPY728 and flanked three of the four cassettes (*hsp-16.2* 5'::FARS-1(N)-Int(N)::*fib-1/rps-16*::*gfp*::*unc-54* 3', *myo-2* 5'::Int(C)-FARS-1(C)::*rpl-16/M01F1.8*::*mCherry*::*let-858* 3', and *rps-27* 5'::*neoR*::*unc-54* 3' but not *hsp-16.41* 5'::*peel-1*::*tbb-2* 3') with modified *Drosophila Mos1* transposon elements (*miniMos*). Jorgensen and coworkers have demonstrated that the *miniMos* transposon can carry large fragments of DNA, even 45-kb fosmids, into the *C. elegans* genome with high insertion frequency and fidelity [160].

To make stable transgenic *C. elegans* lines, we first microinjected young adult *C. elegans* (N2) with a mixture of pKPY728, fluorescent extrachromosomal array marker plasmids pCFJ90 (*myo-2* 5'::*mCherry*::*unc-54* 3', expression of mCherry in pharyngeal muscle), pCFJ104 (*myo-3* 5'::*mCherry*::*unc-54* 3', expression of mCherry in body wall muscle), and pGH8 (*rab-3* 5'::*mCherry*::*unc-54* 3', expression of mCherry in neurons), and a helper plasmid encoding the *Mos1* transposase pCFJ601 (*eft-3* 5'::*Mos*1 Transposase::*tbb-2* 3', expression of *Mos1* transposase in germline) (**Figure 3.20, Top**). Plasmid DNA injected into the syncytial germ line at the distal gonad

(**Figure 3.21**) concatenates to form semistable and multicopy extrachromosomal DNA arrays [161]. Following microinjection, we obtained several independent extrachromosomal G-418-resistant and mCherry-positive (pharyngeal muscle, body wall muscle, and neurons) lines (**Figure 3.22**). Next, we killed array animals by inducing PEEL-1 expression by 1-h exposure to 37°C to distinguish worms with transposase-mediated genomic insertions that have lost the extrachromosomal array from worms still carrying the array (**Figure 3.20, Bottom**). As a result, we identified two independent G-418-resistant and mCherry-positive (pharyngeal muscle) lines with recombinant *miniMos* insertions: PS7055 (*syTi1*) and PS7058 (*syTi2*) (**Figure 3.23**). We mapped the *syTi1* and *syTi2* insertions by inverse PCR [162] to unique insertion sites in the right arm of the X chromosome (**Figure 3.24**) and in the left arm of chromosome II (**Figure 3.26**), respectively. We also verified the insertion fidelity and genotype with chromosome-specific PCRs spanning across the insertion site, genome-insertion junction-specific PCRs, three-primer PCRs and genomic DNA sequencing (**Figures 3.25 and 3.27**).

Next, we investigated whether Azf-labeling of proteins occurs in a temporally resolved (upon heat shock) and a spatially resolved (in pharyngeal muscle) manner in these *C. elegans* lines. We first labeled bacteria by culturing KY33[pKPY514], a phenylalanine-auxotrophic and G-418-resistant strain of *E. coli* that expresses the *E. coli* variant of Thr412Gly-*Ce*PheRS, in M9 minimal medium supplemented with Azf. We next fed Azf-labeled bacteria to PS7055 and PS7058 worms previously maintained on OP50-1[pKPY562], a G-418-resistant strain of *E. coli*, and compared protein labeling after exposure to non-heat-shock and heat-shock conditions (**Figure 3.28**). For both conditions, we transferred worms to solid nematode growth medium without peptone (NGM-peptone) plates containing Azf-labeled bacteria, incubated plates at 24°C for 24 h, and cleared external as well as ingested bacteria by washing worms with S medium over a period of 30 min. For heat-shock conditions, we introduced a

30-min exposure to 37°C immediately after transferring worms to solid NGM-peptone plates. As expected, fluorescence microscopy of live animals revealed that only heat-shocked worms expressed GFP ubiquitously. Following fixation, worms were subjected to strain-promoted conjugation to TAMRA-DBCO to visualize sites of Azf incorporation. Fluorescence microscopy showed that only heat-shocked worms exhibited pharyngeal-muscle-specific Azf-labeling. Importantly, neither non-heat-shocked worms nor the tissues surrounding the pharyngeal muscles in heat-shocked worms exhibited Azf-labeling. These results provided a proof of principle that coupling the combinatorial expression of two promoters with the split-intein-reconstituted split-Thr412Gly-*Ce*PheRS allows precise spatiotemporally selectivity of Azf-labeling of proteins even in living multicellular animals.

3.3 Conclusions

We have developed a previously undescribed way of generating a two-component system involving reconstituted aminoacyl-tRNA synthetase for selective and/or conditional metabolic labeling of targeted cells: individually expressed aminoacyl-tRNA synthetase fragments are brought together by split inteins to produce the active aminoacyl-tRNA synthetase. While this strategy works for the *C. elegans* PheRS, this mode of reconstitution should apply to any aminoacyl-tRNA synthetase. In addition, the finding that split-intein-mediated reconstituted split-Thr412Gly-*Ce*PheRS is active in both *E. coli* and *C. elegans* indicates that this dual-component system will be effective in a broad range of organisms.

3.4 Figures

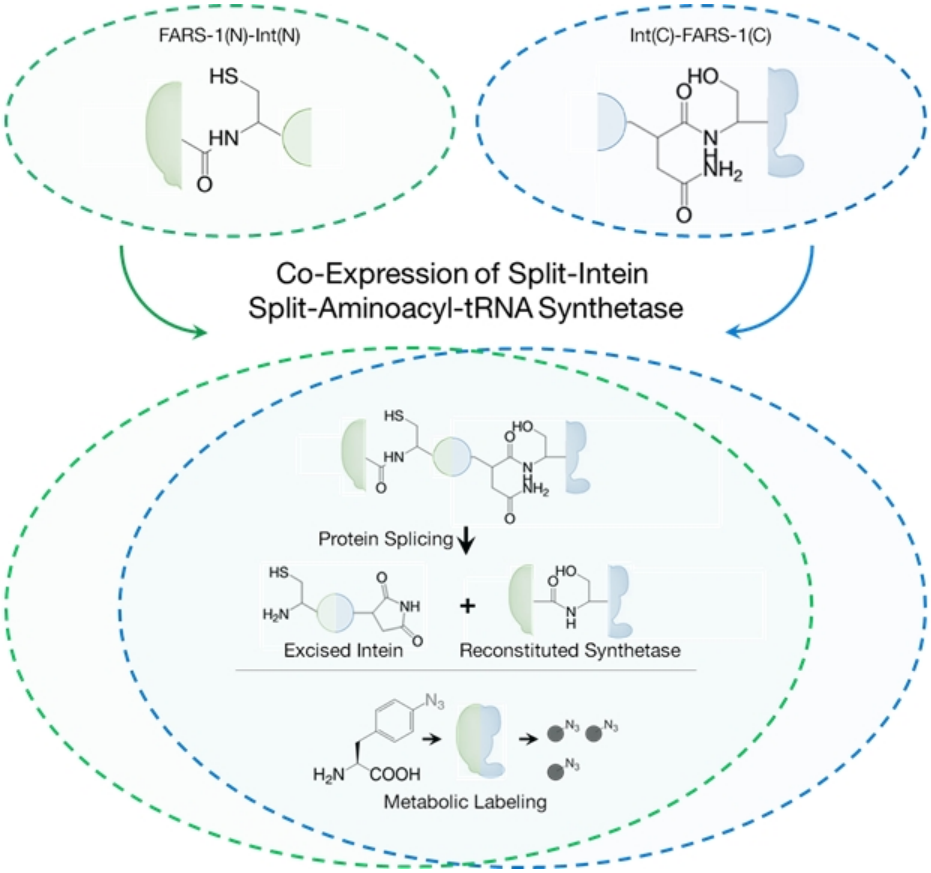

Figure 3.1: *Coupling the combinatorial expression of two promoters with the split-intein-reconstituted split-Thr412Gly-CePheRS allows precise spatiotemporally selectivity of Azf-labeling of proteins.*

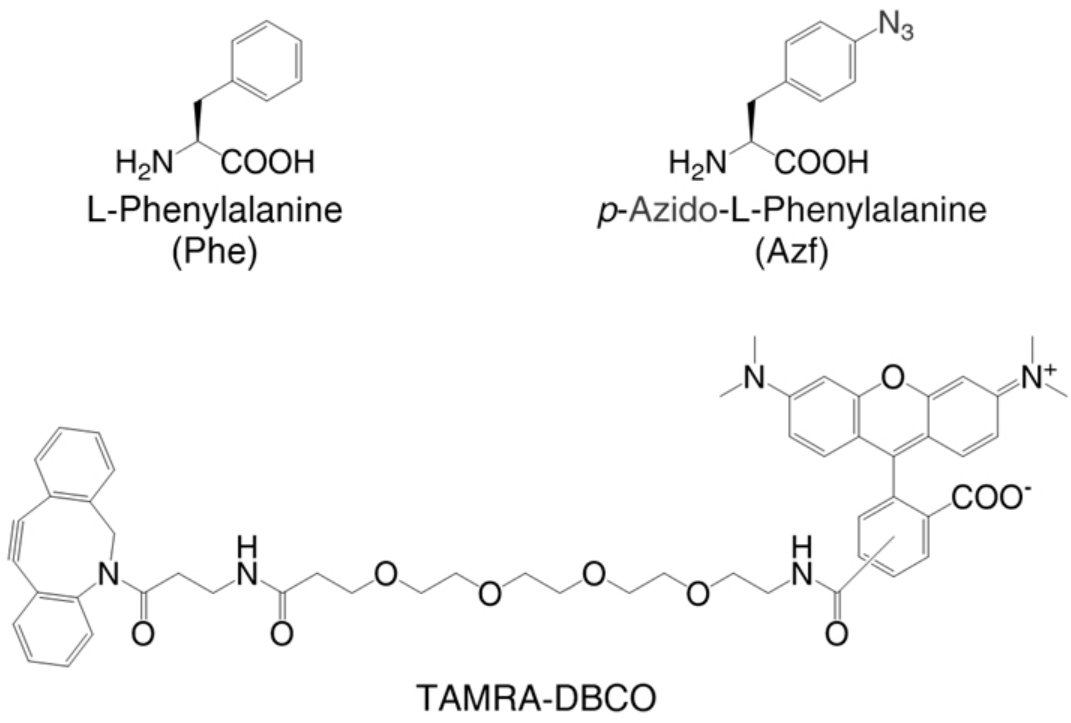

Figure 3.2: *Structures of amino acids and probes used in this study:* L*-phenylalanine (Phe), p-azido-*L*-phenylalanine (Azf), and dibenzocyclooctyne tetramethylrhodamine (TAMRA-DBCO).*

DnaE(N)-Int(N, DnaE);

MSFVPLHIHSDYSLLDGASQLPELVDQAIALGMKAIALT
DHGVMYGAVELIKICRSQNIKPIIGNEMYVINGDIEKQE
RRPKYHQVVLAKNTKGYKNLVKLTTISHLQGVQGKGIFS
RPCINKDLLKQYHEGLIVTSACLGGEVPQAILSNRPDAA
RKVAQWYKDVFGDDYYLEIQDHGSQEDRIVNVEIVKIAR
ELGIKIIATNDSHFISCFDVEAHDALLCIQTGKLIIEDK
RMRYSGTEYLKSGEEMRQLFRDHLPDDVISEAVATTEEV
ADKVEPYHIMGEPQIPTPPIPSGHTADTYAEDVAWNGLL
ERLNRKSRQEVDSVYKERLEYELKMIQQMGFSKYFLVVW
DYIKFARDNNIPVGPGRGSAAGSLVAYAMRITNIDPVHH
GLLFERFLNPERKSMPDIDTDFCIEQRDKVIEYVTEKYG
ADRVAQIITFNRLTSKAVLKDVARVLNIPYGEADKMAKL
IPVVRGKPTKLKVMVSDKTPEPEFKEKYDKEPHVRHWLD
MAMRIEGTNKTFGVHAAGVVISDEPLDEIVPLQKNNDGS
VITQYFMEDLESMGLLKMDFLGLRNLTLIQKTVDLIQET
RGYRVDPDEIPRQERKAQKILAKGEHSSLPKDVQKTYEL
LEAGELEGIFQLESSGMRQIVRDLKPSNIEDISSILALY
RPGPLDAGLIPKFINRKHGRENIDYQHTVLEPILDETYG
IMVYQEQIMKIAQDMAGYSLGQADLLRRAMGKKKVSEMQ
KQREKFVDGAAKNGVPKKVADELFEQMLKFAEYCLSYET
EILTVEYGLLPIGKIVEKRIECTVYSVDNNGNIYTQPVA
QWHDRGEQEVFEYCLEDGSLIRATKDHKFMTVDGQMLPI
DEIFERELDLMRVDNLPN;

Figure 3.3: *dnaE-n contains the N-terminal segment (DnaE(N)) of the DnaE protein followed by a 102-amino acid N-intein (Int(N, DnaE).*

Int(C, DnaE)-DnaE(C);

MIKIATRKYLGKQNVYDIGVERDHNFALKNGFIASNCFN
KSHSTAYGYVTYQTAYLKANYPLEYMAALLTANSGDTDK
VQRYITNCTNMGISIDPPDINRSGVDFTPTLGKILFGFS
AVRNVGQNAIACILEARNETGEFKSLADFCDRVDLRAVN
RRTLESLIYCGAFDKIESNRQQLINDSELVYDWAQSRAK
DRASGQGNLFDLLGDGFSSTQNKRVNNAFETAPKSKPVT
DLPPQKKLQMEKELLGFYVSDHPLKSLRQIAPLLTPINL
SQLGEQREDTRLCAVVMLNNVKKVVTKKGDQMAILQIED
LTTQSEAVVFPKTYERISSLLQVDTRLIIWGKVDRRDEQ
TQFIVEDAEPVETVQMVMVELNPQQAGDMEKLHLLKTIL
QEHSVDKEKAKMPVIGIIQTEKSRKLVRLGWQFSVQDSR
ITVQALQNASFPAHIKSLTGS;

Figure 3.4: *dnaE-c contains a 36-residue C-intein (Int(C, DnaE) followed by the C-terminal segment (C extein, DnaE(C)) of the DnaE protein.*

FARS-1 (Thr412Gly);

MTAETDVRSTENLPQQILDFLQESNEFNSIQLAQQWNLD
HQKVIGAIKSLLANEGVLTTKDVTEKRLELTNEGVQFAN
EGSPEYLVFEFVGTDGAAQADIQKKPFGKIGMAKAMQFK
WVSVDKGRVVRQATEVTDSTRKQLESLRIGSSDVSENEK
KELKKRKLISEVNIKALVVSKGTSFTTSLAKQEADLTPE
MIASGSWKDMQFKKYNFDSLGVVPSSGHLHPLMKVRSEF
RQIFFSMGFSEMATNRYVESSFWNFDALFQPQQHPARDA
HDTFFVSDPAISTKFPEDYLERVKTVHSKGGYGSAGYNY
DWKIEEAQKNVLRTHTTAVSARQLYQLAQEGFRPSKLFS
IDRVFRNETLDATHLAEFHQVEGVIAEKNLSLAHLIGIF
TEFFKKLGITNLRFKPTYNPYGEPSMEIFAYHQGLTKWV
EIGNSGMFRPEMLLPMGLPADVNVAGYGLSLERPTMIKY
GINNIRDLFGSKIDLNVVYNNPICRLDK;

Figure 3.5: *C. elegans FARS-1 Sequence.*

FARS-1(N, Met1-Lys187)-Int(N, DnaE);

MTAETDVRSTENLPQQILDFLQESNEFNSIQLAQQWNLD
HQKVIGAIKSLLANEGVLTTKDVTEKRLELTNEGVQFAN
EGSPEYLVFEFVGTDGAAQADIQKKPFGKIGMAKAMQFK
WVSVDKGRVVRQATEVTDSTRKQLESLRIGSSDVSENEK
KELKKRKLISEVNIKALVVSKGTSFTTSLAKCLSYETEI
LTVEYGLLPIGKIVEKRIECTVYSVDNNGNIYTQPVAQW
HDRGEQEVFEYCLEDGSLIRATKDHKFMTVDGQMLPIDE
IFERELDLMRVDNLPN;

Figure 3.6: *FARS-1(N, Met1-Lys187)-Int(N, DnaE) Sequence.*

Int(C, DnaE)-*Cys-Phe-Asn*-FARS-1(C,
Gln188-Lys496, Thr412Gly);

MIKIATRKYLGKQNVYDIGVERDHNFALKNGFIASN*CFN*
QEADLTPEMIASGSWKDMQFKKYNFDSLGVVPSSGHLHP
LMKVRSEFRQIFFSMGFSEMATNRYVESSFWNFDALFQP
QQHPARDAHDTFFVSDPAISTKFPEDYLERVKTVHSKGG
YGSAGYNYDWKIEEAQKNVLRTHTTAVSARQLYQLAQEG
FRPSKLFSIDRVFRNETLDATHLAEFHQVEGVIAEKNLS
LAHLIGIFTEFFKKLGITNLRFKPTYNPYGEPSMEIFAY
HQGLTKWVEIGNSGMFRPEMLLPMGLPADVNVAGYGLSL
ERPTMIKYGINNIRDLFGSKIDLNVVYNNPICRLDK;

Figure 3.7: *Int(C, DnaE)-Cys-Phe-Asn-FARS-1(C, Gln188-Lys496) Sequence.*

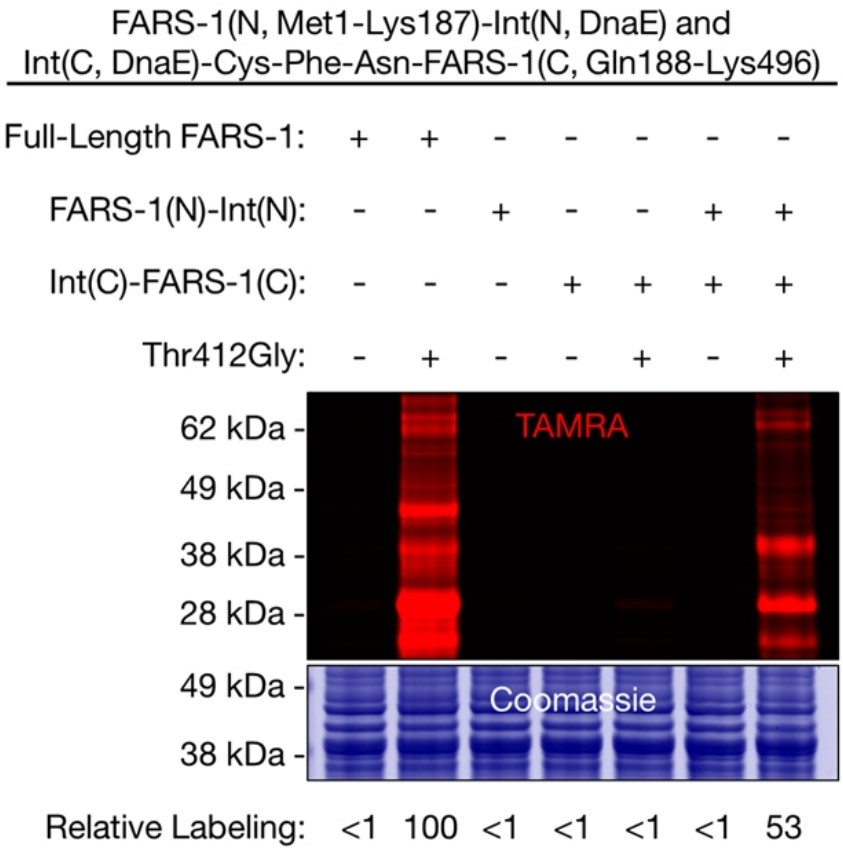

Figure 3.8: *SDS-PAGE and in-gel fluorescence scanning detection of strain-promoted conjugation of TAMRA-DBCO to Azf-labeled protein from E. coli* ***1***, ***2***, ***3***, ***4***, ***5***, ***6***, *and* ***7*** *lysates.*

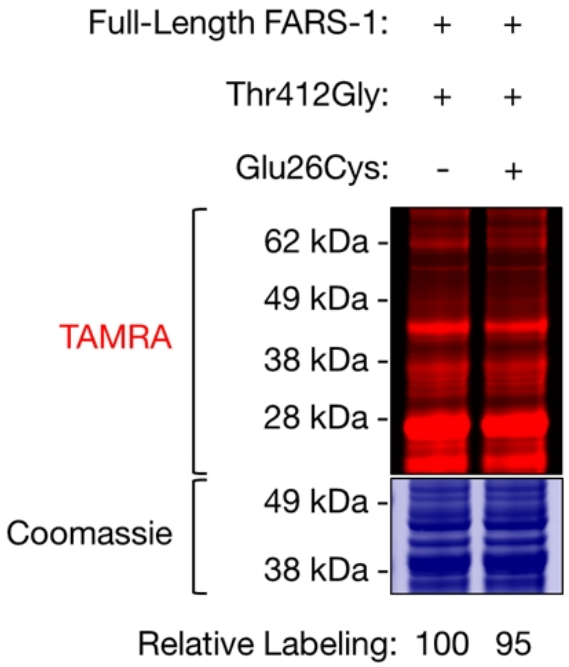

Figure 3.9: *SDS-PAGE and in-gel fluorescence scanning detection of strain-promoted conjugation of TAMRA-DBCO to Azf-labeled protein from E. coli expressing either Thr412Gly-CePheRS or Glu26Cys-Thr412Gly-CePheRS.*

FARS-1(N, Met1-Asn25)-Int(N, DnaE);

MTAETDVRSTENLPQQILDFLQESNCLSYETEILTVEYG
LLPIGKIVEKRIECTVYSVDNNGNIYTQPVAQWHDRGEQ
EVFEYCLEDGSLIRATKDHKFMTVDGQMLPIDEIFEREL
DLMRVDNLPN;

Figure 3.10: *FARS-1(N, Met1-Asn25)-Int(N, DnaE) Sequence.*

Int(C, DnaE)-FARS-1(C, Glu26Cys-Lys496, Thr412Gly);

MIKIATRKYLGKQNVYDIGVERDHNFALKNGFIASNCFN
SIQLAQQWNLDHQKVIGAIKSLLANEGVLTTKDVTEKRL
ELTNEGVQFANEGSPEYLVFEFVGTDGAAQADIQKKPFG
KIGMAKAMQFKWVSVDKGRVVRQATEVTDSTRKQLESLR
IGSSDVSENEKKELKKRKLISEVNIKALVVSKGTSFTTS
LAKQEADLTPEMIASGSWKDMQFKKYNFDSLGVVPSSGH
LHPLMKVRSEFRQIFFSMGFSEMATNRYVESSFWNFDAL
FQPQQHPARDAHDTFFVSDPAISTKFPEDYLERVKTVHS
KGGYGSAGYNYDWKIEEAQKNVLRTHTTAVSARQLYQLA
QEGFRPSKLFSIDRVFRNETLDATHLAEFHQVEGVIAEK
NLSLAHLIGIFTEFFKKLGITNLRFKPTYNPYGEPSMEI
FAYHQGLTKWVEIGNSGMFRPEMLLPMGLPADVNVAGYG
LSLERPTMIKYGINNIRDLFGSKIDLNVVYNNPICRLDK
;

Figure 3.11: *Int(C, DnaE)-FARS-1(C, Glu26Cys-Lys496) Sequence.*

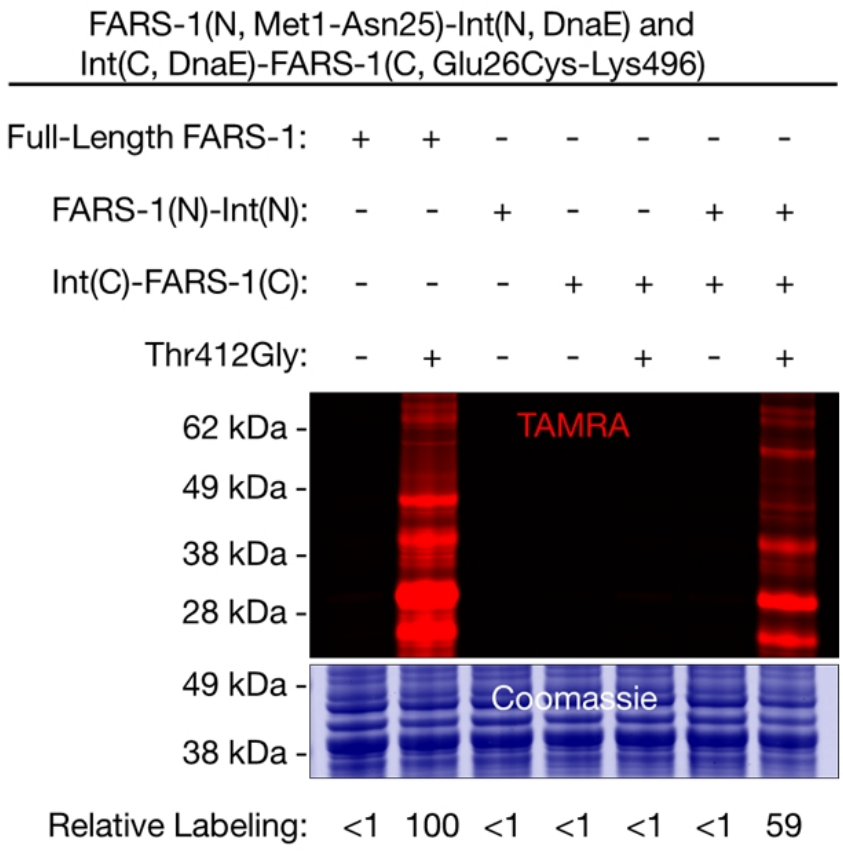

Figure 3.12: *SDS-PAGE and in-gel fluorescence scanning detection of strain-promoted conjugation of TAMRA-DBCO to Azf-labeled protein from E. coli* ***1***, ***2***, ***8***, ***9***, ***10***, ***11***, *and* ***12*** *lysates.*

Gp41-1(N)-Int(N, Gp41-1);

MERIETTILRNLVFNEDFSRKVIPFIEPDYFEERKEKII
FEEVTKFIVKYGSAITVEALNIEIENRTDLNESEIKETR
DISNTLHDSAVEPQWLLDTTEKWCRDRAIYLALMESIHI
ADGEDEQKNRDAIPSILSDALAVSFDSHIGHDYLNDYEE
RYESYHRKEDKIPFDLEYFDKITKGGLPNKTLNIALAGT
GVGKSLFMCHMASSVLLQGKNVLYITLEMAEEKIAERID
ANLLNVNIQNITDLPKPMFENKVSSLTKKTQGSLIIKEY
PTASAHSGHFKSLLQELALKKSFRPDIIFIDYLNICASS
RYRQNASVNSYSFIKAIAEELRGLAVEANLPIVSATQTT
RSGFACLDLKTQVQTPQGMKEISNIQVGDLVLSNTGYNE
VLNVFPKSKKKSYKITLEDGKEIICSEEHLFPTQTGEMN
ISGGLKEGMCLYVKE;

Figure 3.13: *gp41-1-n contains the N-terminal segment (N extein, Gp41-1(N)) of the Gp41-1 protein followed by an 88-amino acid N-intein (Int(N, Gp41-1)).*

Int(C, Gp41-1)-Gp41-1(C);

MMLKKILKIEELDERELIDIEVSGNHLFYANDILTHNSS
SDVDLTDTSESFGLPATADLMFALISTEELEGLNQIMVK
QLKNRYNDPTIFKRFIVGIDRAKMRLYDVEQKAQEDILD
SGKEEEYDPHEEKKPKKSFAGFKFN;

Figure 3.14: *gp41-1-c contains a 38-residue C-intein (Int(C, Gp41-1)) followed by the C-terminal segment (C extein, Gp41-1(C)) of the Gp41-1 protein.*

FARS-1(N, Met1-Gly147)-Int(N, Gp41-1);

MTAETDVRSTENLPQQILDFLQESNEFNSIQLAQQWNLD
HQKVIGAIKSLLANEGVLTTKDVTEKRLELTNEGVQFAN
EGSPEYLVFEFVGTDGAAQADIQKKPFGKIGMAKAMQFK
WVSVDKGRVVRQATEVTDSTRKQLESLRIGCLSYETEIL
TVEYGLLPIGKIVEKRIECTVYSVDNNGNIYTQPVAQWH
DRGEQEVFEYCLEDGSLIRATKDHKFMTVDGQMLPIDEI
FERELDLMRVDNLPN;

Figure 3.15: *FARS-1(N, Met1-Gly147)-Int(N, Gp41-1) Sequence.*

Int(C, Gp41-1)-FARS-1(C, Ser148-Lys496, Thr412Gly);

MMLKKILKIEELDERELIDIEVSGNHLFYANDILTHNSS
SDVSENEKKELKKRKLISEVNIKALVVSKGTSFTTSLAK
QEADLTPEMIASGSWKDMQFKKYNFDSLGVVPSSGHLHP
LMKVRSEFRQIFFSMGFSEMATNRYVESSFWNFDALFQP
QQHPARDAHDTFFVSDPAISTKFPEDYLERVKTVHSKGG
YGSAGYNYDWKIEEAQKNVLRTHTTAVSARQLYQLAQEG
FRPSKLFSIDRVFRNETLDATHLAEFHQVEGVIAEKNLS
LAHLIGIFTEFFKKLGITNLRFKPTYNPYGEPSMEIFAY
HQGLTKWVEIGNSGMFRPEMLLPMGLPADVNVAGYGLSL
ERPTMIKYGINNIRDLFGSKIDLNVVYNNPICRLDK;

Figure 3.16: *Int(C, Gp41-1)-FARS-1(C, Ser148-Lys496) Sequence.*

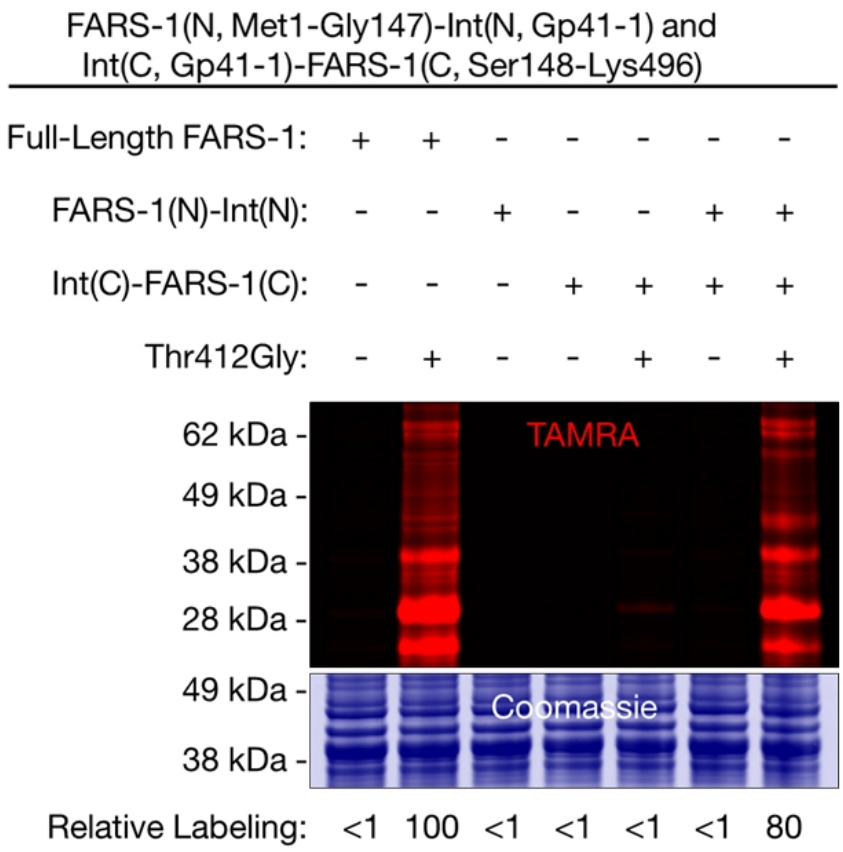

Figure 3.17: *SDS-PAGE and in-gel fluorescence scanning detection of strain-promoted conjugation of TAMRA-DBCO to Azf-labeled protein from E. coli* ***1***, ***2***, ***13***, ***14***, ***15***, ***16***, *and* ***17*** *lysates.*

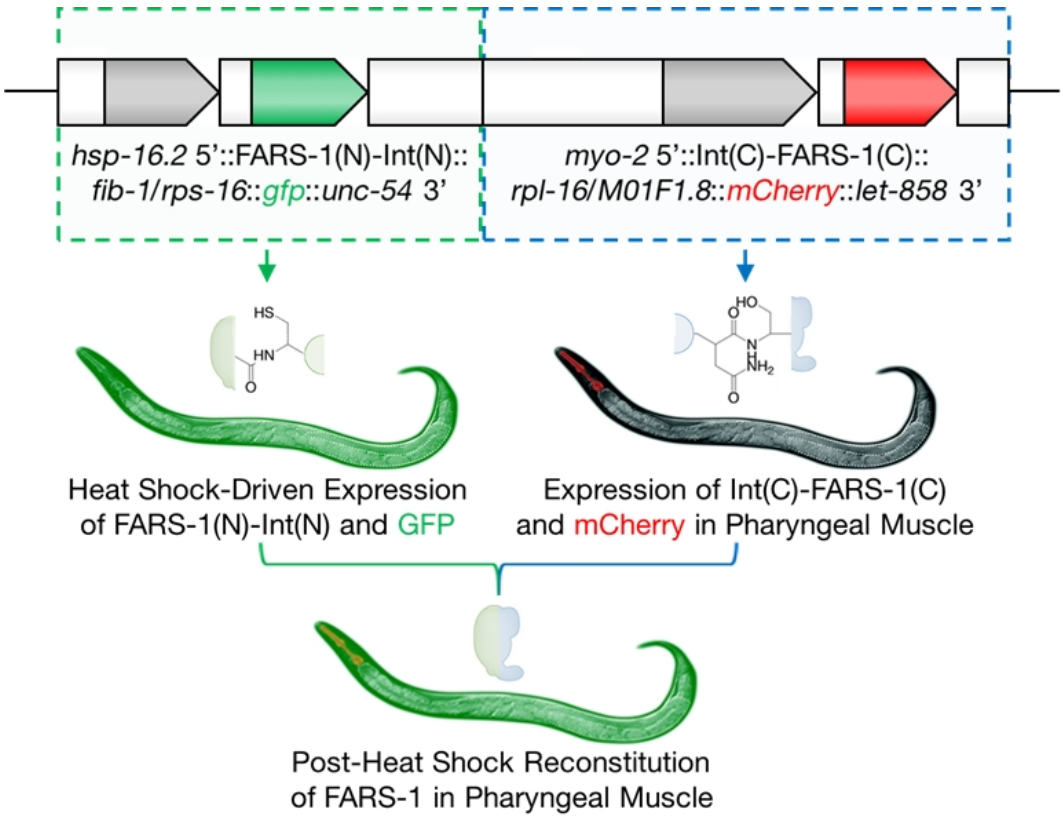

Figure 3.18: *Upon heat shock and only in pharyngeal muscle, we expect that both FARS-1(N)-Int(N) and Int(C)-FARS-1(C) would be expressed, resulting in the recovery of the full-length mutant alpha subunit after intein fragment association and protein splicing in trans.*

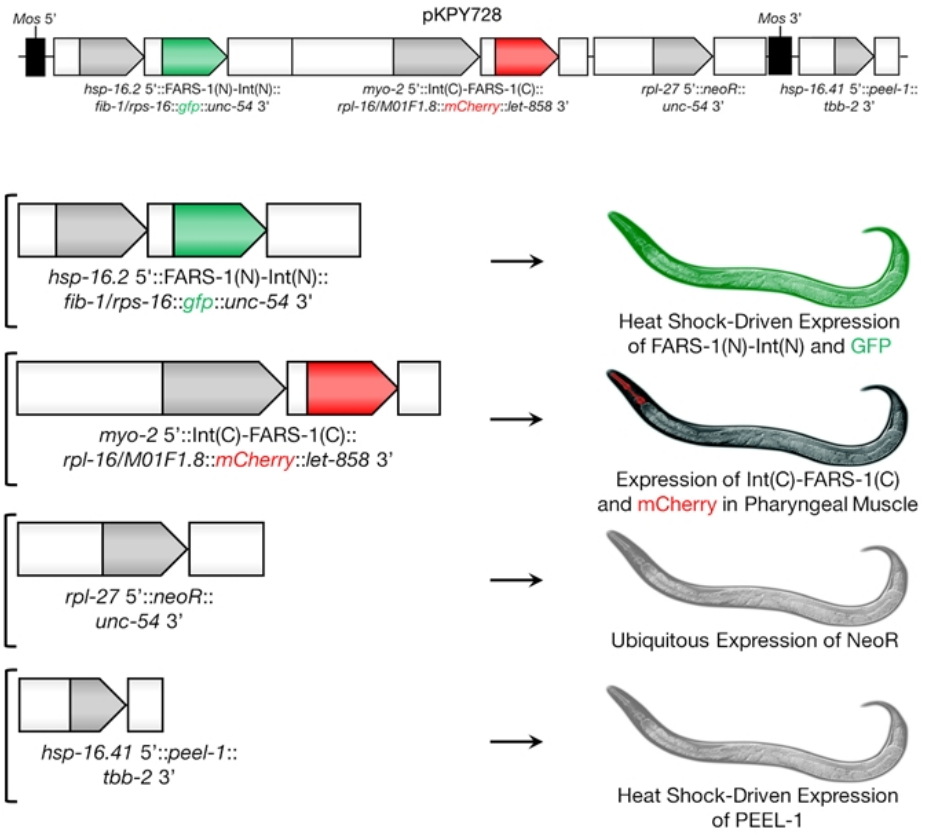

Figure 3.19: *pKPY728 contains four gene cassettes, each consisting of a promoter, a protein-coding segment, and a terminator: (i) hsp-16.2 5'::FARS-1(N)-Int(N)::fib-1/rps-16::gfp::unc-54 3', (ii) myo-2 5'::Int(C)-FARS-1(C)::rpl-16/M01F1.8::mCher ry::let-858 3', (iii) rps-27 5'::neoR::unc-54 3', and (iv) hsp-16.41 5'::peel-1::tbb-2 3'.*

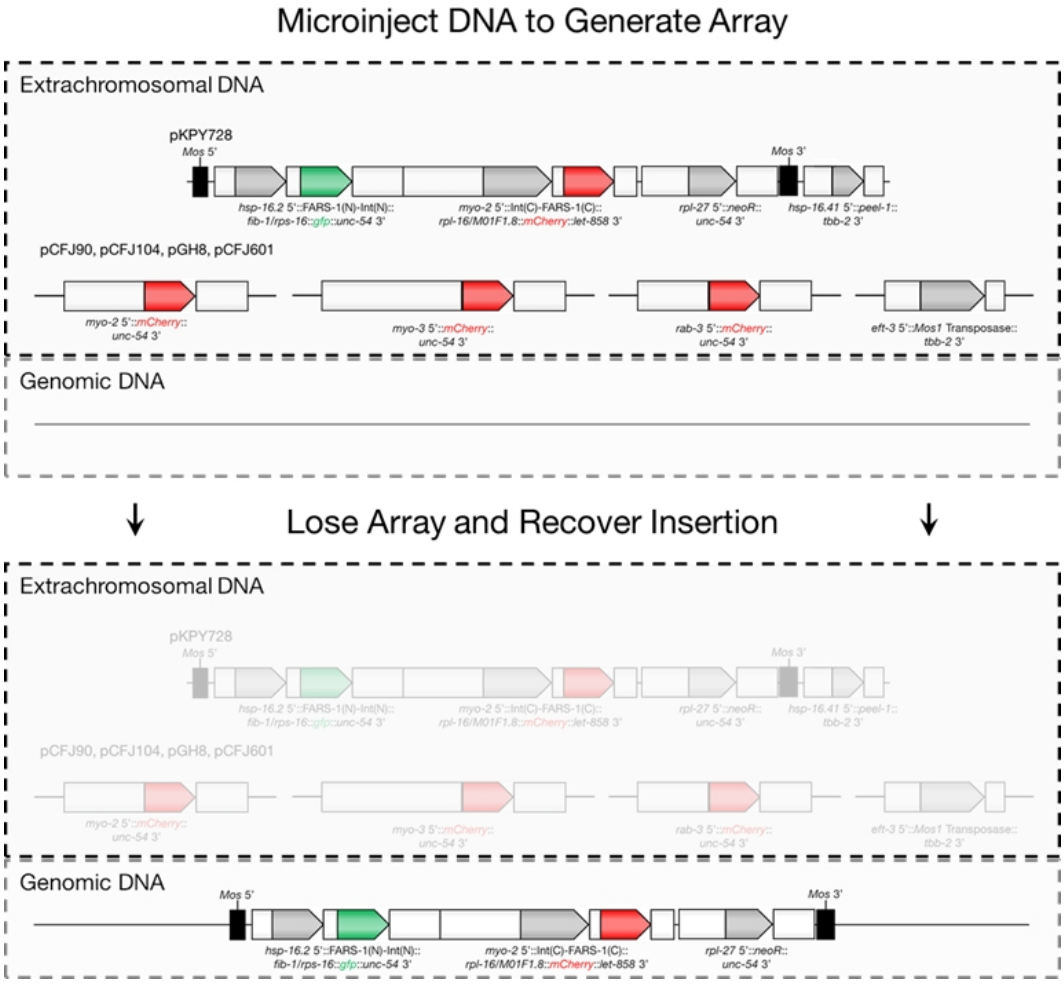

Figure 3.20: *We microinjected worms with a mixture of pKPY728, pCFJ90, pCFJ104, pGH8, and pCFJ601 to generate worms with these plasmids concatenated to semistable and multicopy extrachromosomal DNA arrays. Array animals are killed by inducing PEEL-1 expression by heat shock to distinguish worms with transposase-mediated genomic insertions that have lost the extrachromosomal array from worms still carrying the array.*

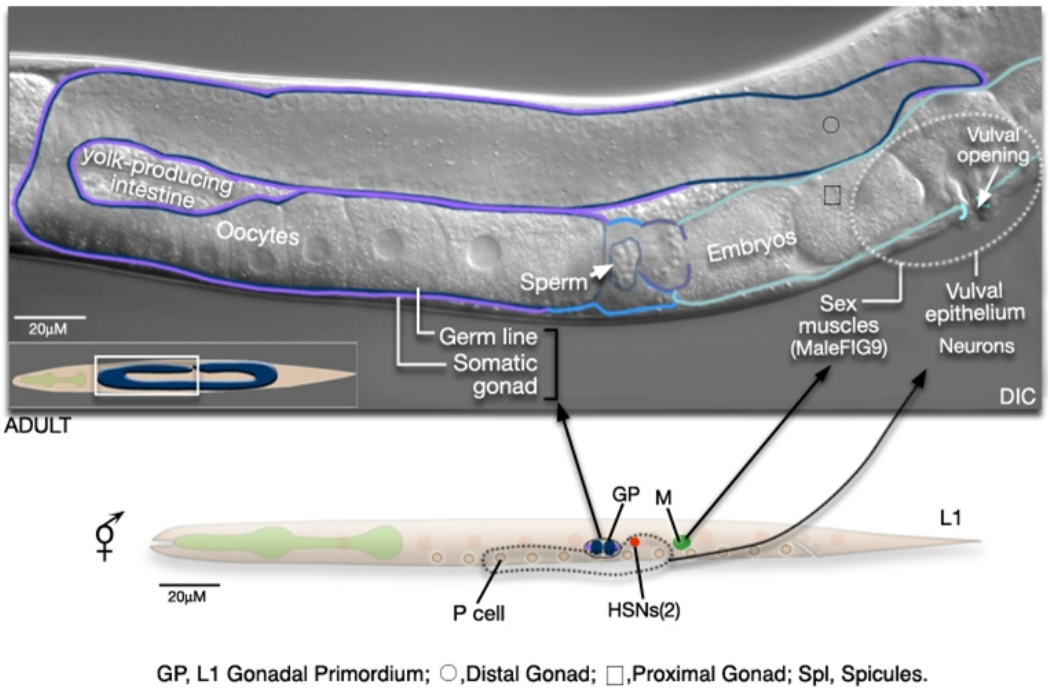

Figure 3.21: *C. elegans Hermaphrodite Gonad.*

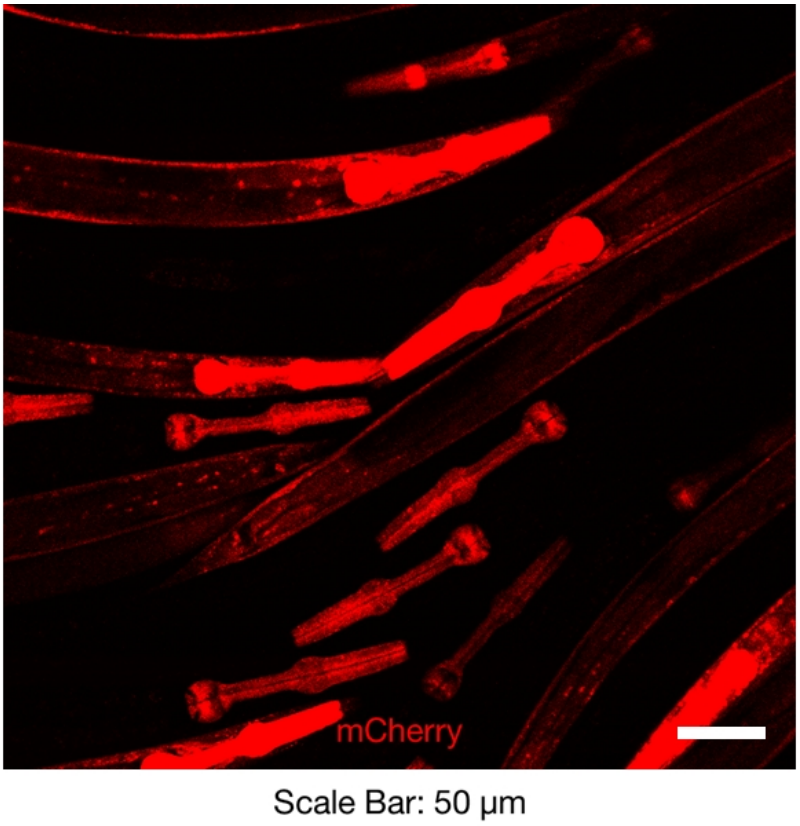

Figure 3.22: *Fluorescence microscopy of an extrachromosomal G-418-resistant and mCherry-positive (pharyngeal muscle, body wall muscle, and neurons; red) line.*

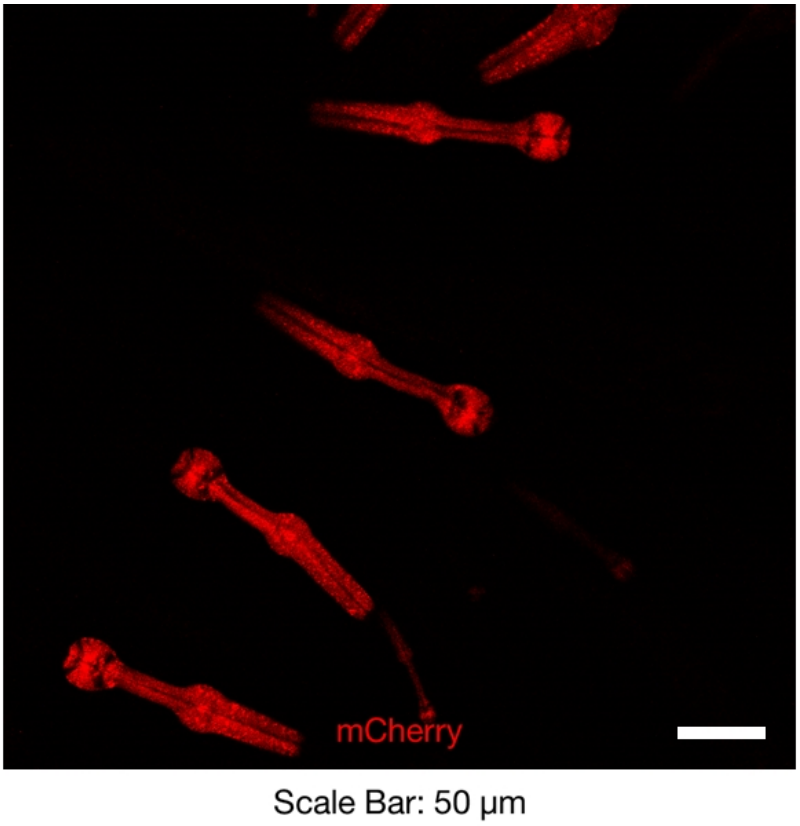

Figure 3.23: *Fluorescence microscopy of the G-418-resistant and mCherry-positive (pharyngeal muscle; red) line PS7055 containing the recombinant miniMos insertions syTi1.*

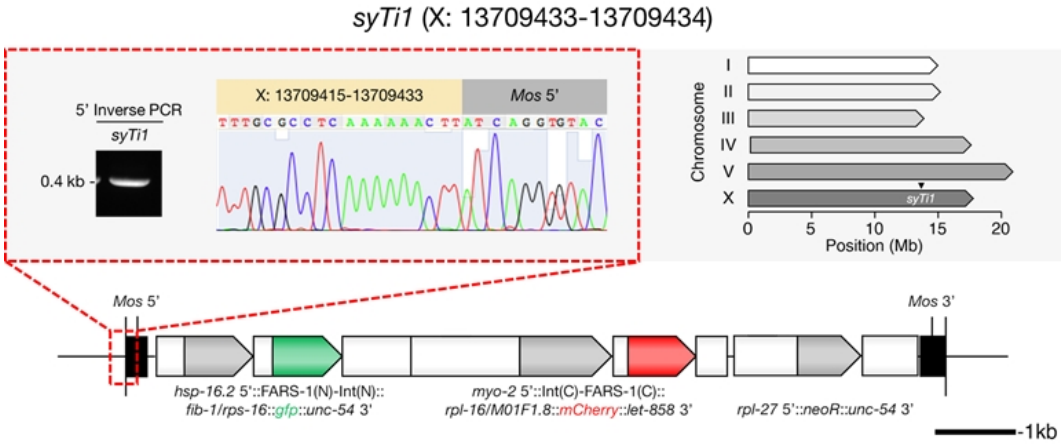

Figure 3.24: *We mapped the syTi1 insertion by inverse PCR to a unique insertion sites in the right arm of the X chromosome.*

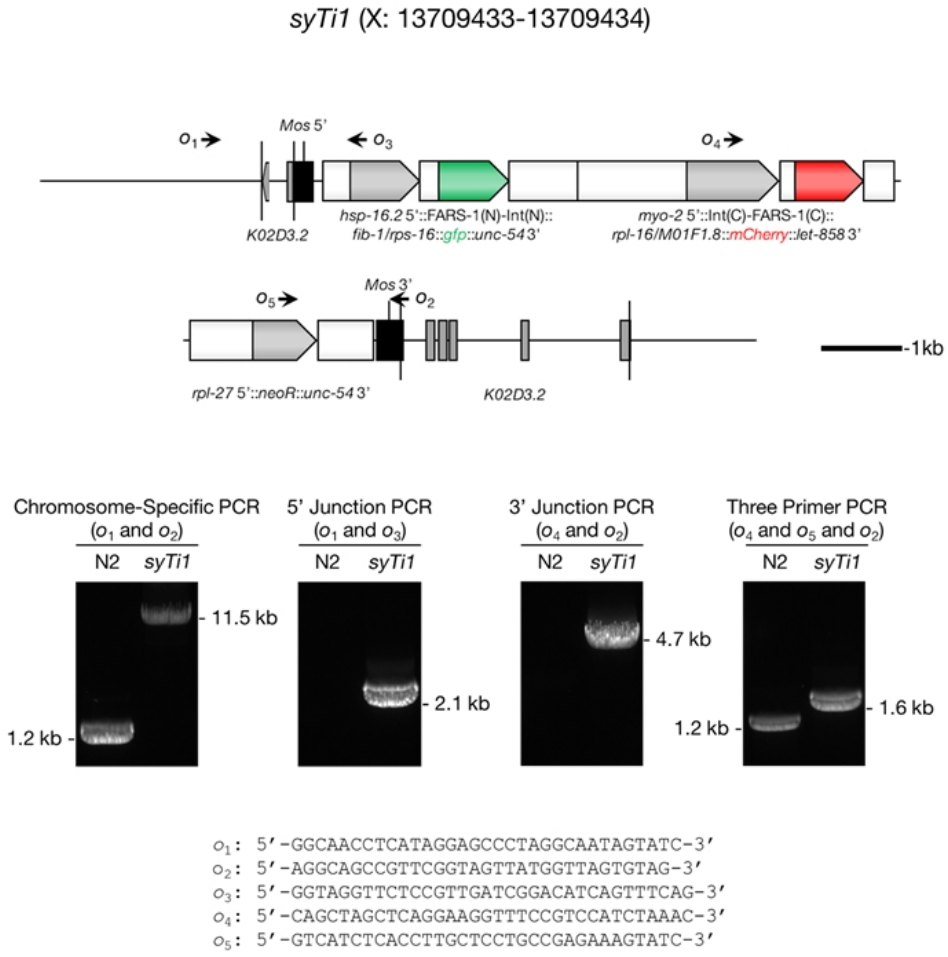

Figure 3.25: *We verified the syTi1 insertion fidelity and genotype with chromosome-specific PCRs spanning across the insertion site, genome-insertion junction-specific PCRs, and three-primer PCRs.*

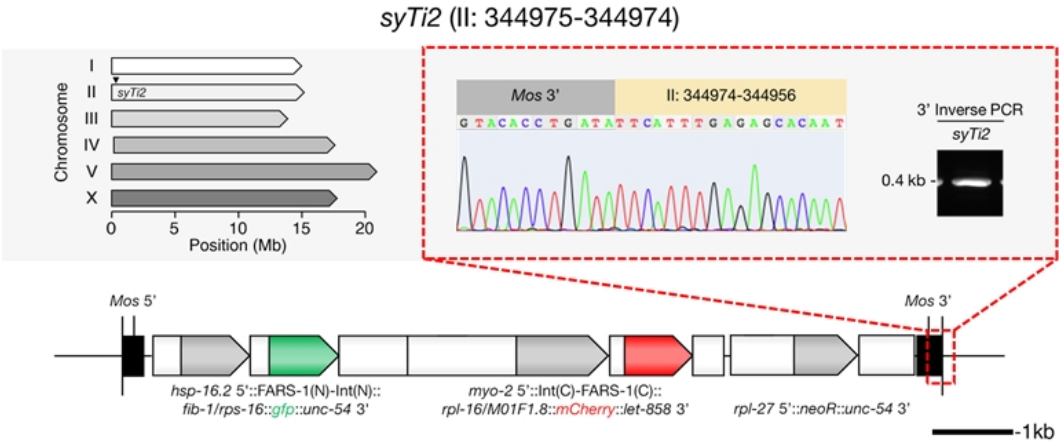

Figure 3.26: *We mapped the syTi2 insertion by inverse PCR to a unique insertion sites in the left arm of chromosome II.*

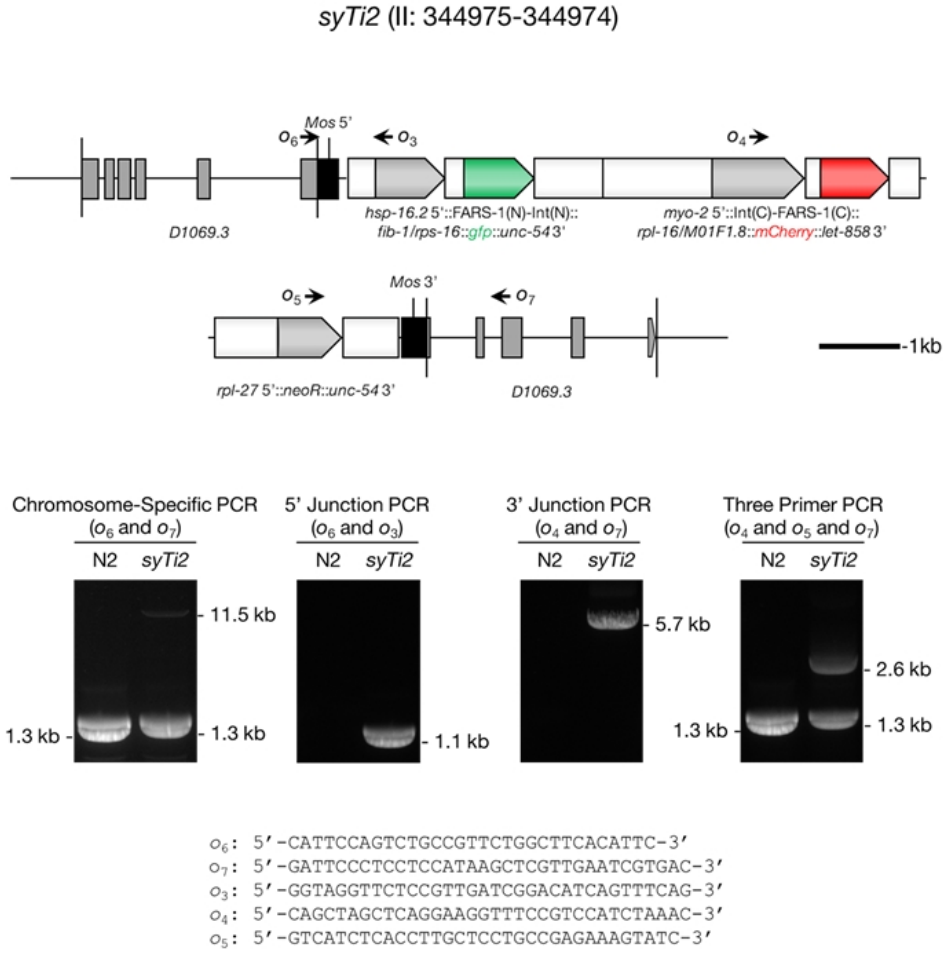

Figure 3.27: *We verified the syTi2 insertion fidelity and genotype with chromosome-specific PCRs spanning across the insertion site, genome-insertion junction-specific PCRs, and three-primer PCRs.*

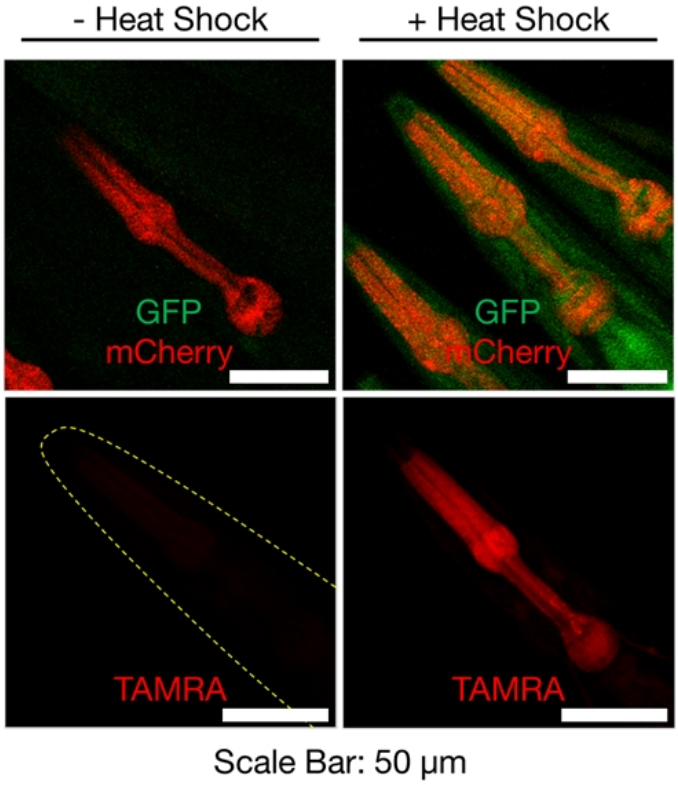

Figure 3.28: *We fed Azf-labeled E. coli strain KY33[pKPY514] to PS7055 and PS7058. These transgenic C. elegans strains (i) express both FARS-1(N)-Int(N) and GFP (green) under control of the hsp-16.2 promoter and (ii) express both Int(C)-FARS-1(C) and mCherry (red) under control of the myo-2 promoter. (Top) Only heat-shocked worms express GFP ubiquitously (PS7055 shown). (Bottom) Azf-labeling in PS7055 and PS7058 were localized to pharyngeal muscle only upon heat shock; the surrounding tissues were not labeled (PS7055 shown).*

3.5 Materials and Methods

3.5.1 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 x 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 x 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.

3.5.2 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.

3.5.3 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 x 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 x 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 x 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 x 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 x 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.

3.5.4 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.

3.5.5 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 Inter-

national), 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 ($^{13}C_6{}^{15}N_2{}^{1}H_{14}{}^{16}O_2$, Cambridge Isotope Laboratories), 40 mg/L L-arginine ($^{13}C_6{}^{14}N_4{}^{1}H_{14}{}^{16}O_2$, 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 ($^{13}C_6{}^{15}N_2{}^{1}H_{14}{}^{16}O_2$, Cambridge Isotope Laboratories), 40 mg/L L-arginine ($^{13}C_6{}^{14}N_4{}^{1}H_{14}{}^{16}O_2$, Cambridge Isotope Laboratories), 35 mg/L kanamycin sulfate (Sigma-Aldrich Corporation)

E. coli **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**, **11**, **12**, **13**, **14**, **15**, **16**, and **17**: 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 OD_{600} of 0.5. Expression of wild-type and mutant *Ce*PheRSs was induced by addition of 1 M L-(+)-arabinose (500 μL, 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 μL, 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.

KY33[pKPY514] 1: 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 OD_{600} 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-*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 x 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] 2: 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 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 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 x 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.

3.5.6 Plasmids and Strains

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**, 41214130 (1995)). The purified fragments were ligated into the NdeI site of pJTN4 (Ngo, J.T., et al. *Nat. Chem. Biol.* **5**, 715717 (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.

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.

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**, 66406645 (2000)) to eliminate the gene *argA* from the *E. coli* strain 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**, 66406645 (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**, 17771781 (2014)).

Worm strains were cultured as described by Brenner (Brenner, S. *Genetics* **77**, 7194 (1974)) and generated as described by Frøkjær-Jensen et al. (Frøkjær-Jensen et al. *Nat. Methods* **11**, 529-534 (2014)). The strains used in this study were as follows:

C. elegans PS7052: *syEx1516[pKPY728 (mos 5-hsp-16.2 5::fars-1(A, N, M1-G203)::gp41-1(N, C1-E88)::fib-1/rps-16::gfp::unc-54 3-myo-2 5::gp41-1(C, M1-S38)::fars-1(A, C, S204-K552, T468G)::rpl-16/M01F1.8::mCherry::let-858 3-rpl-27 5::neoR::unc-54 3-mos 3; hsp-16.41 5::peel-1::tbb-2 3) (5.0 ng/uL) + pCFJ90 (myo-2 5::mCherry::unc-54 3) (2.5 ng/uL) + pCFJ104 (myo-3 5::mCherry::unc-54 3) (5.0 ng/uL) + pGH8 (rab-3 5::mCherry::unc-54 3) (10.0 ng/uL) + pCFJ601 (eft-3 5::mos1::tbb-2 3) (50.0 ng/uL) + pBluescript II SK (127.5 ng/uL)]*

Notes: Extrachromosomal Array Carrying *miniMos* Construct pKPY728 (Injected at 5.0 ng/uL); Maintain with 0.5 mg/mL G-418 (Geneticin);

C. elegans PS7053: *syEx1517[pKPY728 (mos 5-hsp-16.2 5::fars-1(A, N, M1-G203)::gp41-1(N, C1-E88)::fib-1/rps-16::gfp::unc-54 3-myo-2 5::gp41-1(C, M1-S38)::fars-1(A, C, S204-K552, T468G)::rpl-16/M01F1.8::mCherry::let-858 3-rpl-27 5::neoR::unc-54 3-mos 3; hsp-16.41 5::peel-1::tbb-2 3) (10.0 ng/uL) + pCFJ90 (myo-2 5::mCherry::unc-54 3) (2.5 ng/uL) + pCFJ104 (myo-3 5::mCherry::unc-54 3) (5.0 ng/uL) + pGH8 (rab-3 5::mCherry::unc-54 3) (10.0 ng/uL) + pCFJ601 (eft-*

3 5::mos1::tbb-2 3) (50.0 ng/uL) + pBluescript II SK (72.5 ng/uL)]

Notes: Extrachromosomal Array Carrying *miniMos* Construct pKPY728 (Injected at 10.0 ng/uL); Maintain with 0.5 mg/mL G-418 (Geneticin);

C. elegans PS7055: *syTi1[mos 5-hsp-16.2 5::fars-1(A, N, M1-G203)::gp41-1(N, C1-E88)::fib-1/rps-16::gfp::unc-54 3-myo-2 5::gp41-1(C, M1-S38)::fars-1(A, C, S204-K552, T468G)::rpl-16/M01F1.8::mCherry::let-858 3-rpl-27 5::neoR::unc-54 3-mos 3]*

Chromosome: X;

Notes: Derived from *C. elegans* PS7052, Mapped by Inverse PCR to Chromosome X (X: 13709433-13709434), Homozygosity Confirmed by Three-Primer PCR, Maintain w/0.5 mg/mL G-418 (Geneticin);

C. elegans PS7058: *syTi2[mos 5-hsp-16.2 5::fars-1(A, N, M1-G203)::gp41-1(N, C1-E88)::fib-1/rps-16::gfp::unc-54 3-myo-2 5::gp41-1(C, M1-S38)::fars-1(A, C, S204-K552, T468G)::rpl-16/M01F1.8::mCherry::let-858 3-rpl-27 5::neoR::unc-54 3-mos 3]*

Chromosome: II;

Notes: Derived from *C. elegans* PS7053, Mapped by Inverse PCR to Chromosome II (II: 344975-344974), Maintain w/0.5 mg/mL G-418 (Geneticin);