

TOOLS

A collagen IV fluorophore knock-in toolkit reveals trimer diversity in *C. elegans* basement membranes

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The type IV collagen triple helix, composed of three α -chains, is a core basement membrane (BM) component that assembles into a network within BMs. Endogenous tagging of all α -chains with genetically encoded fluorophores has remained elusive, limiting our understanding of this crucial BM component. Through genome editing, we show that the C termini of the *C. elegans* type IV collagen α -chains EMB-9 and LET-2 can be fused to a variety of fluorophores to create a strain toolkit with wild-type health. Using quantitative imaging, our results suggest a preference for LET-2-LET-2-EMB-9 trimer construction, but also tissue-specific flexibility in trimers assembled driven by differences in α -chain expression levels. By tagging *emb-9* and *let-2* mutants that model human Gould syndrome, a complex multitissue disorder, we further discover defects in extracellular accumulation and turnover that might help explain disease pathology. Together, our findings identify a permissive tagging site in *C. elegans* that will allow diverse studies on type IV collagen regulation and function in animals.

Introduction

Basement membranes (BM) are dense, sheetlike extracellular matrices that surround and support most tissues (Fidler et al., 2018; Jayadev and Sherwood, 2017a). Type IV collagen is a triple helical protomer and a core BM component that forms a self-associating network in BMs. The type IV collagen BM network confers resistance to mechanical loads placed on tissues (Fidler et al., 2018; Keeley et al., 2020; Morrissey and Sherwood, 2015), and type IV collagen genetic loss leads to embryonic lethality at the onset of muscle contraction in mice, *Drosophila*, and *Caenorhabditis elegans* (Borchiellini et al., 1996; Gupta et al., 1997; Pöschl et al., 2004). Precise deposition and remodeling of type IV collagen is also required to properly shape tissues, such as the *Drosophila* egg chamber, central nervous system, and wing disk, and the mouse salivary gland (Crest et al., 2017; Harunaga et al., 2014; Isabella and Horne-Badovinac, 2016; Morrissey and Sherwood, 2015; Pastor-Pareja and Xu, 2011). Additionally, type IV collagen networks serve as scaffolds that bind signaling ligands and other BM components that direct cell differentiation and guide cell migration (Brown et al., 2017; Morrissey and Sherwood, 2015; Parkin et al., 2011; Sherwood, 2021; Wang et al., 2008). Further emphasizing its importance to tissue function, pathogenic variants in type IV collagen are linked to at least 10 human genetic disorders that affect muscle, kidney, brain, and vasculature (Fidler et al., 2018). Additionally,

increased type IV collagen BM accumulation is associated with the decline of vascular function in diabetes, in Alzheimer's disease, and during aging (Baum and Bigler, 2016; Candiello et al., 2010; Fidler et al., 2018; Howe et al., 2020; Karttunen et al., 1986; Marshall, 2016; Thomsen et al., 2017; Uspenskaia et al., 2004).

The triple helical type IV collagen protomer is composed of three α -chains, which have a cysteine-rich 7S domain at the N terminus, a central interrupted Gly-X-Y repeat region (X and Y can be any amino acid (aa), but is most often proline), and a noncollagenous NC1 domain at the C terminus (Hudson et al., 1993, 2003). Reconstitution studies have shown that the NC1 domain mediates α -chain selection for type IV collagen trimer assembly, which occurs in the endoplasmic reticulum (ER) (Boutaud et al., 2000; Dölz et al., 1988; Fidler et al., 2018). In vitro studies have also revealed that lysyl and prolyl hydroxylases, along with the cofactor vitamin C, are needed for proper folding and trimerization (Kivirikko and Pihlajaniemi, 1998; Lamandé and Bateman, 1999). Depleting prolyl 4-hydroxylase in *Drosophila* larvae results in the secretion of type IV collagen in its monomeric form and an absence of BM localization, suggesting BM incorporation requires trimerization (Pastor-Pareja and Xu, 2011). Upon secretion into the extracellular space, two type IV collagen protomers connect through the C-terminal

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NC1 domains and four protomers connect through the N-terminal 7S domains (Añazco et al., 2016; Cummings et al., 2016). The interfaces of the NC1 and 7S connections are reinforced with covalent crosslinks (Cummings et al., 2016; Vanacore et al., 2009). Together, the NC1 dimer and 7S tetramer connections establish a polygonal patterned network of type IV collagen in the BM. Electron microscopy studies have also revealed higher order lateral twisting interactions between neighboring protomers, which are thought to provide further stability to type IV collagen networks (Barnard et al., 1992; Ruben and Yurchenco, 1994; Yurchenco and Ruben, 1987).

Based on sequence similarity, type IV collagen α -chains have been divided into $\alpha 1$ -like and $\alpha 2$ -like groups. Most vertebrates have six α -chain-encoding genes (COL4A1–COL4A6 genes encoding the $\alpha 1$ – $\alpha 6$ proteins). The $\alpha 1$ -like chain group contains COL4A1, COL4A3, and COL4A5 genes, and the $\alpha 2$ -like group includes COL4A2, COL4A4, and COL4A6 (Khoshnoodi et al., 2008). *C. elegans* and *Drosophila melanogaster* each possess two type IV collagen α -chains, encoded by *emb-9* and *let-2* in *C. elegans* and *vkg* and *Cg25C* in *Drosophila* (Guo and Kramer, 1989; Natzle et al., 1982; Sibley et al., 1993; Yasothornsrikul et al., 1997). Sequence comparisons with mammalian NC1 domains have suggested that *emb-9* and *vkg* are more similar to $\alpha 1$ -like chains and *let-2* and *Cg25C* are more similar to $\alpha 2$ -like chains (despite *Cg25C* officially being named *Col4a1*) (Guo and Kramer, 1989; Summers et al., 2023).

Chromatography-based protein isolation studies in vertebrates have revealed approximately twice as much $\alpha 1$ compared with the $\alpha 2$ chain in several tissues (Mayne and Zettergren, 1980; Siebold et al., 1988; Trüeb et al., 1982), which is consistent with the existence of the $\alpha 1\alpha 1\alpha 2$ heterotrimer. Characterization of type IV collagen hexamers (the NC1 domains of two cross-linked protomers) isolated from in vivo tissues has also supported the existence of $\alpha 1\alpha 1\alpha 2$, as well as $\alpha 3\alpha 4\alpha 5$ and $\alpha 5\alpha 5\alpha 6$ type IV collagen trimers (Borza et al., 2001; Boutaud et al., 2000; Khoshnoodi et al., 2008). These and other biochemical studies have led to the proposition that only three (out of a possible 56) specific combinations of vertebrate type IV collagen are assembled in vivo— $\alpha 1\alpha 1\alpha 2$, $\alpha 3\alpha 4\alpha 5$, and $\alpha 5\alpha 5\alpha 6$ —with $\alpha 1\alpha 1\alpha 2$ being the most abundant and ubiquitous (Borza et al., 2001; Boutaud et al., 2000; Hudson et al., 1994, 2003; Khoshnoodi et al., 2008).

While there is strong support for the preferential formation of $\alpha 1\alpha 1\alpha 2$, $\alpha 3\alpha 4\alpha 5$, and $\alpha 5\alpha 5\alpha 6$ type IV collagen trimers in mammalian tissues, there is also evidence that other combinations could exist. For example, isolation and characterization of type IV collagen hexamers have not ruled out the existence of $\alpha 1$ homotrimers or $\alpha 1\alpha 2\alpha 2$ heterotrimers (Boutaud et al., 2000; Johansson et al., 1992; Siebold et al., 1988). Indeed, reconstitution experiments using bovine and recombinant NC1 $\alpha 1$ – $\alpha 5$ monomers have found that $\alpha 1$ NC1 domains can form a homohexamer and that the $\alpha 2$ NC1 can assemble in a promiscuous manner with other NC1 domains (Boutaud et al., 2000). Further, rat carcinoma cells generate and secrete $\alpha 1$ homotrimers (Haralson et al., 1985). No studies have yet addressed the overall α -chain levels and possible trimer combinations found in *C. elegans*. Mutations in the *emb-9* or *let-2*, however, result in intracellular retention of

the other (nonmutant) α -chain (Gupta et al., 1997), suggesting a preference for heterotrimer assembly prior to secretion. The recombinant expression of forced predetermined NC1 trimers in *Drosophila* larvae has shown that only the Cg25C–Cg25C–Vkg NC1 heterotrimer combination is recruited to the BM. Yet, NC1 domains expressed as individual monomers in mammalian cells assemble in almost all possible combinations. Additionally, analysis of endogenous hexamers did not rule out the formation of homotrimers (Summers et al., 2023). Together, these observations suggest that flexibility in the α -chain composition of type IV collagen trimers might exist in BMs in vivo.

Endogenous tagging of BM components with genetically encoded fluorophores allows quantitative comparisons of component abundance at the tissue, cell, and subcellular level (Jayadev et al., 2022; Keeley et al., 2020). A key challenge for tagging type IV collagen is identifying a fluorophore insertion site common to all α -chains that does not disrupt type IV collagen assembly and function. To date, endogenous tagging in mice, *Drosophila*, and *C. elegans* has predominantly used the *Drosophila* Vkg–GFP protein trap insertion site, which inserts GFP within the start of the 7S domain at the N terminus (Morin et al., 2001). While insertion of fluorophores at this site is viable for *Drosophila vkg*, *C. elegans emb-9*, and mouse COL4A2 (Keeley et al., 2020; Wuergeszen et al., 2025), insertion at this site is not viable in *C. elegans let-2* or mouse COL4A1 (Jones et al., 2024; Keeley et al., 2020). A site within an interruption of the Gly–X–Y repeats in the collagenous domain of *C. elegans emb-9* has also been reported to be viable (Matsuo et al., 2019), but the health of this strain has not been fully explored.

Here, we use genome editing, fitness assays, and live-cell imaging to compare sites for fluorophore insertion into the *C. elegans emb-9* and *let-2* type IV collagen α -chains. We show that fluorophore insertion at the Vkg–GFP site (renamed internal site 1, IS1) and an internal site within the collagenous domain of EMB-9 (internal site 2, IS2) lead to accumulation of the tagged α -chains in the ER and reduced organism fitness. In contrast, we use multiple fluorophores and show that insertion at the C terminus of EMB-9 and LET-2 proteins leads to dramatically less intracellular retention and phenotypically wild-type fitness. Using these new strains with mass spectrometry on whole animals, we reveal there is approximately twofold more LET-2 protein than EMB-9, suggesting predominantly a LET-2–LET-2–EMB-9 (LLE) heterotrimer. However, using quantitative live imaging of mNeonGreen (mNG)-tagged strains, we find that the LET-2/EMB-9 protein ratio present at various BMs is not precisely 2:1, but instead is ~ 1.7 on most tissues, and at the spermathecal BM is over 2.2. These results suggest a preference for the LLE heterotrimer, but that there is also flexibility in α -chain trimer makeup. Using tissue-specific translational reporters, RNAi knockdown, and overexpression, we provide evidence suggesting that α -chain availability during assembly can drive trimer diversity. Finally, we show that the C-terminal site for type IV collagen allows endogenous tagging of type IV collagen variants found in the COL4A1/A2 syndrome (also known as the Gould syndrome), a genetically dominant heterogeneous multisystem disorder predominantly caused by substitutions of glycine aa in the Gly–X–Y motifs of the triple helical

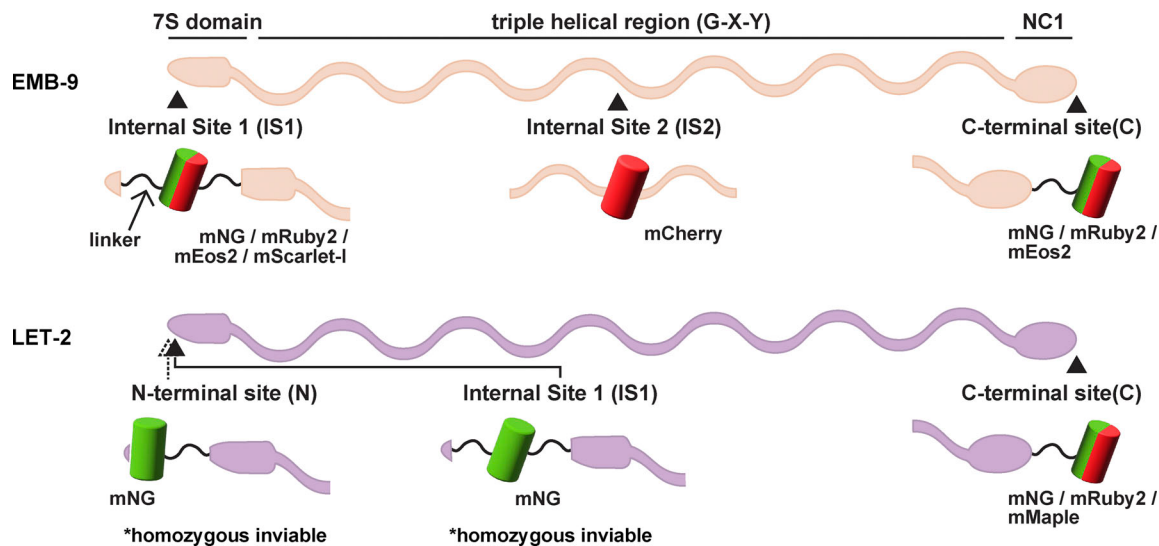


Figure 1. **Fluorophore insertion sites in the *C. elegans* type IV collagen α -chains.** The schematic illustrates *C. elegans* type IV collagen α -chains EMB-9 and LET-2 with the N-terminal 7S domains, the triple helical regions, and the C-terminal NC1 domains annotated above the proteins. Arrowheads mark fluorophore insertion sites. Each site is labeled with the fluorophores that were inserted at the location and whether the fluorophore was flanked by a linker sequence (thin black wavy lines; see Fig. S1 A). Insertions that were homozygous-inviable are denoted.

domain (Jeanne and Gould, 2017; Labelle-Dumais et al., 2024). Through localization and photoconversion analysis, we find that in addition to defects in type IV collagen secretion, these mutant forms affect extracellular accumulation and turnover. Together, our findings identify a permissive site for tagging type IV collagen α -chains in *C. elegans*, which will allow wide-ranging studies.

Results

Healthy C-terminal fluorophore knock-ins of EMB-9 and LET-2 type IV collagen α -chains

The inability to comprehensively label and visualize type IV collagen α -chains in animals has limited our understanding of type IV collagen regulation, function, and composition in the BM. Fluorophore insertion at the C terminus through genome editing has successfully labeled 37 of the 56 BM components tagged in *C. elegans* to date, but has not yet been attempted for the type IV collagen α -chain-encoding genes *emb-9* and *let-2* (Fan et al., 2020; Hastie et al., 2019; Jayadev et al., 2022; Keeley et al., 2020; Naegeli et al., 2017). Thus, we knocked in genetically encoded fluorescent proteins with an 18-aa linker within the endogenous *emb-9* and *let-2* genes at the C terminus (C site) of the protein-encoding regions using CRISPR/Cas9 gene editing (Fig. 1 and Fig. S1 A). The resulting gene-edited animals were homozygous-viable and fertile. To compare the health of these new strains to animals with fluorescent proteins inserted at IS1 (*Drosophila* Vkg-GFP site) and IS2 of type IV collagen (Fig. 1), we plated four L4 larvae from each strain onto a pre-determined amount of food and recorded how long it took for the food to be depleted (time to starvation). This served as a collective estimate of growth, feeding, and fertility. Eight strains with six distinct knock-ins at the C site of *emb-9* and *let-2* were analyzed: *emb-9* and *let-2* each tagged with mNG or mRuby2,

strains harboring both *let-2* and *emb-9* tagged with mNG and mRuby2 and vice versa, *emb-9* tagged with photoconvertible mEos2, and *let-2* tagged with photoconvertible mMaple (Heppert et al., 2016; McEvoy et al., 2012; Zhang et al., 2012) (Fig. 1; and Fig. S1, A and B). All strains were indistinguishable from wild-type animals, apart from *let-2* tagged with mMaple, which showed a slight delay in the time to starvation (Table 1). In contrast, *emb-9* strains tagged at IS1 (mRuby2, mScarlet-I, mEos2, mNG) and IS2 (mCherry) (Bindels et al., 2017; Heppert et al., 2016) all showed delays to starvation (Table 1; Fig. 1; and Fig. S1, A and B). It is notable that all fluorophore insertions at IS1 (Vkg site), which has been used most extensively to label type IV collagen (Keeley et al., 2020; Morin et al., 2001; Wuergeszen et al., 2025), caused delayed growth.

We next wanted to compare protein trafficking and localization of the type IV collagen tagged in distinct sites. Type IV collagen is expressed in several tissues in *C. elegans*, but the predominant site of production is the *C. elegans* body wall muscle, where type IV collagen is made, secreted into the BM, and also trafficked from the body wall muscles to many other tissue BMs (Graham et al., 1997; Morrissey et al., 2016). Confocal imaging of L1-staged larvae (0–12 h after hatch) showed large and extensive body wall muscle intracellular aggregates of EMB-9 tagged at IS1 with mNG, mEos2, mRuby2, or mScarlet-I, and aggregates of EMB-9 tagged at IS2 with mCherry (Fig. 2 A and Table S1). In contrast, EMB-9 and LET-2 tagged with mNG or mRuby2 at the C site possessed few if any aggregates (Fig. 2 A and Table S1). Previous immunolocalization studies also found limited intracellular aggregates of EMB-9 and LET-2 in wild-type L1 animals (Graham et al., 1997; Kang and Kramer, 2000), suggesting tagging at the C site permits normal intracellular trafficking. Imaging of worms tagged at the C site from the L1 through L4 stage and into the gravid adult revealed the gradual appearance of small type IV collagen aggregates starting at the L3 larval stage that

Table 1. Health of fluorescently tagged type IV collagen strains

Strain	Fluorophore insertion site	Additional days to starvation relative to wild type ^a		
		Replicate 1	Replicate 2	Replicate 3
<i>let-2::mNG</i>	C	0	0	0
<i>let-2::mRuby2</i>	C	0	0	0
<i>emb-9::mNG</i>	C	0	0	0
<i>emb-9::mRuby2</i>	C	0	0	0
<i>emb-9::mRuby2; let-2::mNG</i>	C; C	0	0	0
<i>emb-9::mNG; let-2::mRuby2</i>	C; C	0	0	0
<i>emb-9::mEos2</i>	C	0	0	0
<i>let-2::mMaple</i>	C	1	1	1
<i>emb-9::mRuby2</i>	IS1	1	1	1
<i>emb-9::mScarlet-I</i>	IS1	1	1	1
<i>emb-9::mCherry</i>	IS2	2	2	2
<i>emb-9::mEos2</i>	IS1	5	5	5
<i>emb-9::mNG</i>	IS1	6	8	8

^aFor each strain, four L4 larvae were plated on 400 μ l of OP50 *E. coli* and allowed to grow at 20°C (replicates of 3). With every batch of experiments, wild-type N2 animals were also plated. Plates were checked twice per day, and the time at which food was depleted was recorded (time to starvation). Numbers above indicate how much longer strains took to starve than N2 animals. No strains starved faster than N2 animals.

continued to the adult (Fig. S2 A). Given the wild-type health of these strains, this might represent natural intracellular accumulation.

Type IV collagen folding and trimerization occur in the ER, which has quality control mechanisms that retain misfolded proteins (Ellgaard and Helenius, 2003). We thus hypothesized that the type IV collagen aggregates found in strains tagged at IS1 and IS2 were likely localized to the ER. Colocalization of type IV collagen aggregates tagged at IS1 (EMB-9::mScarlet-I [IS1]) with an ER marker (ELO-1::mNG [Park et al., 2024]) revealed that the EMB-9::mScarlet-I (IS1) aggregates were always enclosed by the ER membrane (Fig. 2 B). Similarly, EMB-9::mCherry (IS2) aggregates were also contained within the ER (Fig. S2 B). This suggests that fluorophore insertion at IS1 or IS2 interferes with proper protein folding or trimerization, leading to ER accumulation and possibly reduced secretion. Consistent with this idea, measurements at the pharyngeal BM in L1 larvae showed that less EMB-9::mNG (IS1) localized to the BM compared with EMB-9::mNG (C) (Fig. 2 C). Decreased EMB-9::mNG (IS1) in the BM might also be caused by changes in the rates of addition to the BM, removal from the BM, or both (often referred to as turnover) (Stramer and Sherwood, 2024). However, fluorescence recovery in a photo-bleached region of EMB-9::mNG (IS1) compared with EMB-9::mNG (C) and LET-2::mNG (C) revealed similar recovery dynamics: LET-2::mNG (C) recovered ~32%, and both EMB-9::mNG (C) and EMB-9::mNG (IS1) recovered ~25% of bleached fluorescence over a 5-h period (Fig. S2 C). Taken together, the wild-type health and greatly reduced intracellular aggregation offer compelling evidence that the C terminus is a permissive site for fluorescently tagging type IV collagen α -chains in *C. elegans*.

LET-2 is approximately twice as abundant as EMB-9 in whole animals

We next wanted to determine the overall levels of EMB-9 and LET-2 proteins in the extracellular matrix of whole animals using the C site-tagged strains as material for liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Comparisons of abundance of distinct proteins using LC-MS/MS are usually challenging because differences in the physical and chemical properties of distinct peptides can affect processing and detection (Bantscheff et al., 2007; Xie et al., 2011). However, the tagging of LET-2 and EMB-9 with the same fluorophore allowed us to use the fluorophore proteins as a proxy of LET-2 and EMB-9 abundance to determine their levels using label-free quantitative proteomics. We collected mixed populations of L1-L3 larval-staged worms expressing the knock-in proteins EMB-9::mRuby2 (C); LET-2::mNG (C) (Strain 1) and EMB-9-mNG (C); LET-2::mRuby2 (C) (Strain 2) and chemically fractionated them to enrich for extracellular matrix proteins as described previously (Morais et al., 2022, 2023). Measuring the protein abundance of mRuby2 in the extracellular matrix-enriched fraction of these strains using LC-MS/MS revealed an approximately twofold higher level of LET-2 compared with EMB-9 (Fig. 3 A). mNG was not detected in the matrix fraction, which may be due to differing trypsin susceptibilities between the two fluorophores since mNG is only 27% similar to mRuby2 (Fig. S1 B). Nonetheless, we found that in the cellular fraction, the levels of mNG indicated LET-2 was ~1.7-fold higher than EMB-9::mNG (Fig. S3 A). Similar results were observed in the cellular fraction for LET-2 and EMB-9 tagged with mRuby2 (Fig. S3 B). Finally, comparison of EMB-9 with EMB-9 and LET-2 with LET-2 protein levels between Strain 1 (EMB-9::mRuby2 [C]; LET-2::mNG [C]) and Strain 2 (EMB-9-mNG [C]; LET-2::mRuby2 [C]) matrix

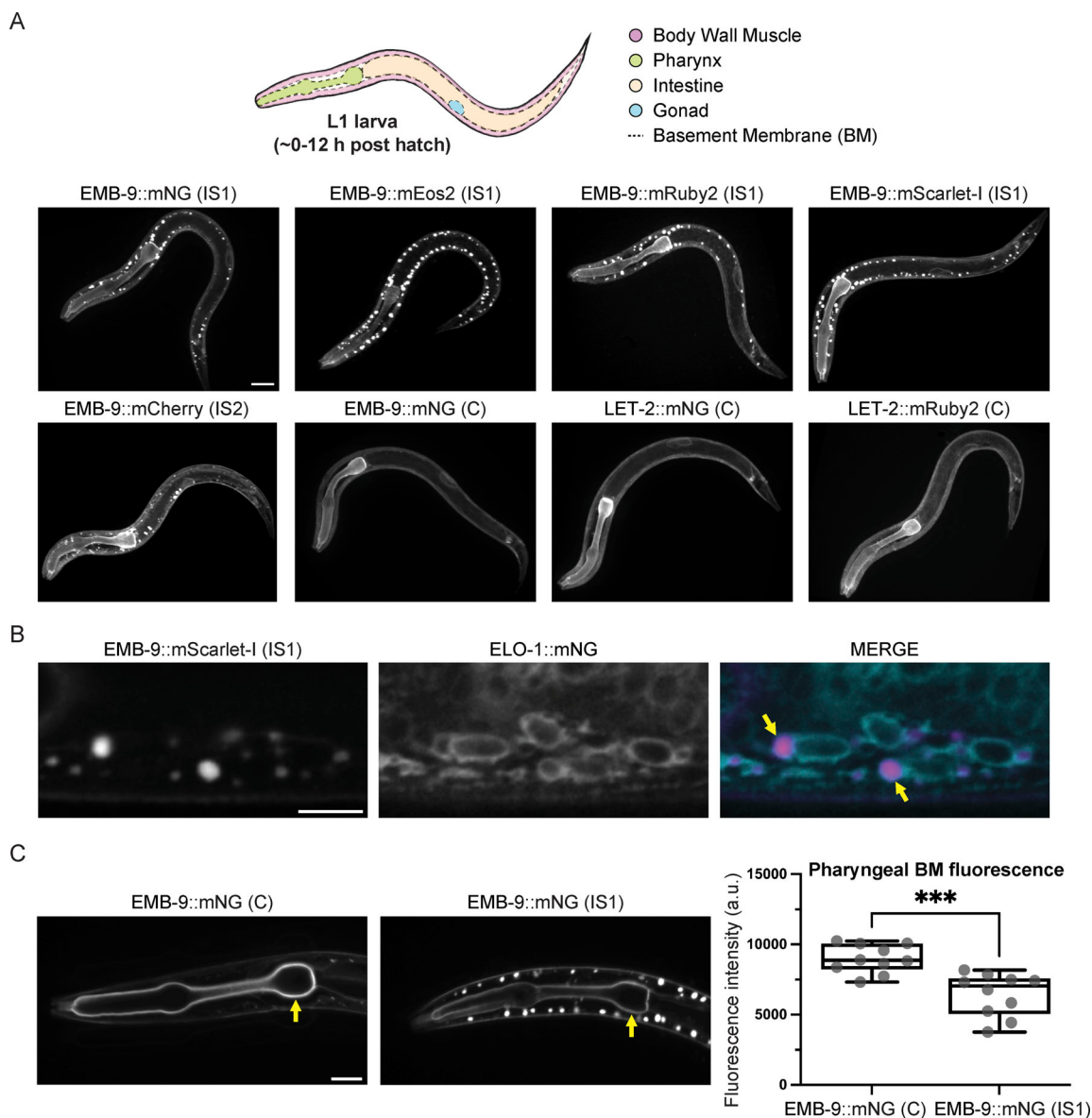


Figure 2. C-terminal EMB-9 and LET-2 fluorophore knock-ins are secreted and incorporated into BMs more efficiently than internal knock-ins.

(A) Top: the schematic indicates location of tissues and surrounding BMs (dashed lines) analyzed in an L1 larva. Bottom: representative max projections from confocal fluorescence z-stacks of L1 larvae with fluorescently tagged type IV collagen α -chains. Internally tagged α -chains aggregate in body wall muscles, while C-terminally tagged α -chains do not ($n = 10$ animals per strain). The scale bar is 10 μ m. **(B)** Single z-slice confocal images show that aggregates of EMB-9::mScarlet-I (IS1) (magenta in merge) are localized within the ER membrane (visualized with ELO-1::mNG, cyan in merge) in L1 larvae. Yellow arrows indicate aggregates in the ER (100/100 aggregates localized to the ER, 10 aggregates counted per animal). The scale bar is 5 μ m. **(C)** Left: Single z-slice confocal images show reduced EMB-9::mNG (IS1) localized to the pharyngeal BM compared with EMB-9::mNG (C) in L1 larvae (~28% reduction; yellow arrows indicate pharyngeal BM). Right: Boxplots show mean fluorescence intensity measurements (a.u.) of the pharyngeal BM surrounding the proximal bulb (median of data denoted by horizontal line within each box for this and all subsequent boxplots) for EMB-9::mNG (C) versus EMB-9::mNG (IS1) animals ($n = 10$ animals each, *** $P < 0.001$, unpaired two-tailed Student's t test). The scale bar is 10 μ m.

fractions confirmed that the protein abundance between the two strains was comparable (Fig. S3, C and D). Taken together, these studies reveal that LET-2 is present at approximately twice the levels of EMB-9 in larval stage *C. elegans*.

LET-2/EMB-9 α -chain BM ratios are not precisely 2:1 and vary between tissues

The 2:1 whole-body protein ratio of LET-2/EMB-9 suggested an LLE heterotrimer combination is assembled in the ER and is

present in BMs (Fig. 3, B and C). We thus next wanted to use our endogenously tagged EMB-9 and LET-2 strains to determine whether a 2:1 LET-2/EMB-9 ratio is indeed present in BMs. We measured the average fluorescence intensity of LET-2::mNG (C) and EMB-9::mNG (C) in a specific region of the BM in the pharynx (feeding organ), the body wall muscle, the distal tip cell (DTC, the cell that leads gonad migration), and the spermatheca (where oocyte fertilization and ovulation occur) (Fig. 4 A) (Gieseler et al., 2017; Keeley et al., 2020; Kelley and Cram, 2019;

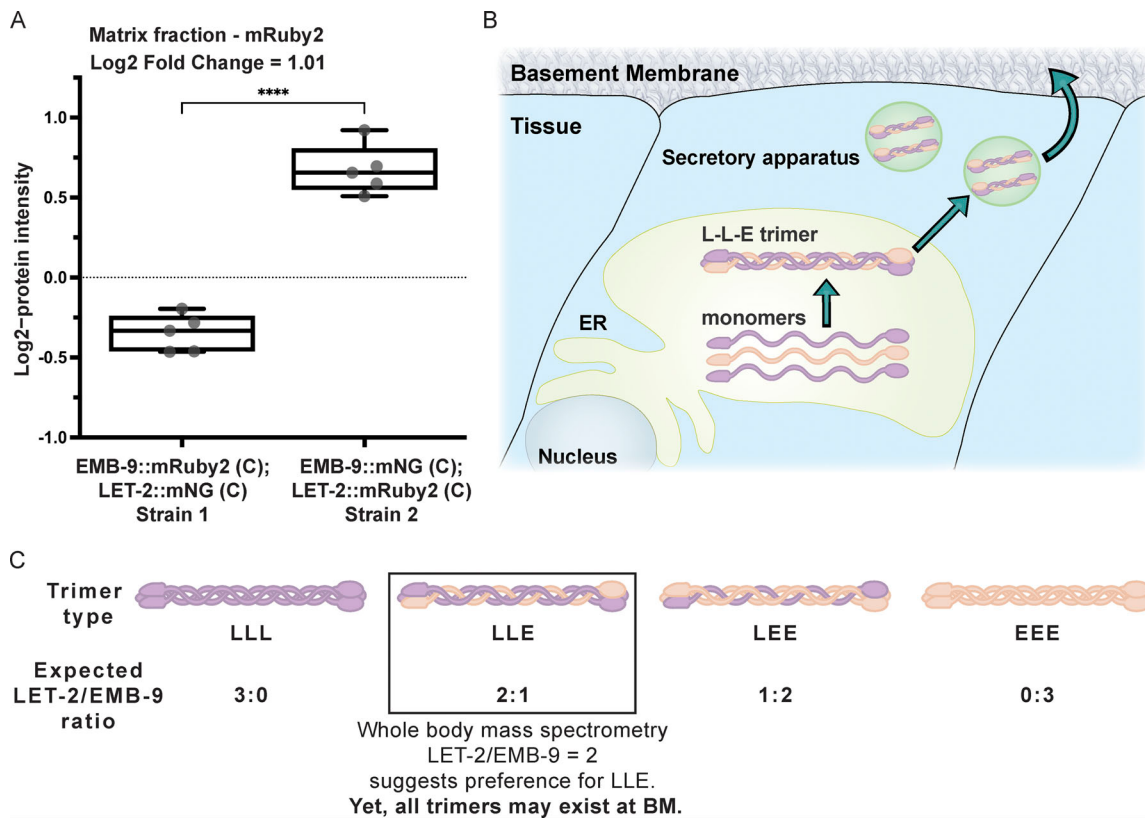


Figure 3. LET-2 is the more abundant type IV collagen α -chain. (A) Boxplots display the mRuby2 abundance represented by the $\log_2$ protein intensity (a.u.) determined using LC-MS/MS. The samples displayed are the larval matrix fractions of Strain 1 (EMB-9::mRuby2 [C]; LET-2::mNG [C]) and Strain 2 (EMB-9::mNG [C]; LET-2::mRuby2 [C]). The $\log_2$ fold change from the comparison between strains is listed above the graph. mRuby2 (LET-2) was ~ 2 times more abundant in Strain 2 than mRuby2 (EMB-9) in Strain 1 ($n = 5$ samples for each strain, $P < 0.0001$, unpaired two-tailed Student's t test). (B) Schematic demonstrates trimerization of *C. elegans* EMB-9 and LET-2 α -chains in the ER prior to secretion into the BM. (C) Schematic showing possible types of collagen IV trimers and the LET-2/EMB-9 ratios expected for each trimer type. Mass spectrometry on whole animals suggests a preference for the LLE trimer type but does not rule out different trimers being made and localized to the BM.

Sherwood and Plastino, 2018). The ratio of LET-2 to EMB-9 across the BMs of these tissues in L4.8–L4.9 worms (1-h window in larval development) (Mok et al., 2015) revealed an average ratio of ~ 1.7 within the pharynx, muscle, and DTC, but a significantly greater ratio of ~ 2.2 in the spermathecal BM (Fig. 4, A and B; and Fig. S4 A). While all tissue BMs had a LET-2/EMB-9 ratio close to 2:1, strongly suggesting the presence and perhaps a preference for the LLE trimer, ratios of LET-2 to EMB-9 lower than 2:1 indicated the existence of LEE or EEE or both trimers. Further, the ratio of $>2:1$ in the spermathecal BM implied the presence of LET-2 LLL homotrimers (Fig. 4 C).

To confirm the validity of these measurements, we assessed both the stability of fluorophore association with EMB-9 and LET-2 C termini and the possibility of fluorophore self-quenching, as type IV collagen networks associate end to end at the C terminus (NC1 domain) possibly placing multiple fluorophores in close proximity (Bae et al., 2021). Western blot analysis using mNG antibodies confirmed that EMB-9 and LET-2 C-terminal mNG fusion proteins were present almost exclusively as full-length proteins (Fig. S4 B). To test for quenching, we reasoned that reducing the number of fluorophores by half in heterozygous animals would reduce quenching (if present) and result in $>50\%$ fluorescence of the homozygous strain.

Fluorescence intensity measurements of EMB-9::mNG (C) and LET-2::mNG (C) L4.8- to L4.9-staged heterozygous worms, however, were not $>50\%$ of the levels of homozygous animals (Fig. S4 C), suggesting that fluorescence quenching was not occurring in homozygous animals. We conclude that mNG is stably associated with C-terminally tagged EMB-9 and LET-2 and that C-terminal-inserted mNG does not show quenching in the BM. Further, the levels of LET-2/EMB-9 are close to 2:1 for all tissue BMs, consistent with a predominant LLE heterotrimer composition. However, the diversity in α -chain ratios also suggests some flexibility in the α -chain makeup of trimers.

Tissue-specific translation levels of LET-2 and EMB-9 mirror the BM α -chain ratios

We hypothesized that differences in levels of translation (either from differences in transcription or from post-translational regulation) could alter the availability of LET-2 and EMB-9 α -chains for trimerization in the ER and influence the tissue-specific differences in α -chain ratios at the BM. We thus used CRISPR/Cas9 to insert a P2A peptide sequence followed by PEST::mNG immediately before the *emb-9* and *let-2* terminal codons (*emb-9*::P2A::PEST::mNG and *let-2*::P2A::PEST::mNG). The P2A peptide causes the ribosome to fail in making a peptide bond

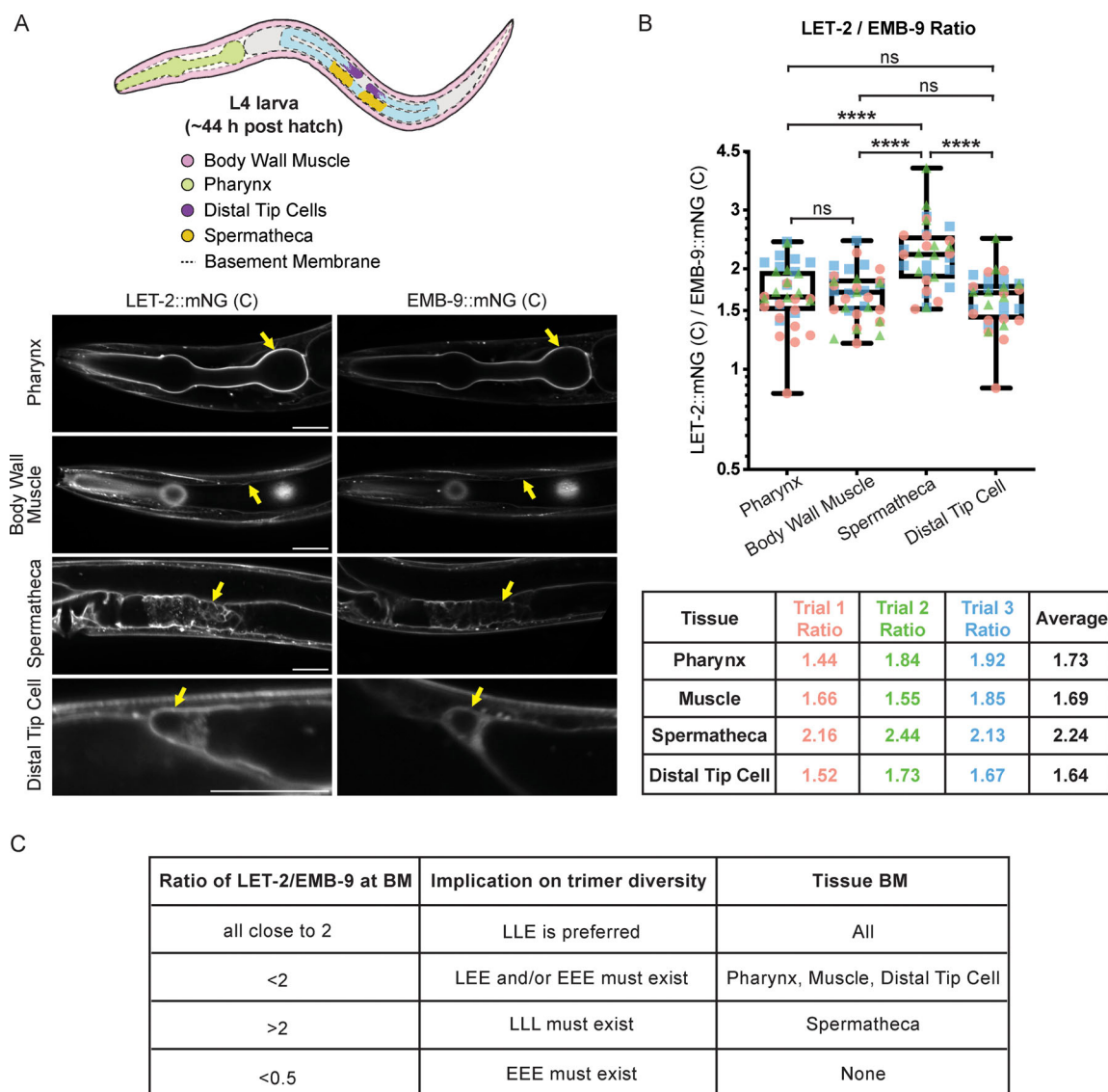


Figure 4. LET-2/EMB-9 BM ratios are not precisely 2:1 and differ between tissues. (A) Top: a schematic diagram of a late L4 larva with analyzed tissues and surrounding BMs (dashed lines) shown. Bottom: single z-slice confocal fluorescence images showing LET-2::mNG (C) and EMB-9::mNG (C) in tissues analyzed. Yellow arrows indicate BMs measured (see Materials and methods). The scale bar is 20 μ m. **(B)** Top: boxplot of the mean BM fluorescence intensity measurements of LET-2::mNG (C) normalized to the average of all mean BM fluorescence intensity measurements of EMB-9::mNG (C) for the same tissue and trial (see Materials and methods, $n > 10$ animals for each of three trials, **** $P < 0.0001$, ns, not significant, Brown–Forsythe ANOVA tests followed by Dunnett’s T3 multiple comparisons test). Colors of the data points indicate different trials. Bottom: table showing the ratios of the average BM fluorescence levels of LET-2::mNG (C)/EMB-9::mNG (C) for each tissue and trial. Colors in the table correspond to data points in the boxplot. Ratios at the spermathecal BM were consistently over 2 (LET-2::mNG [C] levels were more than double EMB-9::mNG [C] levels), while ratios at other BMs were consistently under 2. **(C)** Table showing what the composite ratio of LET-2/EMB-9 BM protein levels suggests for trimer diversity and the tissues where those ratios were found.

between the glycine and proline near the C terminus of the 2A peptide during translation, resulting in the separate synthesis of EMB-9 or LET-2 and mNG::PEST from the same mRNA (Lartey et al., 2024; Sharma et al., 2012). The PEST-destabilized mNG that remains within the cell cytoplasm can be used as a sensitive reporter of active EMB-9 and LET-2 translation levels (Li et al., 1998; Stevenson et al., 2023). We examined the average fluorescence intensity of PEST::mNG in the cytosol of three tissues that express and secrete type IV collagen—the body wall muscle, spermatheca myoepithelium, and the DTC (Fig. 5 A) (Graham et al., 1997). The ratio of LET-2 to EMB-9

protein translation in the body wall muscle and DTC was ~ 1.5 (Fig. 5 B and Fig. S4 D), similar to the ratio of the LET-2 and EMB-9 proteins in the body wall muscle and DTC BMs (Fig. 4 B and Fig. S4 A). In contrast, the ratio of LET-2 to EMB-9 protein translation in the spermatheca was ~ 2.5 (Fig. 5 B and Fig. S4 D), which mirrored the higher LET-2/EMB-9 protein ratio specifically found in the spermathecal BM (Fig. 4 B and Fig. S4 A). Together, these findings support the idea that the levels of α -chains available for trimerization influence the α -chain composition of trimers assembled and then incorporated into BM.

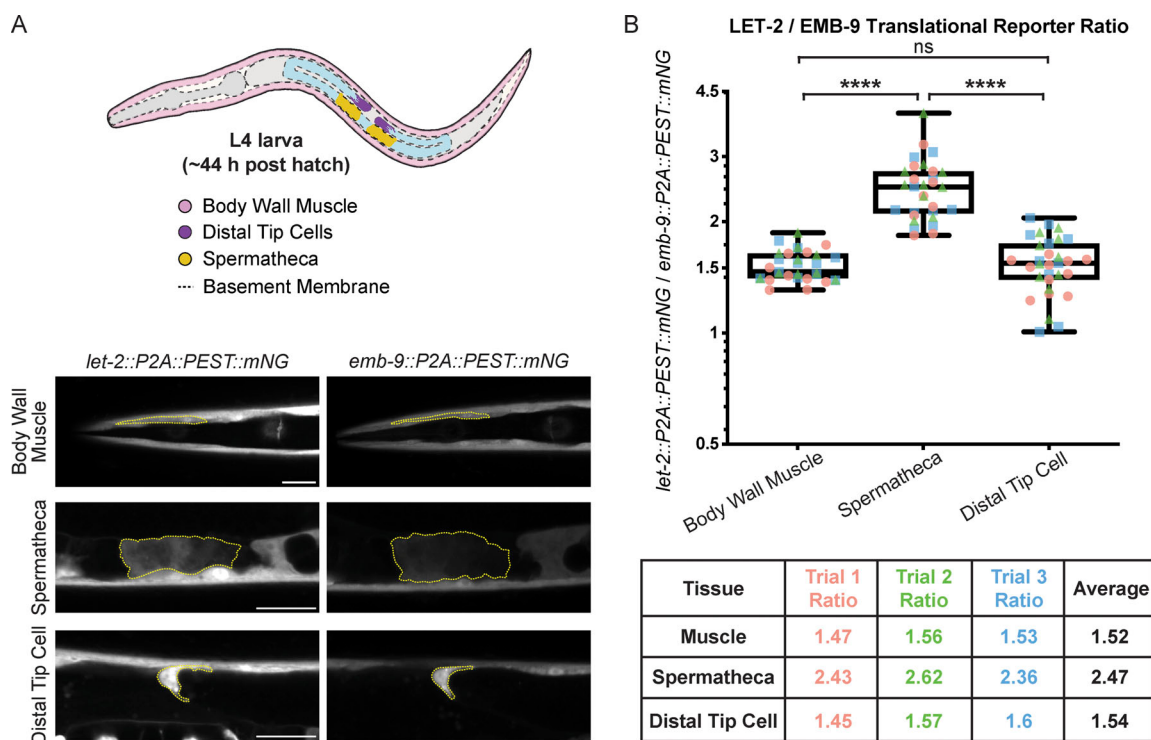


Figure 5. **LET-2/EMB-9 translation reporter ratios in tissues mirror BM ratios.** (A) Top: a schematic diagram of a late L4 larva with tissues analyzed for LET-2 and EMB-9 translation reporters shown. Bottom: single z-slice confocal fluorescence images of translation reporters for LET-2 (*let-2::P2A::PEST::mNG*) and EMB-9 (*emb-9::P2A::PEST::mNG*) in the body wall muscle, spermatheca, and DTC in late L4 larvae. Yellow dotted lines indicate where measurements were taken. The scale bar is 20 μ m. (B) Top: boxplot of the mean fluorescence intensity measurements of the LET-2 translational reporter normalized to the average of all mean fluorescence intensity measurements of the EMB-9 translational reporter in the same tissue and trial (see Materials and methods, $n > 10$ animals for each of three trials). **** $P > 0.0001$, ns, not significant, Kruskal–Wallis test followed by Dunn’s multiple comparisons test). Colors of the data points indicate different trials. Bottom: table showing the ratios of the average fluorescence levels of LET-2 translation reporter to EMB-9 translation reporter in each trial. Colors in the table correspond to data points in the boxplot.

Changes in type IV collagen α -chain expression are sufficient to alter α -chain BM ratios

Our results suggest that although the ratio of LET-2 to EMB-9 is close to 2:1 in tissue BMs, and thus likely predominantly contains an LLE trimer, there is also flexibility in the α -chain composition of trimers assembled and incorporated into the BM based on α -chain availability during trimer assembly. We next wanted to directly test both the preference for heterotrimer formation and whether changing α -chain levels is sufficient to alter the ratios of α -chains deposited in the BM. We first reduced *emb-9* expression in EMB-9::mNG (C) and LET-2::mNG (C) knock-in worms by feeding RNAi targeting *emb-9* beginning at the L1 larval stage (Jayadev et al., 2019). We then measured the mean fluorescence intensity of EMB-9::mNG and LET-2::mNG in the body wall muscle BM of day 1 adults (72 h post-RNAi initiation). RNAi targeting of *emb-9* resulted in a 75% loss of the EMB-9 protein at the body wall muscle BM (Fig. 6, A and B; and Fig. S5 A). LET-2 levels in the BM were decreased by ~50%, and there was increased internal aggregation of LET-2::mNG in body wall muscle (Fig. 6, A and B; Fig. S5, A and C; and Table S1). The reduction in LET-2 at the BM and increase in intracellular accumulation are consistent with a preference for heterotrimer assembly. Yet, a

greater proportion of LET-2 than EMB-9 was incorporated into the BM—the LET-2/EMB-9 ratio went from ~1.8 to ~3.5 after *emb-9* knockdown (Fig. 6 C and Fig. S5 B), also indicating flexibility in trimer α -chain composition based on expression levels of the α -chains.

To complement RNAi-mediated reduction of EMB-9, we also increased EMB-9 protein levels by overexpressing a tandem-tagged *emb-9p::EMB-9::mNG::mRuby2* in EMB-9::mNG (C) knock-in animals and we overexpressed *emb-9p::EMB-9::mRuby2* in LET-2::mNG (C) knock-in animals. We used the mRuby2 tag to ensure that the overexpressed EMB-9 (marked with mRuby2) was being incorporated into the BM of these strains. The overexpression of EMB-9 increased EMB-9 levels ~36% in the BM of day 1 adults, and there was no significant change in LET-2 levels (Fig. 7, A and B; and Fig. S5 D). The LET-2/EMB-9 ratio decreased from 1.7 to 1.3 (Fig. 7 C and Fig. S5 E). There was also an increase in EMB-9::mNG aggregation in the body wall muscle but not LET-2::mNG (Fig. S5 F and Table S1), consistent with a preference for heterotrimer formation. Taken together, these experiments support the idea that there is a preference for LLE heterotrimer assembly, but also indicate that differences in α -chain expression can drive flexibility in trimer α -chain makeup at the BM.

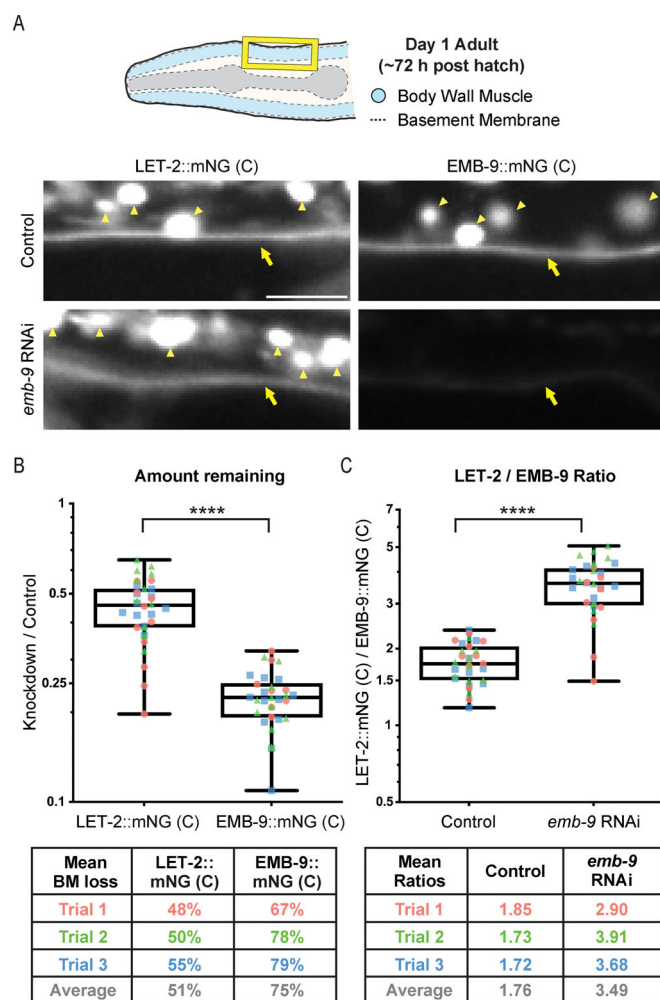


Figure 6. Reduction of *emb-9* expression increases the LET-2/EMB-9 BM ratio. (A) Top: a schematic diagram of a day 1 adult showing the head region and the location of the body wall muscle BM analyzed (yellow box). Below: single z-slice confocal images of LET-2::mNG (C) and EMB-9::mNG (C) along the body wall muscles of day 1 adults with and without *emb-9* RNAi treatment. The yellow arrows indicate body wall muscle BM and yellow arrowheads indicate intracellular aggregates. Aggregates were more prevalent in LET-2::mNG (C) animals after *emb-9* RNAi treatment (see Fig. S5 C). The scale bar is 5 μ m. (B) Top: boxplots show the percentage of LET-2::mNG (C) and EMB-9::mNG (C) protein (BM mean fluorescence intensity) remaining at the BM after *emb-9* RNAi-mediated knockdown. For each trial, mean fluorescence intensity measurements of LET-2::mNG (C) and EMB-9::mNG (C) with *emb-9* RNAi were normalized to the average of all mean fluorescence intensity measurements of LET-2::mNG (C) and EMB-9::mNG (C) with control RNAi (L4440 empty RNAi vector) from that trial (see Materials and methods, $n > 8$ animals for each of the three trials). **** $P > 0.0001$, unpaired two-tailed Student's t test with Welch's correction). Colors of the data points indicate different trials. Bottom: the percent loss of LET-2::mNG (C) and EMB-9::mNG (C) mean fluorescence intensity for each trial is listed in the table. Colors in the table correspond to data points in the boxplot. (C) Top: boxplots show the LET-2::mNG (C)/EMB-9::mNG (C) mean fluorescence ratios in the body wall muscle BM with *emb-9* RNAi and control RNAi (L4440 empty vector) treatment. LET-2::mNG (C) mean fluorescence intensity measurements were normalized to the average of all EMB-9::mNG (C) mean fluorescence intensity measurements within each trial ($n > 8$ animals for each of three trials). **** $P > 0.0001$, unpaired two-tailed Student's t test with Welch's correction). Bottom: table showing the ratios of the average BM fluorescence levels of LET-2::mNG (C)/EMB-9::mNG (C) for each trial. Colors in the table correspond to data points in the boxplot.

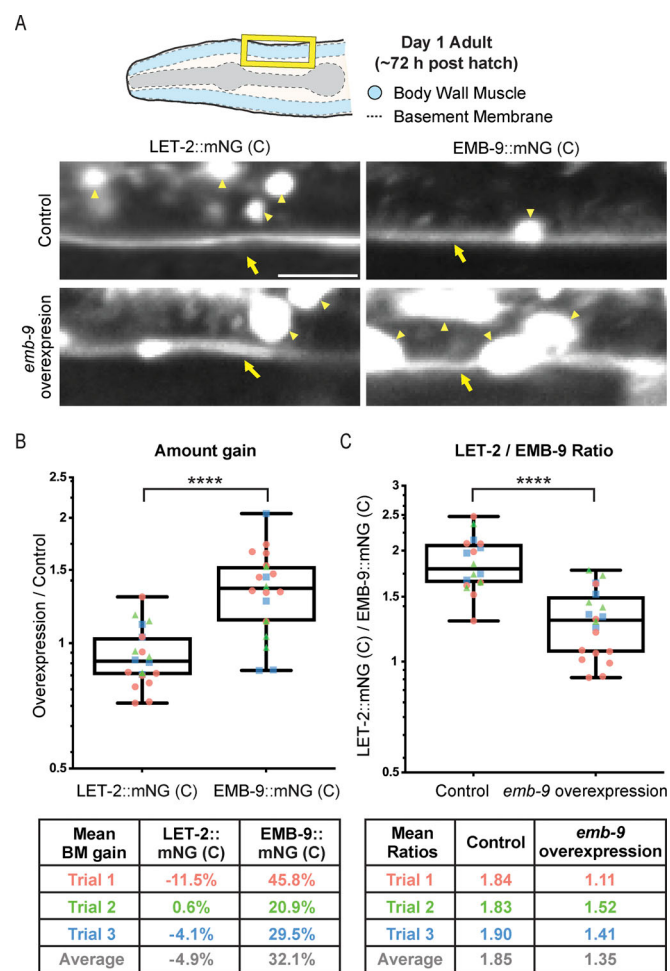


Figure 7. Increasing *emb-9* expression decreases the LET-2/EMB-9 BM ratio. (A) Top: a schematic diagram of a day 1 adult showing the head region and the location of the body wall muscle BM analyzed (yellow box). Below: LET-2::mNG (C) with and without *emb-9::mRuby2* overexpression and EMB-9::mNG (C) with and without *emb-9::mRuby2* overexpression in the body wall muscles of day 1 adults. The yellow arrows indicate body wall muscle BM and yellow arrowheads indicate intracellular aggregates. Aggregates were more prevalent in EMB-9::mNG (C) animals after *emb-9* overexpression (see Fig. S5 F). The scale bar is 5 μ m. (B) Top: boxplots show percent gain of LET-2::mNG (C) and EMB-9::mNG (C) protein (mean fluorescence intensity) in the body wall muscle BM after *emb-9* overexpression. For each trial, mean fluorescence intensity measurements of LET-2::mNG (C) and EMB-9::mNG (C) with *emb-9* overexpression were normalized to the average of all mean fluorescence intensity measurements of LET-2::mNG (C) and EMB-9::mNG (C) without overexpression (see Materials and methods, $n > 5$ animals for each of three trials). **** $P > 0.0001$, unpaired two-tailed Student's t test with Welch's correction). Bottom: the percent gain of both proteins for each trial is listed in the table. Colors in the table correspond to data points in the boxplot. (C) Top: boxplots show the LET-2::mNG (C)/EMB-9::mNG (C) mean fluorescence ratios in the body wall muscle BM with and without *emb-9* overexpression. LET-2::mNG (C) mean fluorescence intensity measurements were normalized to the average of all EMB-9::mNG (C) mean fluorescence intensity measurements within each trial ($n > 5$ animals for each of the three trials). **** $P > 0.0001$, unpaired two-tailed Student's t test with Welch's correction). Bottom: table showing the ratios of the average BM fluorescence levels of LET-2::mNG (C)/EMB-9::mNG (C) for each trial. Colors in the table correspond to data points in the boxplot.

Type IV collagen glycine substitution mutant alleles cause extracellular turnover defects

Pathogenic variants in *COL4A1* and *COL4A2* lead to the multi-system disorder known as Gould syndrome (Labelle-Dumais et al., 2024). The most common variants found in Gould syndrome are substitutions of glycine aa in the Gly-X-Y motifs of the triple helical domain, which lead to ER retention, reduced secretion, and defective BMs in mice and humans (Gould et al., 2007; Jeanne and Gould, 2017; Kuo et al., 2014; Murray et al., 2014). Similar to humans and mice, immunofluorescence studies in *C. elegans* have revealed that *emb-9* and *let-2* glycine missense mutant alleles that mimic Gould syndrome result in intracellular accumulation and decreased BM levels (Gupta et al., 1997). Treatment of mouse and human fibroblast cells with the chemical chaperone 4-phenylbutyrate (4PBA) increases secretion of mutant type IV collagen and increases incorporation into mouse BMs, but does not alleviate disease severity for all tissues, and can even increase skeletal myopathy for some *COL4A1* mutant alleles (Gould et al., 2007; Jones et al., 2019; Kuo et al., 2014; Labelle-Dumais et al., 2019). This suggests that type IV collagen harboring glycine substitutions might not function normally extracellularly. How these mutant forms may alter type IV collagen extracellular regulation and function, however, is unknown.

We next wanted to assess whether the ability to endogenously visualize fluorophore-tagged type IV collagen with quantitative live imaging could reveal new insights into how glycine substitutions affect type IV collagen extracellular regulation. Previous immunolocalization studies in *C. elegans* glycine substitution mutants in *emb-9* and *let-2* have reported apparent decreased type IV collagen α -chain BM levels, but these levels were not quantified (Gupta et al., 1997). We thus used genome editing to tag the temperature-sensitive glycine substitution mutants *emb-9* (*b117*) (EMB-9 G1173D) and *let-2* (*b246*) (LET-2 G1287D) with mNG at the C terminus (Fig. 8 A) (Gupta et al., 1997; Sibley et al., 1994). Animals harboring *emb-9* (*b117*) or *let-2* (*b246*) are homozygous-viable at the permissive temperature of 15°C but mothers shifted to 25°C have progeny that die as embryos or arrest as larvae (Gupta et al., 1997; Sibley et al., 1994).

We examined type IV collagen localization at the body wall muscle BM, where most type IV collagen is generated and secreted, and at the pharynx—a tissue that does not produce type IV collagen, but instead recruits it from extracellular fluid (Fig. 8, B and C) (Graham et al., 1997; Morrissey et al., 2016). We examined mNG (C)-tagged EMB-9 G1173D and LET-2 G1287D mutants arrested at the L1 larval stage and compared localization with mNG (C)-tagged EMB-9 and LET-2 wild-type L1 larvae. In line with previous studies, we saw extensive intracellular accumulation in mutant strains at nonpermissive temperatures (Fig. S5 G and Table S1). Interestingly, we found that the levels of mutant EMB-9 (G1173D) and LET-2 (G1287D) protein were higher in the body wall muscle BM compared with nonmutant control proteins, but the mutant protein levels were lower in the pharyngeal BM (Fig. 8, D and E). Consistent with a preference for heterotrimers, we found that when wild-type mNG-tagged EMB-9 and LET-2 α -chains were crossed into the respective untagged *let-2* (*b246*) and *emb-9* (*b117*) mutant backgrounds, we

observed similar intracellular accumulation, but also increased body wall muscle BM levels and decreased pharyngeal BM levels (Fig. 8, D and E; Fig. S5 G; and Table S1). As type IV collagen is trafficked from the body wall muscles to other tissues such as the pharynx, these results suggest that mutant type IV collagen might not be properly removed from the body wall muscle BM to allow for its distribution to other tissues.

To examine removal dynamics of mutant type IV collagen trimers from the body wall muscle BM, we crossed animals with endogenously tagged photoconvertible EMB-9::mEos2 and LET-2::mMaple into untagged *let-2*(*b246*) and *emb-9*(*b117*) mutants, respectively. We examined the removal of type IV collagen from the body wall muscle BM in wild-type and mutant animals by photoconverting the body wall muscle BM and assessing reduction in the photoconverted signal after 5 h. Approximately 46% and 56% of the photoconverted signal were removed in wild-type EMB-9 and LET-2 controls, respectively, after 5 h, while 30% and 33% were removed in EMB-9 (G1173D) and LET-2 (G1287D) mutants, respectively (Fig. 9, A–C). Together, these results indicate that glycine missense mutant α -chains cause BM accumulation defects that might result from decreased removal from BMs surrounding tissues that produce type IV collagen.

Discussion

Type IV collagen is built as a helical trimer, composed of three interweaved α -chains. Comparative studies in animals suggest that type IV collagen is a founding and core BM component with crucial structural and signaling roles throughout animal life (Fidler et al., 2018; Pöschl et al., 2004). Most vertebrates encode six α -chains, while *C. elegans* and *Drosophila* each has two (Fidler et al., 2017). Genetic and regulatory defects in type IV collagen are implicated in numerous human diseases and aging (Gatseva et al., 2019; Mao et al., 2015; Uspenskaia et al., 2004; Zhang et al., 2022). Thus, there is broad interest in understanding type IV collagen regulation and function. Endogenous fluorophore tagging has powerfully advanced the understanding of BM protein regulation and function in *Drosophila* and *C. elegans*, and more recently in zebrafish and mice (Stramer and Sherwood, 2024). Endogenous tagging of type IV collagen α -chains in *Drosophila*, *C. elegans*, and mice has almost exclusively used the *Drosophila* Vkg-GFP protein trap insertion site, where GFP is inserted at the start of the 7S domain near the N terminus of the protein (Jones et al., 2024; Keeley et al., 2020; Morin et al., 2001; Stramer and Sherwood, 2024). Insertion of fluorophores at this site is viable for *Drosophila* *vkg*, *C. elegans* *emb-9*, and mouse *COL4A2* α -chains (Keeley et al., 2020; Morin et al., 2001; Wuergeszen et al., 2025), but has not yet been attempted for *Drosophila* *Cg25C*, and is not viable for *C. elegans* *let-2* or mouse *COL4A1* α -chains (Jones et al., 2024; Keeley et al., 2020). As a result, there has been an inability to comprehensively examine type IV collagen regulation and composition using live imaging in any animal to date.

Using a health assay and localization analysis, we found that *C. elegans* *emb-9* tagged at the Vkg-GFP site, which we have named IS1, causes reduced health and extensive aggregation of the fluorophore-tagged protein in the ER of body wall muscle

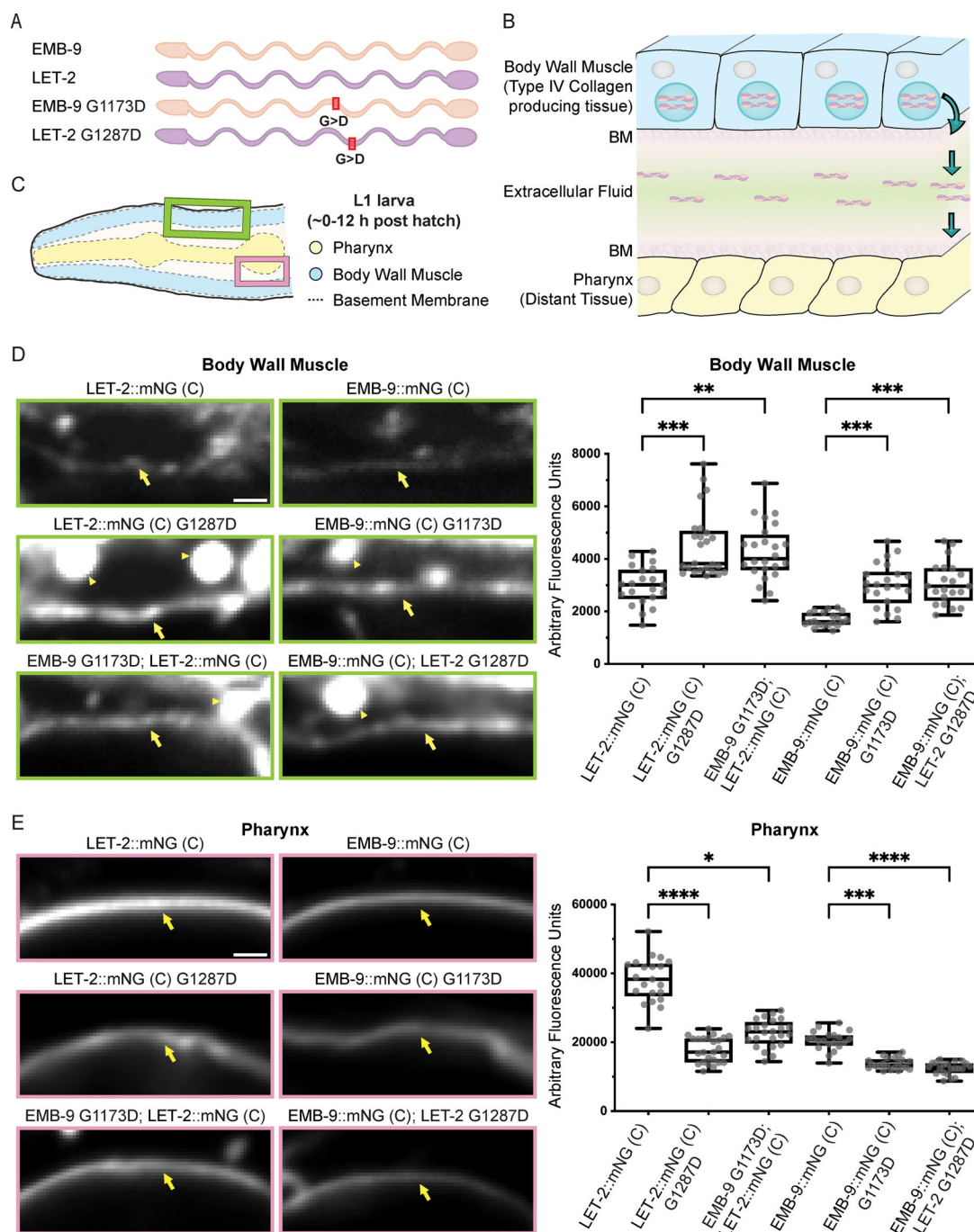


Figure 8. **EMB-9 and LET-2 glycine substitution mutants mimicking Gould syndrome accumulate at higher levels in body wall muscle BM.** **(A)** Schematic indicating the location of glycine-to-aspartic acid mutations in EMB-9 and LET-2 proteins. **(B)** Schematic of the *C. elegans* body wall muscles, which are the primary site of type IV collagen production. Secreted type IV collagen is incorporated into body wall muscle BM or trafficked in extracellular fluid and incorporated into the BM of distant tissues (such as the pharyngeal BM). **(C)** Schematic showing BM locations analyzed in L1 larvae. The green box corresponds to images in Fig. 8 D, and the pink box corresponds to images in Fig. 8 E. **(D)** Left: Single z-slice confocal fluorescence images in the body wall muscles of LET-2::mNG (C) and EMB-9::mNG (C) wild-type animals, LET-2::mNG G1287D and EMB-9::mNG (C) G1173D mutant animals, and animals with a wild-type fluorescently tagged protein paired with an untagged mutant protein: EMB-9 G1173D; LET-2::mNG (C) and EMB-9::mNG (C); LET-2 G1173D in the body wall muscles. Yellow arrows indicate body wall muscle BM, and yellow arrowheads indicate aggregates in body wall muscle cells (see Fig. S5 G). Right: Boxplots showing mean fluorescence intensity measurements of the body wall muscle BM ($n > 10$ animals per condition, $**P < 0.01$, $***P < 0.001$, Kruskal–Wallis test followed by Dunn’s multiple comparisons test). **(E)** Left: Single z-slice confocal fluorescence images in pharynx of LET-2::mNG (C) and EMB-9::mNG (C) wild-type animals, LET-2::mNG (C) G1287D and EMB-9::mNG (C) G1173D mutant animals, and animals with a wild-type fluorescently tagged protein paired with an untagged mutant protein: EMB-9 G1173D; LET-2::mNG (C) and EMB-9::mNG (C); LET-2 G1173D. Yellow arrows indicate the body wall muscle BM (note lack of intracellular aggregates in the pharynx, which does not express type IV collagen). Right: Boxplots showing mean fluorescence intensity measurements of pharyngeal BM ($n > 10$ animals per condition, $*P < 0.1$, $***P < 0.001$, $****P < 0.0001$, Kruskal–Wallis test followed by Dunn’s multiple comparisons test). The scale bars are 1 μ m, and all animals were grown at 25°C (restrictive temperature for *emb-9* and *let-2* mutants).

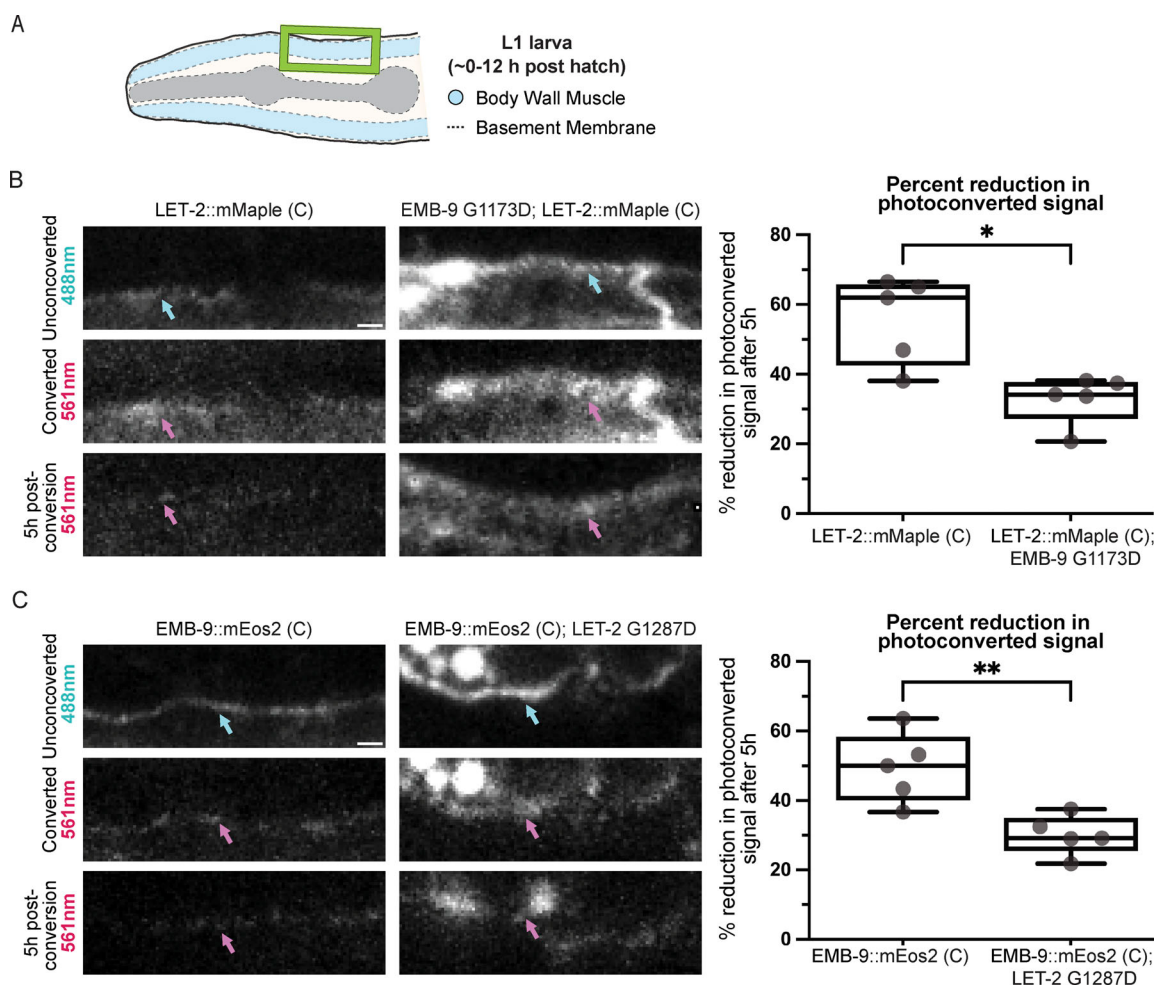


Figure 9. EMB-9 and LET-2 glycine substitution mutants have slower removal rates from body wall muscle BM. (A) Schematic indicates body wall muscle BM region analyzed (yellow box) in L1 larvae. (B) Left: Single z-slice representative confocal fluorescence images of the LET-2::mMaple (C) signal at the body wall muscle BM in wild-type animals (LET-2::mMaple [C]) or *emb-9* glycine mutant animals (EMB-9 G1173D; LET-2::mMaple [C]). Unconverted fluorophore signal (488-nm excitation), and converted fluorophore signal (561-nm excitation) immediately after photoconversion and 5 h after photoconversion (561-nm excitation) are shown. Cyan and magenta arrows point to unconverted and converted signals at the body wall muscle BM, respectively. Right: Boxplots show the percentage of the photoconverted signal reduced after 5 h ($n > 5$ animals, $P > 0.05$, unpaired two-tailed Student's *t* test). (C) Left: Single z-slice representative confocal fluorescence images of the EMB-9::mEos2 (C) signal at the body wall muscle BM in wild-type animals (EMB-9::mEos2 [C]) or *let-2* glycine mutant animals (EMB-9::mEos2; LET-2 G1287D). Unconverted fluorophore signal (488-nm excitation), and converted fluorophore signal (561-nm excitation) immediately after photoconversion and 5 h after photoconversion (561-nm excitation) are shown. Cyan and magenta arrows point to unconverted and converted signals at the body wall muscle BM, respectively. Right: Boxplots show the percentage of the photoconverted signal reduced after 5 h ($n > 5$ animals, $P > 0.05$, unpaired two-tailed Student's *t* test followed by the Mann-Whitney test). All animals were grown and recovered at 25°C (restrictive temperature for *emb-9* and *let-2* mutants). The scale bars are 1 μ m.

cells, where type IV collagen is predominantly expressed and secreted (Graham et al., 1997). Tagging *emb-9* at IS1 also reduced the amount of the EMB-9 protein localized to BM. We also found that *emb-9* tagged at an internal site within an interruption of the Gly-X-Y repeats (IS2) (Matsuo et al., 2019) had reduced health and had ER-localized aggregation of the tagged EMB-9 protein. In contrast, we discovered that tagging the *C. elegans* type IV collagen α -chains EMB-9 and LET-2 at the C terminus with a linker and many different fluorophores resulted in animals that were indistinguishable from wild-type animals in our health assay. Even animals that were homozygous for both labeled α -chains (e.g., EMB-9::mRuby2 [C]; LET-2::mNG [C]) were similar to wild-type animals. Further, these type IV collagen strains showed little to no internal protein aggregation in early

larval-staged worms. Western blot analysis also confirmed that the fluorophore mNG was stably associated with both EMB-9 and LET-2. One reason that the C terminus of type IV collagen α -chains might not have been selected for endogenous tagging previously is that the NC1 domain, which both selects for heterotrimer assembly and mediates dimerization of type IV collagen molecules extracellularly through sulfilimine bonds, is located at the C terminus (Fidler et al., 2018; Khoshnoodi et al., 2008). C-terminally tagged type IV collagen trimers could also cluster multiple fluorophores—up to six when both α -chains are tagged. Yet, we did not detect fluorophore quenching (Bae et al., 2021), suggesting that C-terminal site tagging, along with the 18-aa linker we used, does not cause quenching in native *C. elegans* BM.

One unexpected finding from our studies was the dramatic influence of different fluorophores inserted at IS1 (Vkg) on strain health. Insertion of the red fluorophores mRuby2 or mScarlet-I resulted in only mild delays in the time to starvation health assay for worm populations (1-day delay). In contrast, the insertion of mEos2 or mNG resulted in dramatically slower growth and long delays to starvation (5–8 days). All these genetically encoded fluorescent proteins are similar in size, share the characteristic β -barrel structure of the Aequorea GFP, and have similar mechanisms of fluorophore formation (Rodriguez et al., 2017). However, red fluorescent proteins and photoconvertible proteins have been constructed through extensive engineering of genes from a variety of anthozoan species and are thus encoded by significantly different aa (Bindels et al., 2017; Kredel et al., 2009; Thorn, 2017; Zhang et al., 2012). As a result, the folding rates and fluorophore formation rates vary significantly between fluorescent proteins (Balleza et al., 2018; Thorn, 2017). Our results suggest that these differences might impact the ability of type IV collagen α -chains to fold and trimerize in the ER, particularly when tagged at IS1 (Vkg), and negatively affect animal health.

Using our endogenous C-terminal fluorophore-tagged LET-2 and EMB-9 α -chains, our proteomics findings indicated that LET-2 is approximately twofold more abundant than EMB-9 in whole animals. This observation suggests that the *C. elegans* type IV collagen trimer is composed predominantly of two LET-2 (α 2-like chain) and one EMB-9 (α 1-like chain)—an LLE heterotrimer composition (Guo and Kramer, 1989). Notably, this composition does not follow the proposed mammalian rule of combining two odd-numbered (α 1-like) and one even-numbered (α 2-like) α -chain (Khoshnoodi et al., 2008). *Drosophila* also appears to not follow the mammalian rule. Hexamer isolation, NC1 recombination experiments, and transgenic NC1 domain expression suggest that the *Drosophila* type IV collagen trimer is predominantly Cg25C-Cg25C-Vkg (i.e., two α 2 [Cg25C] and one α 1 [Vkg] chain). This discrepancy, however, might simply be due to the difficulty of assigning clear homology of *C. elegans* and *Drosophila* NC1 sequences into vertebrate α 1- and α 2-like groups (Guo and Kramer, 1989; Summers et al., 2023).

A critical step in type IV collagen trimer maturation is catalyzed by prolyl 4-hydroxylase. In the absence of prolyl 4-hydroxylase or its cofactor ascorbate/vitamin C, type IV collagen α -chains cannot form trimers (Lamandé and Bateman, 1999). Work in *Drosophila* using the GFP-tagged Vkg protein showed that in prolyl 4-hydroxylase-deficient larvae, type IV collagen is not incorporated into BMs (Pastor-Pareja and Xu, 2011). These results strongly suggest that all type IV collagens within BMs are trimers and that fluorescence intensity of individual α -chains tagged with the same fluorophore at the same site can be used to assess the composite picture of BM trimers within the BM. Importantly, our quantitative fluorescence analysis of tissue-specific BMs revealed BMs did not have a strict LET-2/EMB-9 ratio of 2.0 (i.e., are not stringently LLE); instead, most were $\sim$ 1.7, whereas the spermathecal BM had a ratio of $\sim$ 2.2. These observations suggest a predominance of the LLE trimer, likely because LLE heterotrimer assembly is more efficient (i.e., higher molecular affinity), but also some flexibility in

trimer composition. LET-2/EMB-9 ratios >2 suggest the existence of LLL homotrimers, while ratios <2 indicate LEE and possibly EEE trimers. Analysis of translational reporters within specific tissues and experimentally increasing and decreasing α -chain expression showed that trimer compositional variability within BMs can be driven by differences in α -chain expression levels. This implies that the relative availability of α -chains for trimerization in the ER influences trimer makeup. Notably, our studies also revealed dependence between α -chains for secretion—decreasing the levels of EMB-9 led to the retention of LET-2 in cells, while increasing EMB-9 expression led to EMB-9 intracellular accumulation. This observation is consistent with studies showing that mutations in *emb-9* and *let-2* result in intracellular retention of the other (nonmutant) α -chain (Gupta et al., 1997). Together, these results suggest a preference for the in vivo assembly of the LLE heterotrimer, but also some flexibility in building other trimers in *C. elegans*.

Based on α -chain abundance and hexamer associations of the six α -chains in vertebrates, it has been proposed that only three (out of a possible 56) type IV collagen trimers are built— α 1 α 1 α 2, α 3 α 4 α 5, and α 5 α 5 α 6 (Khoshnoodi et al., 2008). Yet, studies on the α 1 α 1 α 2 trimer, the most prevalent and most investigated type IV collagen in vertebrates, suggest that other trimer combinations could exist—carcinoma cells secrete α 1 homotrimers (Haralson et al., 1985), reconstitution of NC1 domains has revealed many possible trimer combinations (Boutaud et al., 2000), and in vivo hexamer analysis has not excluded the existence of α 1 homotrimers or α 1 α 2 α 2 heterotrimers (Boutaud et al., 2000; Johansson et al., 1992; Siebold et al., 1988). Studies in *Drosophila* have also not ruled out variable trimer formation (Summers et al., 2023). Thus, flexible type IV collagen trimer combinations might occur in other organisms.

When assessing if trimer flexibility exists in other species, an important aspect to consider is the varied gene arrangement in *C. elegans* wherein the two type IV collagen α -chain-encoding genes are located on different chromosomes (Guo and Kramer, 1989). In other animals, including *Drosophila* and mammals, type IV collagen genes are arranged on chromosomes in a head-to-head orientation and share a common promoter (Khoshnoodi et al., 2008; Yasothornsrikul et al., 1997). The alternative arrangement in *C. elegans* may allow for unique regulatory mechanisms that permit variable transcriptional expression levels for each α -chain and therefore trimer flexibility. However, despite having a shared promoter, COL4A5 and COL4A6 show variable expression between tissues in humans. One possible mechanism driving this difference is that COL4A6 may have an alternative promoter as evidenced by the identification of two separate COL4A6 transcripts that vary in their 5' regions (Khoshnoodi et al., 2008; Sugimoto et al., 1994). Additionally, different microRNAs can regulate the expression of distinct type IV collagen α -chains. For example, in vivo and in vitro RNA expression profiles indicate that microRNA 29a downregulates the expression of five type IV collagen α -chains (COL4A1–COL4A5), while microRNA 29b specifically downregulates COL4A2 expression (Ma et al., 2023; Wang et al., 2012). Therefore, type IV collagen genes that share a promoter may still be differentially

expressed, potentially allowing for trimer flexibility in other species.

Trimer compositional variability likely expands the possible functions of type IV collagen and could allow for tissue-specific tailoring of type IV collagen trimer construction. For example, it has been hypothesized that the more restricted and tissue-specific vertebrate $\alpha3\alpha4\alpha5$ heterotrimer might have increased mechanical strength relative to that of the $\alpha1\alpha1\alpha2$ heterotrimer because of additional internal cross-linking (Gunwar et al., 1998; Naylor et al., 2021). Interestingly, we found that the spermathecal BM had a LET-2/EMB-9 ratio of ~ 2.2 , whereas other tissue BMs had a significantly lower ratio of ~ 1.7 . The spermatheca tissue is unique in that it undergoes repeated stretching every ~ 20 min during peak egg laying in *C. elegans* (Kelley and Cram, 2019). The distinct type IV collagen composition found in the spermathecal BM might mechanically support the ability of the spermatheca to repetitively stretch. Trimer α -chain compositional diversity could also increase the variety and specificity of interactions with other BM components, signaling molecules, and cell surface receptors (Khoshnoodi et al., 2008; Wang et al., 2008) to support many tissue-specific functions.

The most common human pathogenetic variants in the COL4A1 and COL4A2 genes are those that result in substitutions of glycine residues with other aa within the triple helical domain (Jeanne and Gould, 2017). These variants often impair the formation and secretion of COL4A1 and COL4A2 heterotrimers ($\alpha1\alpha1\alpha2$) and lead to Gould syndrome, which is a multisystem disorder involving variable defects in many tissues, including cerebrovascular, eye, kidney, brain, skeletal, and neuromuscular tissues (Labelle-Dumais et al., 2024; Mao et al., 2015). Notably, not all glycine mutations affect type IV collagen secretion (Kuo et al., 2014). Further, pharmacological treatment of mice harboring COL4A1 mutations with the chemical 4PBA, which enhances secretion and BM incorporation of mutant forms of type IV collagen, can decrease, increase, or have no effect on tissue pathology depending upon the mutation and tissue (Hayashi et al., 2018; Jones et al., 2019; Labelle-Dumais et al., 2019). These observations highlight the complex roles of type IV collagen in tissue function and suggest that mutations in type IV collagen can affect its extracellular functions. Through C-terminal tagging with mNG, we show that temperature-sensitive mutants harboring glycine substitutions in *emb-9* and *let-2* can be successfully labeled with fluorophores. We found that mutant forms of both α -chains led to type IV collagen retention in cells at the nonpermissive temperature, as previously reported using immunofluorescence (Gupta et al., 1997). However, we also discovered increased accumulation of mutant type IV collagen in the BM on the body wall muscle, which is a site of type IV collagen production and secretion, and decreased accumulation of type IV collagen on the pharynx—a tissue that does not produce type IV collagen (Graham et al., 1997). Through optical highlighting of photoconvertible mEos2- and mMaple-tagged type IV collagen trimers, we found that type IV collagens harboring glycine substitution mutations were more stably associated with the body wall muscle BM. This decrease in the type IV collagen BM on-off rate might not only increase accumulation in BMs surrounding cells and tissues

that secrete type IV collagen, but also decrease levels in BMs surrounding tissues such as the pharynx that depend upon type IV collagen trafficking away from secreting tissues (Duncan et al., 2020; Pastor-Pareja and Xu, 2011; Stramer and Sherwood, 2024). Consistent with this notion, a similar increase of type IV collagen in the body wall muscle BM and a decrease in the pharyngeal BM occur after loss of the extracellular collagen chaperone SPARC, which promotes type IV collagen trafficking from sites of synthesis (Morrissey et al., 2016; Pastor-Pareja and Xu, 2011). As type IV collagen levels can affect BM biomechanical and signaling properties (Crest et al., 2017; Jayadev and Sherwood, 2017b; Tian and Jiang, 2014), these alterations in type IV collagen BM accumulation could in part explain extracellular dysfunction of COL4A1 and COL4A2 variants.

Collagens are a major ECM superfamily composed of 46 α -chains that form 28 different collagens in mammals (I-XXVIII). All collagens share a triple helical domain, but they take on many forms, ranging from fibrils, to networks, and even membrane-spanning proteins, which together play essential roles in cell adhesion, mechanical support, and signaling (Ricard-Blum, 2011; Wakabayashi, 2021). Variants in collagens are implicated in nearly 40 human genetic diseases, most of which are glycine substitutions in the triple helical domain (Fidler et al., 2018). Although generally viewed as stable, static scaffoldings, work in *Drosophila* and *C. elegans* has revealed that type IV collagen has dynamic properties, including rapid polymerization, remodeling, and association and disassociation from the BM (Serna-Morales et al., 2023; Stramer and Sherwood, 2024). We expect that the tagging at the C terminus of mature α -chains (either extreme C terminus or prior to cleaved propeptide) will be broadly applicable to many collagens. Supporting this notion, the type XVIII collagen, the membrane-spanning COL-99, and the BLI-2 and BLI-6 cuticular collagens in *C. elegans* have been endogenously tagged with mNG at the C terminus and are homozygous-viable (Adams et al., 2023; Jayadev et al., 2022; Keeley et al., 2020). Endogenously tagging diverse collagen molecules with genetically encoded fluorophores offers to provide important insight not only into human disease, but also into the fascinating functions and likely underappreciated dynamics of this crucial ECM superfamily.

Materials and methods

C. elegans strains and growth conditions

C. elegans were maintained according to standard procedures on nematode growth medium (NGM) agar plates seeded with OP50 *E. coli* at 16°C or 20°C (Stiernagle, 2006). Strains used in this study are listed in Table S2.

DNA cloning

Endogenous knock-in constructs

All genome-edited strains were generated using CRISPR/Cas9-mediated homologous recombination with a self-excising cassette (SEC) for drug selection (hygromycin) as previously described (Dickinson and Goldstein, 2016; Keeley et al., 2020). For all C-terminally tagged strains, we placed an 18-aa flexible linker in frame and directly upstream of the fluorophore. The

flexible linker and fluorophore were inserted at the endogenous locus, just upstream of the stop codon. For *emb-9::mScarlet-I*, flexible linkers were placed on both sides of the fluorophore and the construct was inserted in frame at the same location as previously published IS1 constructs (Jayadev et al., 2022; Keeley et al., 2020). Primers used in CRISPR repair plasmid construction are listed in Table S3. We generated single guide RNA (sgRNA) plasmids by inserting the sequences into the pDD122 plasmid (Table S4) (Dickinson et al., 2013). Nucleic acid sequences of sgRNAs and aa sequences of flexible linkers are listed in Fig. S1.

Translational reporter constructs

A *C. elegans* codon-optimized P2A sequence was built using primers listed in Table S3. The PEST sequence was amplified from the plasmid pCFJ150 (Table S4) (Kaymak et al., 2016) using primers listed in Table S3. *let-2::mNG* (C) and *emb-9::mNG* (C) CRISPR repair plasmids were linearized by ClaI digestion. The P2A and PEST sequences were assembled with both linearized CRISPR plasmids by the Gibson assembly. C-site sgRNAs of *emb-9* or *let-2* were used to direct Cas9 cleavage for homologous insertion.

Transcriptional reporter construct

The ~2-kb *let-2* upstream regulatory sequence was amplified from wild-type genomic DNA using the primers listed in Table S3. The mNG sequence was amplified from the *emb-9::mNG* (C) CRISPR repair plasmid. Both fragments were ligated into the pAPO88 starter repair plasmid (Pani and Goldstein, 2018) containing homology arms for the tTt4348 site using the Gibson assembly. This single-copy transgene was inserted into the tTt4348 transposon insertion site on chromosome I (Frøkjær-Jensen et al., 2012) using the SEC CRISPR repair plasmid as described above. The pCFJ352 plasmid (Table S4) containing the sgRNA was used to direct Cas9 cleavage near this region for insertion.

Overexpression constructs

The *emb-9* overexpression construct *emb-9p::emb-9::mRuby2::emb-9 3'utr* (pQD03) was built by Gibson assembling by ligating the following fragments into pBluescript SK(-): the ~3.8-kb *emb-9* upstream regulatory sequence, the full-length *emb-9* gene, an ~500-bp *emb-9 3'utr* (all amplified from N2 genomic DNA sequence), and mRuby2 amplified from the *emb-9::mRuby2*(C) CRISPR repair plasmid (using primers listed in Table S3). To create the *emb-9p::emb-9::mNG::mRuby2::emb-9 3'utr* (pQD04) construct, the mNG sequence amplified from the *emb-9::mNG* (C) CRISPR repair plasmid was inserted into pQD03 using the Gibson assembly.

Creation of transgenic strains

Mixtures of the sgRNA plasmid (5–10 ng/μl), the SEC repair template plasmid (100 ng/μl), and co-injection marker plasmids (pCFJ90 [*myo-2p::mCherry*] and pCFJ104 [*myo-3p::mCherry*], 5 ng/μl each) (Table S4) were injected into the gonads of young adult N2 hermaphrodites as previously described (Dickinson and Goldstein, 2016). mNG knock-in strains harboring glycine

mutation alleles for *emb-9* and *let-2* were generated using the respective C-site mNG CRISPR repair plasmid and C-site sgRNA sequence (described in *Endogenous knock-in constructs*). Translational reporters for *emb-9* and *let-2* were generated using the respective C-site sgRNA sequence (described in *Endogenous knock-in constructs*). The *let-2* transcriptional reporter was generated using the sgRNA sequence in the pCFJ352 plasmid (Table S4) that targets the tTt4348 transposon insertion site on chromosome I. Following injection, animals were singled onto OP50 plates and allowed to lay eggs at 20°C for 2–3 days before treating with 500 μl of hygromycin (2 mg/ml). After 3–4 days at 20°C posthygromycin treatment, surviving candidate knock-in animals were identified by the dominant roller phenotype (*sqt-1(el350)*) and an absence of co-injection marker fluorescence. To excise the SEC from homozygous-viable lines, 4–8 L3/L4 animals were heat-shocked in a 34°C water bath for 4 h and then left to grow at 25°C for 3–4 days. Progeny lacking the roller phenotype were singled, and edited lines were identified by visualizing the fluorophore at the BM and via PCR genotyping DNA using the primers listed in Table S3.

emb-9 overexpression strains

Transgenic worms expressing *emb-9p::emb-9::mRuby2::emb-9 3'utr* or *emb-9p::emb-9::mNG::mRuby2::emb-9 3'utr* were created by injecting pQD03 or pQD04 (200 ng/μl) with the plasmid pRF4 containing *rol-6(su1006)* that causes a dominant “roller” phenotype (50 ng/μl), EcoRI-digested salmon sperm DNA (30 ng/μl), and the empty pBluescript II vector (40 ng/μl) into the gonads of young adult LET-2::mNG or EMB-9::mNG hermaphrodites, respectively (Yochem and Herman, 2003). The pQD03 and pQD04 were expressed as extrachromosomal arrays, which often have mosaic somatic expression (Yochem and Herman, 2003). mRuby2 fluorescence was used to confirm transgene expression and BM localization of the overexpressed protein. The dominant roller phenotype was used to track transgene propagation. Young adult rollers (48 h after hatch) were imaged for the experiments.

RNAi

RNAi experiments were performed using the feeding method (Timmons and Fire, 1998), and the *emb-9* RNAi construct was obtained from the Ahringer library (Fraser et al., 2000; Kamath et al., 2003). RNAi bacterial cultures were grown in selective media at 37°C for 18 h and then for one additional hour after adding IPTG (1 mM final concentration) (Table S4) to induce double-stranded RNA expression. Cultures were then used to seed NGM plates that had been treated with a 1:1 mixture of 1 M IPTG and 100 mg/ml ampicillin (18 μl per plate) (Table S4). Plates were allowed to dry overnight after seeding. Synchronized L1 larvae were placed on the seeded plates and allowed to grow for 72 h at 20°C. The L4440 empty vector was used as a control (Timmons and Fire, 1998).

Image acquisition

Confocal fluorescence imaging

Confocal images (excluding photoconversion experiments) were acquired at 20°C on Zeiss Axio Imager A1 microscopes, fitted

with either a Yokogawa CSU-10 or a Yokogawa CSU-W1 spinning disk, and either an ORCA-Fusion CMOS or ORCA-Quest qCMOS camera using 488-, 505-, and 561-nm excitation lasers. All microscopes were controlled with μ Manager software v1.4.23 or v2.0.1 (Edelstein et al., 2010). Zeiss 40 $\times$, 63 $\times$, and 100 $\times$ Plan Apochromat (1.4 numerical aperture) oil-immersion objectives were used. Animals were mounted on 5% noble agar pads with 0.002 M sodium azide (Table S4) for L1s and 0.01 M sodium azide for all other stages (Kelley et al., 2017).

Fluorescence recovery after photobleaching experiments

For fluorescence recovery after photobleaching (FRAP) experiments, photobleaching was performed using an iLas² targeted laser system from BioVision equipped with an Omicron Lux 60 mW 405 nm continuous wave laser and controlled with MetaMorph version 7.10.4.407 software (Keeley et al., 2020). Laser power and duration of photobleach were consistent for all animals within an experiment. Imaging intervals and duration allowed for fluorescence recovery were determined empirically to capture quantifiable recovery. For all FRAP experiments, worms were immobilized on 5% agar pads with 0.002 M sodium azide. The half of the pharyngeal terminal bulb opposite to the L1 gonad was photobleached. Animals were then rescued from the slide and washed with M9 to dilute the sodium azide treatment. Recovery was assessed using the same imaging settings after allowing the worms to develop normally for 5 h. Animals rescued after posterior pharynx photobleaching all developed normally and had no pharyngeal morphological defects ($n = 30/30$ animals).

Optical highlighting/photoconversion experiments

Photoconversion experiments were performed at 20°C on an inverted Zeiss 880 LSM confocal mounted to a Zeiss Axio Observer Z1 microscope using a Zeiss 63 $\times$ Plan Apochromat (1.4 numerical aperture) oil-immersion objective and a pinhole size of 57 μ m. The photoconvertible mMaple and mEos2 fluorophores were photoconverted with a 405-nm laser, and photoconversion settings were the same between samples. Unconverted mMaple and mEos2 were imaged using a 488-nm laser and converted mMaple and mEos2 with a 561-nm laser. For all optical highlighting experiments, worms were immobilized on 5% agar pads with 0.002 M sodium azide. An $\sim 350\text{-}\mu\text{m}^2$ section of the body wall muscle BM EMB-9::mEos2 or LET-2::mMaple between the pharyngeal bulbs was photoconverted. Animals were then rescued from the slide and washed with M9 to dilute the sodium azide. Recovery was assessed using the same imaging settings after allowing the worms to develop normally for 5 h. Animals rescued all developed normally and had no morphological defects ($n = 20/20$ animals).

Image analysis, processing, and quantification

LET-2 and EMB-9 BM fluorescence measurements and protein translation assessment

Single z-slice confocal images were quantified using FIJI (2.0) (Schindelin et al., 2012). BM fluorescence intensity was measured by drawing 3 pixel-wide lines along the BM of interest outlined below and recording the mean fluorescence value.

Pharyngeal BM measurements were recorded from both sides of the terminal bulb and averaged. Body wall muscle BM measurements were recorded on both sides of the head region flanking the pharynx and averaged. The spermathecal BM was measured on the side proximal to unfertilized eggs. Measurements were recorded on both dorsal and ventral sides of the organ (in regions where it was not folded in on itself) and averaged together. DTC BM measurements were taken around the entire cell. To account for autofluorescence, background measurements were acquired by drawing a line either next to the pharynx (for pharyngeal or body wall muscle BM measurements) or inside the gonad (DTC and spermathecal BM measurements), where no type IV collagen signal was apparent. mNG translational reporter measurements were taken by recording the mean fluorescence value in a single z-slice confocal image for the entire DTC, the entire spermatheca, or two body wall muscles flanking both sides of the pharynx (which were then averaged together). To account for autofluorescence, background measurements were acquired by drawing a line either next to the pharynx (for body wall muscle translation measurements) or inside the vulva (for DTC and spermatheca translation measurements), where no translation reporter signal was apparent.

Normalization of data for LET-2/EMB-9 BM protein and translation reporter ratios

In Fig. 4 B, Fig. 6 C, and Fig. 7 C, to directly compare LET-2/EMB-9 ratios between tissues, LET-2::mNG mean fluorescence intensity data points were normalized to the mean value of EMB-9::mNG within that tissue for each trial. Specifically, each data point in the boxplots was normalized with the following formula: $(L/\text{mean } E)$, where “L” is a single mean fluorescence intensity value measurement of LET-2::mNG in a tissue BM, and “mean E” is the mean value of all EMB-9::mNG measurements within the same tissue BM for the trial. For Fig. S4 A, Fig. S5 B, and Fig. S5 E, the EMB-9::mNG data points were normalized in a similar way to display the spread of data around the mean. Specifically, the formula used was $(E/\text{mean } E)$, where “E” is a single measurement of EMB-9::mNG mean fluorescence in a tissue BM. For Fig. 5 B and Fig. S4 D, the same normalization methods were used, substituting measurements of *let-2* translation reporter for L and *emb-9* translation reporter for E.

Normalization of data for RNAi and overexpression experiments

To compare the percent loss or gain of EMB-9::mNG or LET-2::mNG in RNAi or overexpression experiments, the data points in Fig. 6 B and Fig. 7 B were normalized by dividing each treatment mean fluorescence value measurement by the mean value of the control measurements within that trial. Specifically, the formula used was $(T/\text{mean } C)$, where “T” is a single fluorescence intensity measurement from the treatment animal, and “mean C” is the mean value of all measurements from the control animals within a trial. For Fig. S5 A and Fig. S5 D, data points were normalized in a similar way to show the spread of control measurements around the mean. Specifically, the formula used was $(C/\text{mean } C)$, where “C” is a single measurement from the control animals.

Quantification of ER-localized type IV collagen muscle aggregates

The number of EMB-9::mScarlet-I (IS1) and EMB-9::mCherry (IS2) fluorescent aggregates that localized within the ER (marked with the ER membrane protein ELO-1::mNG) in body wall muscles (Fig. 2 B and Fig. S2 B) was scored in a blinded manner—aggregates were first identified, and then, it was determined whether they were within the ER. A total of 10 aggregates were counted per animal, and 10 animals were analyzed for the EMB-9::mScarlet-I (IS1) and EMB-9::mCherry (IS2) strains.

Quantification of type IV collagen muscle aggregates

Sum projections of the first four slices of the body wall muscle from confocal stacks of animals from Figs. 2, 6, 7, and 9 were generated. A manual threshold was applied to these images to create a binary mask of the aggregates. The aggregate area in the body wall muscle was estimated by drawing a polygon of area ~4,000 pixels in the body wall muscle and measuring the raw integrated density. The aggregate area measured was then divided by the total area of the polygon to report the percent area occupied by aggregates in the body wall muscle.

FRAP analysis

For FRAP analysis, the mean fluorescence intensity values were generated by drawing an ~0.3-μm-wide and 3.825-μm-long line through a clearly defined section of the midplane of a single z-slice of the pharyngeal terminal bulb. The background was calculated by drawing an equivalent line within the terminal bulb of the pharynx, where no type IV collagen is produced or localized. Mean fluorescence intensity was measured within the photobleached region and within an equivalent region on the unbleached side of the terminal bulb before bleach (prebleach), immediately after bleach (bleach), and 5 h after bleach (5 h postbleach). A bleach correction factor was used to account for general photobleaching during image acquisition across the duration of the experiment (Gianakas et al., 2023). In brief, the fluorescence intensity measurement in the unbleached region at the 5-h postbleach time point was divided by the value at the prebleach time point to obtain the bleach correction factor. Fluorescence intensity measurements at the prebleach and bleach time points in the photobleached region were then multiplied by the bleach correction factor to normalize these values to the 5-h postbleach time point. The background signal was then subtracted from bleach-corrected values and used in analysis. Recovery was calculated by dividing the measurement at the 5-h postbleach time point by that at the prebleach time point.

Photoconversion/optical highlighting

For photoconversion analysis, mean fluorescence intensity values were generated by drawing an ~0.3-μm-wide and 3.825-μm-long line through a clearly defined section of the body wall muscle BM located between the two pharyngeal bulbs in a single z-slice image. The background was calculated by drawing an equivalent line next to the pharynx. Mean fluorescence intensity of the red channel was measured within the converted region immediately after photoconversion (conversion) and 5 h after

photoconversion (5 h postconversion). The mean background signal was then subtracted from mean fluorescence intensity values and used for analysis. Percent reduction in the photoconverted signal was calculated by dividing the value at the 5-h postphotoconversion time point by the value at the conversion time point and subtracting that fraction from 1.

Percent reduction in photoconverted signal

$$= 1 - \frac{\text{Fluorescence value at 5 h postconversion}}{\text{Fluorescence value at conversion}}$$

Time to starvation assay

For each replicate, four L4 hermaphrodites were placed on an NGM plate seeded with 400 μl of OP50 *E. coli*. Plates were maintained at 20°C and checked twice daily for starvation. For every experiment, N2 wild-type animals were plated on the same day, using the same batch of plates. The time it took for experimental strains to starve was reported relative to the time it took for the corresponding N2 plates to starve.

Western blots

Mixed populations of worms were washed off NGM plates with sterile water and placed into 1.5-ml Eppendorf tubes. Animals were then washed three times with sterile water and frozen in liquid nitrogen. Frozen animals were then ground to a powder under cryogenic conditions with a mortar and pestle and then resuspended in cooled RIPA buffer plus 1X SIGMAFAST Protease Inhibitor Cocktail (Table S4). Laemmli sample buffer and β-mercaptoethanol (Table S4) were then added to a final concentration of 1X and 4%, respectively. The final concentration of sample powder to buffer was 0.5 mg/ml. Samples were boiled for 5 min, and then, 15 μl of resuspended samples was loaded onto 4–15% gradient Mini-PROTEAN TGX Gels (Table S4) and run at 100 V. Wet transfers to nitrocellulose were carried out overnight in a cold room at 30 V for 16 h. Nitrocellulose membranes were blocked with 5% milk in TBST for 1 h, incubated with primary antibody against mNG in 1% milk and TBST overnight at 4°C (antibody dilution 1:1,000), and then treated with secondary peroxidase-conjugated antibody (Table S4) for 1 h at room temperature. Blots were treated with SuperSignal West Atto Ultimate Sensitivity Chemiluminescent Substrate (Table S4) for 5 min before imaging. Blots were imaged on an iBright 1500 imaging system (Invitrogen).

Sample fractionation and processing for mass spectrometry

Samples were processed by chemical fractionation to enrich for BM proteins for mass spectrometry-based proteomics as described previously (Morais et al., 2023). Briefly, a mixed population of L1, L2, and L3 larvae were collected in a Tris lysis buffer (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 25 mM EDTA added of EDTA-free Roche cOmplete Protease Inhibitor Cocktail) and homogenized to extract soluble proteins. After centrifugation at 14,000 × g, the protein supernatant was collected. This fraction is enriched for soluble cellular proteins. The remaining pellet was then resuspended in an alkaline detergent buffer (20 mM NH₄OH and 0.5% Triton X-100 in 0.1 M PBS) to disrupt cell-matrix bonds and centrifuged again, and the supernatant

was removed. The final pellet was resuspended in TEAB/SDS lysis buffer (50 mM TEAB and 5% SDS in LC-MS grade water). This matrix-enriched fraction was sonicated with Covaris LE220+ Focused Ultrasonicator (Covaris). The cellular fraction was added to 2X TEAB/SDS lysis buffer, and then, both soluble and insoluble protein lysates were reduced with 5 mM dithiothreitol and further alkylated with 15 mM iodoacetamide and processed for overnight digestion at 37°C in S-Trap columns (ProtiFi) using Sequencing Grade Modified Trypsin (Catalog # V5111; Promega). The peptide mixtures were eluted from the columns, desalted, and dried to completeness by vacuum centrifugation for mass spectrometry.

Mass spectrometry data acquisition and analysis

Peptide samples were analyzed by LC-MS/MS with a Thermo Exploris 480 mass spectrometer (Thermo Fisher Scientific). The raw spectra data were analyzed using MaxQuant version 2.5.0.0 (Cox and Mann, 2008). MS/MS data were searched against a modified version of the UniProt *C. elegans* proteome (v. 2024_01; containing 26,706 reviewed and unreviewed entries, with the addition of the protein sequence for mRuby2 and mNG [downloaded from <https://www.fpbases.org/>]) using MaxQuant's integrated search engine, Andromeda. Carbamidomethylation of cysteine was specified as a fixed modification, and oxidation of methionine, proline, and lysine, and N-terminal acetylation as variable modifications. Up to two missed tryptic cleavage sites were allowed. Mass tolerance settings for precursor peptide ions were 20 ppm for the first MS/MS search and 4.5 ppm for the main MS/MS search, with 0.5 Da for fragment ions. A target-decoy search strategy was employed to control false-positive identifications, maintaining a 1% false discovery rate (FDR) for both peptide-spectrum matches and protein groups. The match-between-runs algorithm was enabled to increase the number of identifications. Contaminants were identified from matches to the MaxQuant contaminants database. Data processing and analysis were performed using RStudio version 2023.9.1.494 (<https://rstudio.com>). Peptide data obtained with MaxQuant were processed using the QFeatures package version 1.12.0 (Gatto, 2024). Peptides from decoy and contaminant proteins, nonunique, and detected in only one sample were removed. Peptide intensities were log₂-transformed and normalized through median centering. Peptide data were summarized to protein groups via robust summarization, and statistical comparisons were performed with multiple *t* tests with the Benjamini-Hochberg FDR correction using the msqrob2 package (Goeminne et al., 2016, 2020).

Statistical analysis

Statistical analysis was performed in GraphPad Prism 10. The distribution of data was assessed for normality using the Kolmogorov-Smirnov test. To compare fluorescence intensities or peptide levels between two populations, we used an unpaired *t* test for normally distributed data or a Mann-Whitney test for data that were not normally distributed. For Fig. 6, we used a *t* test with Welch's correction because the variance between datasets was unequal. To compare fluorescence intensities between three or more populations, we

used a one-way ANOVA followed by Tukey's multiple comparisons test for normally distributed data with equal variance between groups or a Brown-Forsythe and Welch ANOVA test followed by a Dunnett's test for normally distributed data with unequal variance between groups. For data that were not normally distributed, we used a Kruskal-Wallis test followed by Dunn's test. Sample sizes were determined before each experiment. Sample sizes of at least 10 were chosen for each biological replicate of experiments in Fig. 2, A and B; Fig. 4 A; Fig. 5; and Fig. 8. Where protein ratios were calculated, three biological replicates of those experiments were carried out to ensure reproducibility of the results. Sex was not a factor in analyzing data because *C. elegans* primarily exist as hermaphrodites. Data points were only excluded if the animal moved during acquisition or developed vacuoles and died.

Online supplemental material

Fig. S1, related to Fig. 1, shows the endogenous locus of fluorophore insertion for all genome-edited strains. Flexible linker sequences and Cas9 guide RNA sequences are listed. The fluorophore aa length and aa sequence similarity between fluorophores are shown. Fig. S2, related to Fig. 2, shows intracellular aggregates of LET-2::mNG (C) and EMB-9::mNG (C) in the body wall muscles of *C. elegans* as they develop from L1 larvae to gravid adults. Also shown are the localization of EMB-9::mCherry (IS2) aggregates within the ER and FRAP analysis of LET-2::mNG (C), EMB-9::mNG (C), and EMB-9::mNG (IS1) at the pharyngeal BM. Fig. S3, related to Fig. 3, shows the abundance of mNG and mRuby2 proteins determined by LC-MS/MS in the larval cellular fractions of strains expressing the knock-in proteins Strain 1 (EMB-9::mRuby2 [C]; LET-2::mNG [C]) and Strain 2 (EMB-9-mNG [C]; LET-2::mRuby2 [C]). Also shown is the comparable abundance of the LET-2 and EMB-9 protein in the matrix fractions of both strains. Fig. S4, related to Figs. 4 and 5, shows the normalized LET-2::mNG (C) data with the normalized EMB-9::mNG (C) data and the normalized LET-2 translation reporter (*let-2::P2A::PEST::mNG*) data with the normalized EMB-9 translation reporter (*emb-9::P2A::PEST::mNG*) data for all tissues and trials. Also shown is a western blot confirming the stability of mNG associated with the LET-2::mNG (C) and EMB-9::mNG (C) fusion proteins and mean fluorescence intensity measurements of both LET-2::mNG (C) and EMB-9::mNG (C) at the pharyngeal BM of homozygotes and heterozygotes. Fig. S5, related to Figs. 6, 7, and 8, shows the normalized LET-2::mNG (C) data with the normalized EMB-9::mNG (C) data and the normalized treatment (RNAi or overexpression) data with the normalized control (L4440 empty vector RNAi or absence of overexpression) data for all trials. Also shown are intracellular aggregates of LET-2::mNG (C) and EMB-9::mNG (C) in the muscles of day 1 adult *C. elegans* after *emb-9* RNAi and *emb-9* overexpression. Additionally, sum projections from confocal z-stacks of L1 larvae carrying *let-2* or *emb-9* glycine mutant alleles grown at the restrictive temperature are shown. Table S1 reports the quantification of intramuscular aggregates. Table S2 outlines the strains used in this study. Table S3 shows the primer sequences used in this study. Table S4 lists the chemical and DNA reagents used.

Data availability

Plasmids generated, *C. elegans* strains generated, and data are available upon request. Strains will be donated to the Caenorhabditis Genetics Center (CGC). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al., 2022) with the dataset identifier PXD055575 and <https://doi.org/10.6019/PXD055575>. All other data are included in the manuscript and supplemental materials.

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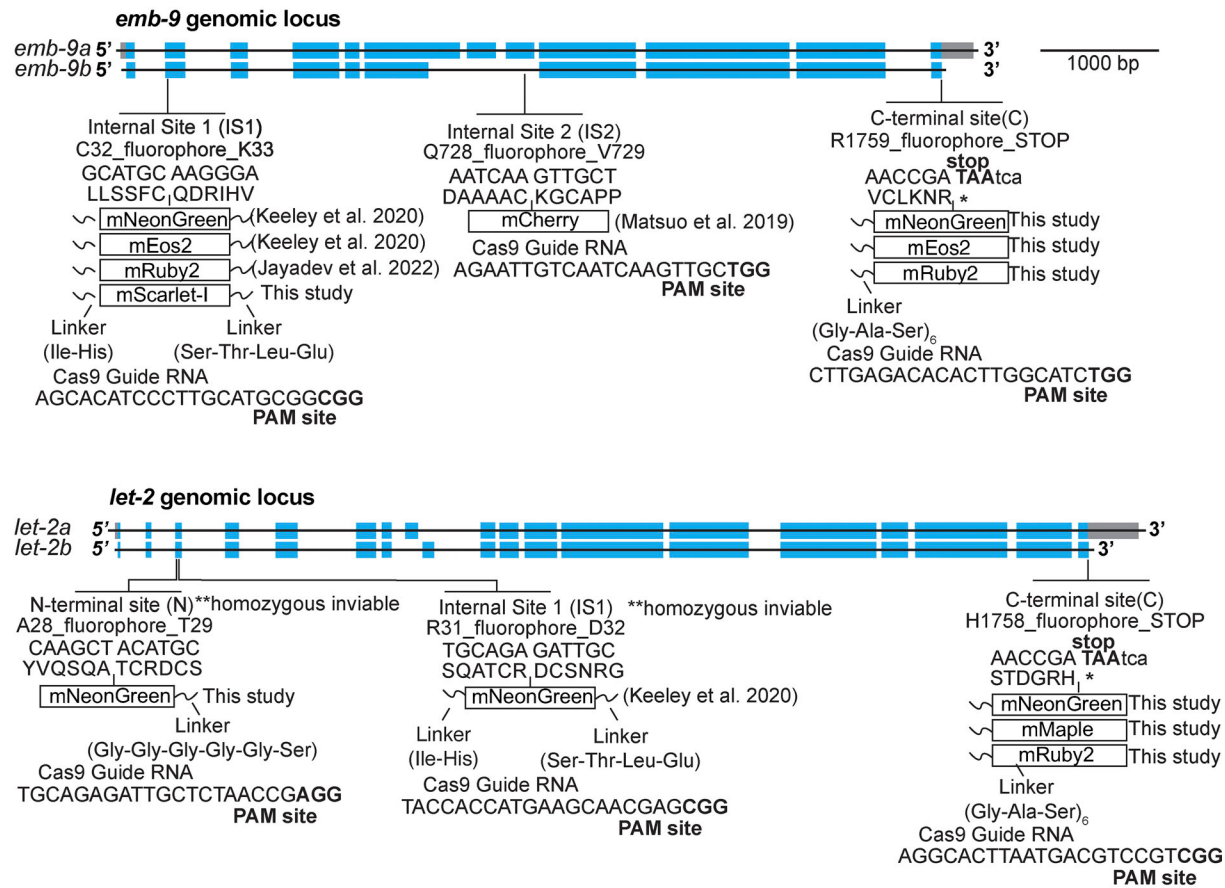
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Supplemental material

A



B

Fluorophore	Length (aa)	Fluorophore	mNG	mEos2	mMaple	mScarlet-I	mCherry	mRuby2
mNG	236	mNG	100.00	24.18	29.92	29.10	31.15	27.05
mEos2	226	mEos2		100.00	74.59	48.36	44.67	48.36
mMaple	237	mMaple			100.00	54.92	53.69	50.82
mScarlet-I	232	mScarlet-I				100.00	85.66	59.84
mCherry	236	mCherry					100.00	55.74
mRuby2	237	mRuby2						100.00

Figure S1. **Knock-in sites and comparisons between different genetically encoded fluorophores. (A)** Schematic illustrates the *C. elegans* type IV collagen genes *emb-9* and *let-2*. Exons are depicted as blue boxes. The lines indicate the endogenous sites where the indicated fluorophores were inserted, and listed underneath are the two aa the fluorophore was inserted between. The precise nucleic acid sequence at the insertion site and translated aa sequence are listed. Linkers used to flank fluorophores are depicted as wavy black lines, and their sequences are listed. For each knock-in site, the sgRNA sequence(s) and PAM site used to target the gene for CRISPR-mediated homologous recombination are shown. Strains with mNG knocked in at the *let-2* N-terminal site (N) or *let-2* IS1 were homozygous-inviable and were not further evaluated in this study. **(B)** Left: The table shows the aa length of each fluorophore used in this study. Right: The table shows the aa sequence similarity between each pair of fluorophores.

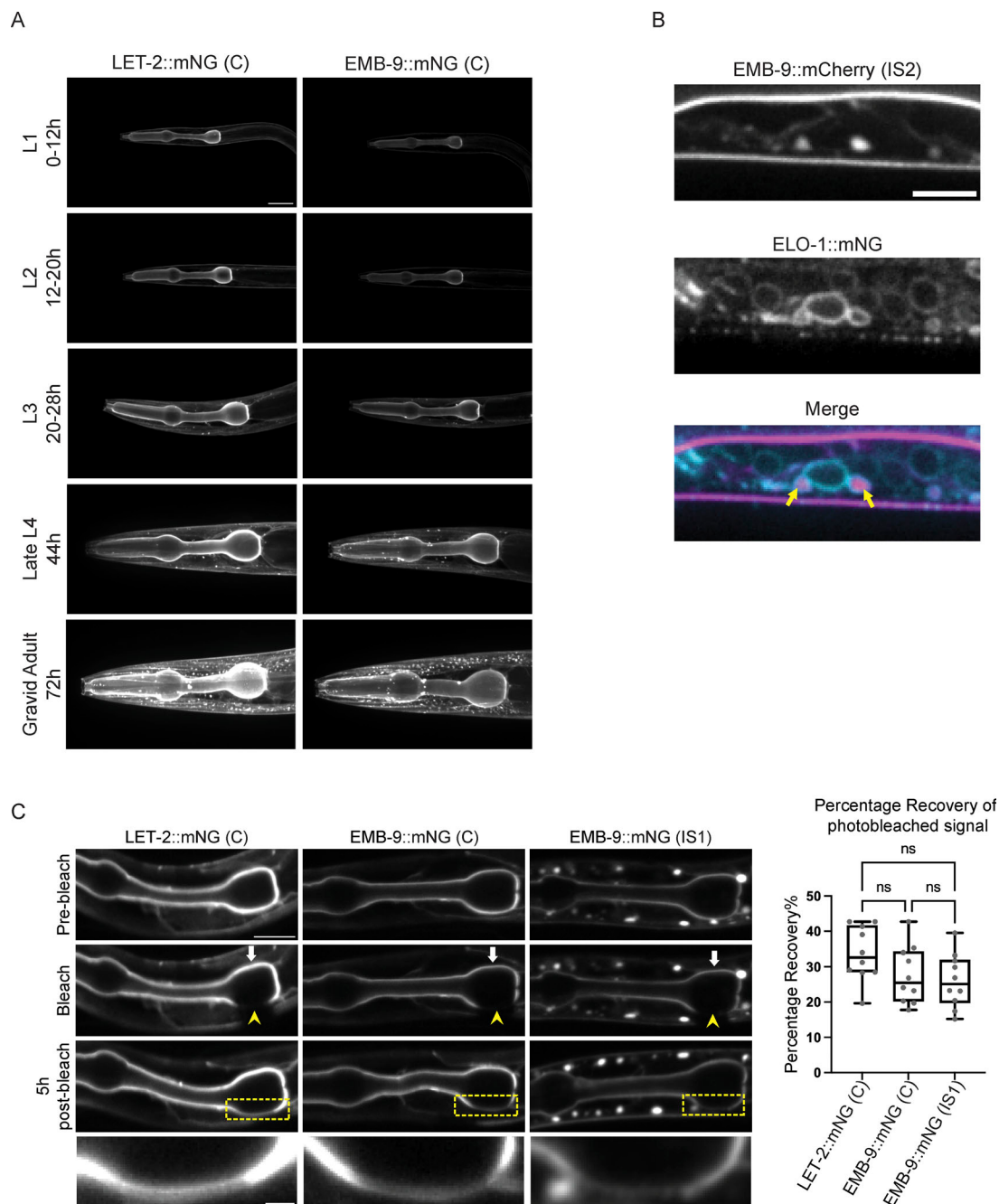


Figure S2. Internalization and FRAP analysis of LET-2 and EMB-9 knock-ins. **(A)** Representative sum projections of confocal fluorescence z-stacks of LET-2::mNG (C) and EMB-9::mNG (C) at the L1, L2, L3, late L4, and gravid adult stages. Small aggregates in the body wall muscle were first detected in L3 larvae (47% of LET-2::mNG [C] and 73% of EMB-9::mNG [C], $n > 10$ each) and continued into gravid adult (100% for LET-2::mNG and EMB-9::mNG, $n > 10$ each). The scale bar is 20 μ m. **(B)** Single z-slice fluorescence image of EMB-9::mCherry (IS2) muscle aggregates (magenta in merge) localized within the ER (visualized with ELO-1::mNG; cyan in merge) in L1 larvae. Yellow arrows point to aggregates in the ER ($n = 10$ animals; 10 aggregates counted per animal; 99/100 aggregates localized to the ER). The scale bar is 5 μ m. **(C)** Left: Single z-slice images of LET-2::mNG (C), EMB-9::mNG (C), and EMB-9::mNG (IS1) prior to, immediately after, and 5 h after FRAP at the L1-proximal pharyngeal bulb. Yellow arrowheads point to photobleached regions. White arrows point to unbleached regions used as the control region for analysis. Dotted boxes indicate the location of magnified regions shown in the panel below. The scale bar is 1 μ m for magnified panels. The scale bar is 10 μ m for all other panels. Right: Boxplots show the percentage of fluorescent signal recovered after 5 h ($n = 10$ animals, $P > 0.05$, ANOVA with Tukey's multiple comparisons).

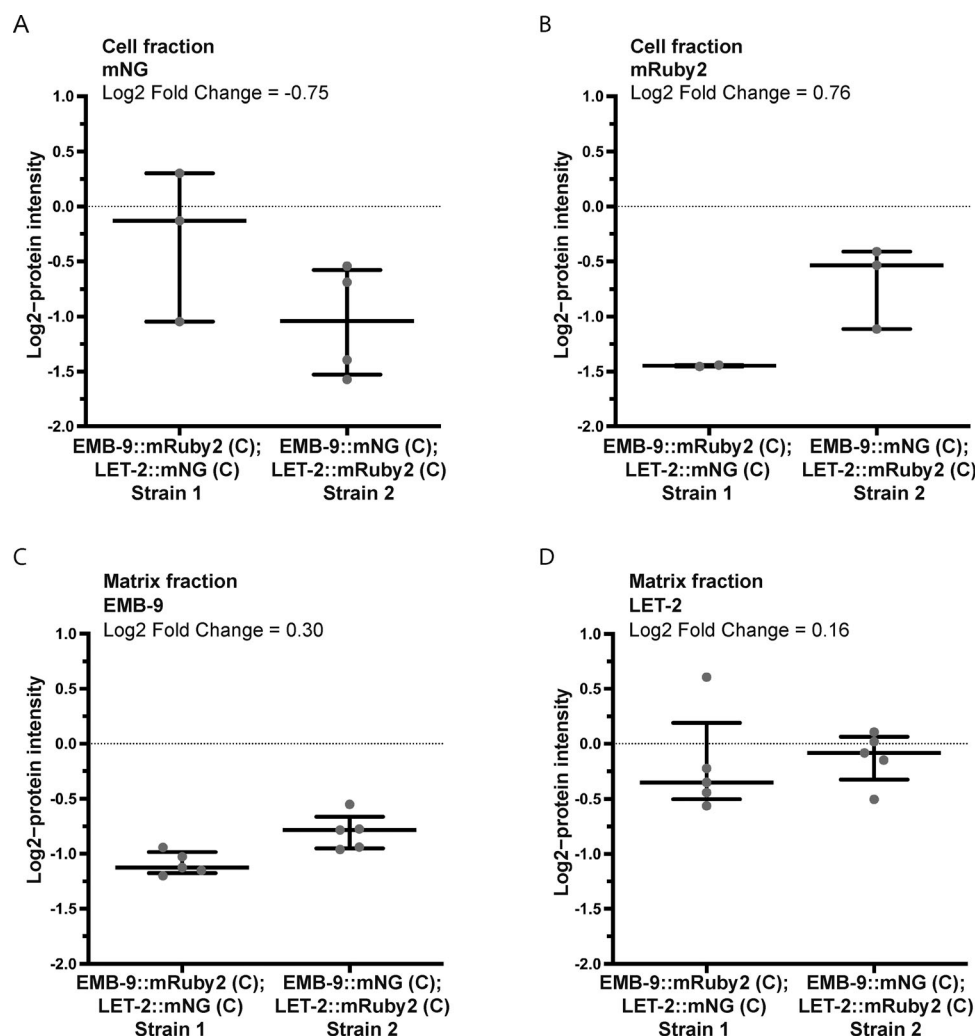


Figure S3. LET-2 is approximately twice as abundant as EMB-9 in larva, and the total EMB-9 and LET-2 proteins are comparable between strains. (A and B) Boxplots display the abundance of mNG and mRuby2 represented by their respective log₂ protein intensity (a.u.) determined using LC-MS/MS. The samples displayed are the larval (L1–L3 stages) cellular fractions of Strain 1 (EMB-9::mRuby2 [C]; LET-2::mNG [C]) and Strain 2 (EMB-9-mNG [C]; LET-2::mRuby2 [C]). The log₂ fold changes from the comparison between strains are listed above the boxplots. The mNG (LET-2) in Strain 1 ($n = 3$) was ~1.7 times the abundance of mNG (EMB-9) in Strain 2 ($n = 4$), while mRuby2 (LET-2) was ~1.7 times more abundant in Strain 2 ($n = 3$) than mRuby2 (EMB-9) in Strain 1 ($n = 2$). **(C and D)** Boxplots display the abundance of EMB-9 and LET-2 represented by their respective log₂ protein intensity (a.u.). The samples displayed are the larval matrix fractions of Strain 1 and Strain 2 ($n = 5$ each). Log₂ fold changes from the comparison between strains are listed as well.

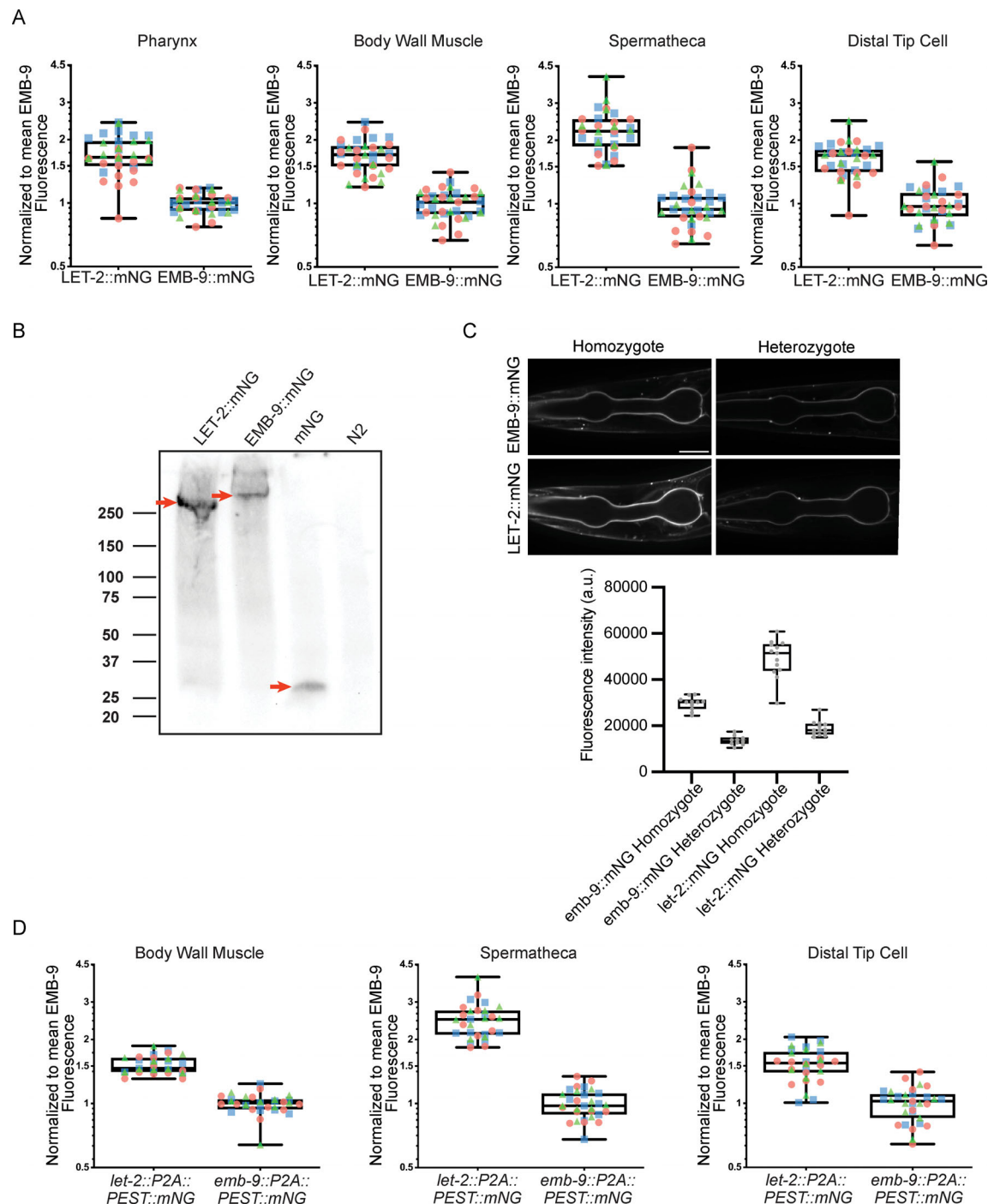


Figure S4. Normalization of LET-2/EMB-9 ratios, absence of quenching, and stability of fluorophore tagging at the C terminus. (A) Boxplots show the normalized LET-2::mNG (C) data with the normalized EMB-9::mNG (C) data for every tissue shown in Fig. 4 B. Colors of the data points indicate different trials. Normalization was performed by dividing the mean BM fluorescence intensity measurement of each LET-2::mNG (C) or EMB-9::mNG (C) animal by the average of all mean BM fluorescence intensity measurements of EMB-9::mNG (C) for the same tissue and trial (see Materials and methods, $n > 10$ animals for each of three trials). **(B)** Western blot using anti-mNG primary antibody on nonsonicated total lysate from *let-2::mNG* (C) and *emb-9::mNG* (C) animals (LET-2::mNG and EMB-9::mNG protein), transgenic animals expressing mNG driven by promoter for *let-2* (*let-2p>mNG*), and wild-type N2 animals lacking mNG. Red arrows indicate predominant bands in each lane. LET-2::mNG and EMB-9::mNG bands are larger than the expected 197 and 200 kDa protein sizes. This is likely due to samples not being sonicated to break up cross-linked protomers. **(C)** Top: single z-slice confocal fluorescence images of pharynxes from late L4-staged EMB-9::mNG (C) and LET-2::mNG (C) homozygotes and heterozygotes. The scale bar is 20 μ m. Bottom: the boxplots show the mean fluorescence intensity measurements from the BM surrounding the proximal pharyngeal bulb ($n > 10$ animals). **(D)** Boxplots show the normalized LET-2 translation reporter (*let-2::P2A::PEST::mNG*) data with the normalized EMB-9 translation reporter (*emb-9::P2A::PEST::mNG*) data plotted for each tissue shown in Fig. 5 B. Colors of the data points indicate different trials. Normalization was performed by dividing the mean BM fluorescence intensity measurement of each LET-2 translation reporter or EMB-9 translation reporter animal by the average of all mean BM fluorescence intensity measurements of EMB-9 translation reporter for the same tissue and trial (see Materials and methods, $n > 10$ animals for each of three trials). Source data are available for this figure: SourceData FS4.

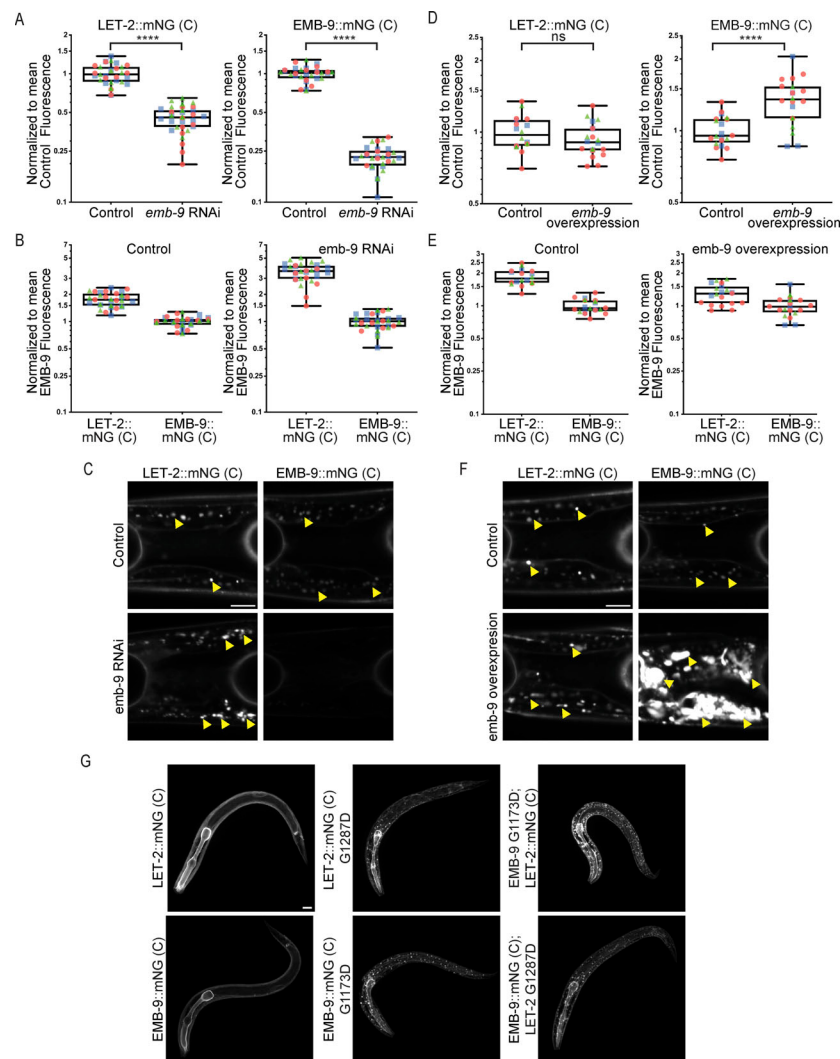


Figure S5. Normalization of LET-2/EMB-9 ratios and internal aggregates after *emb-9* reduction or overexpression and in *let-2* or *emb-9* glycine substitution mutants. (A) Boxplots show the normalized *emb-9* RNAi knockdown and control RNAi (L4440 empty vector) data in LET-2::mNG (C) and EMB-9::mNG (C) animals shown in Fig. 6 B. Colors of the data points indicate different trials. Normalization was performed by dividing the mean BM fluorescence intensity (LET-2::mNG [C] or EMB-9::mNG [C]) measurement from each *emb-9* RNAi or control animal by the average of all mean BM fluorescence intensity measurements from control animals for the same trial (see Materials and methods, $n > 8$ animals for each of three trials, t tests with Welch's correction, **** $P < 0.0001$). (B) Boxplots show the normalized LET-2::mNG (C) and normalized EMB-9::mNG (C) data for control RNAi (L4440 empty vector) and *emb-9* RNAi knockdown animals shown in Fig. 6 C. Colors of the data points indicate different trials. Normalization was performed by dividing the mean BM fluorescence intensity measurement of each LET-2::mNG (C) or EMB-9::mNG (C) animal by the average of all mean BM fluorescence intensity measurements of EMB-9::mNG (C) for the same trial (see Materials and methods, $n > 8$ animals for each of three trials). (C) Single z-slice images of LET-2::mNG (C) (left) and EMB-9::mNG (C) (right) in day 1 adult worms (72 h after hatch) with and without *emb-9* RNAi knockdown. Yellow arrowheads indicate body wall muscle intracellular aggregates. Increased intracellular aggregation was seen in LET-2::mNG (C) *emb-9* RNAi-treated worms, and decreased intracellular aggregation was seen in EMB-9::mNG (C) *emb-9* RNAi-treated worms ($n = 28/28$ worms observed for each). The scale bar is 10 μ m. (D) Boxplots show the normalized *emb-9* overexpression and control (no overexpression) data in LET-2::mNG (C) and EMB-9::mNG (C) animals shown in Fig. 7 B. Colors of the data points indicate different trials. Normalization was performed by dividing the mean BM fluorescence intensity (LET-2::mNG [C] or EMB-9::mNG [C]) measurement of each overexpression or control animal by the average of all mean BM fluorescence intensity measurements of control animals for the same trial (see Materials and methods, $n > 5$ animals for each of three trials, unpaired t test [left] and a t test with Welch's correction [right], **** $P < 0.0001$, ns, not significant). (E) Boxplots show the normalized LET-2::mNG (C) and normalized EMB-9::mNG (C) data after *emb-9* overexpression and in control (no overexpression) animals shown in Fig. 7 C. Colors of the data points indicate different trials. Normalization was performed by dividing the mean BM fluorescence intensity measurement of each LET-2::mNG (C) or EMB-9::mNG (C) animal by the average of all mean BM fluorescence intensity measurements of EMB-9::mNG (C) for the same trial (see Materials and methods, $n > 5$ animals for each of three trials). (F) Single z-slice images of LET-2::mNG (C) (left) with and without *emb-9::mRuby2* overexpression and EMB-9::mNG (C) (right) with and without *emb-9::mRuby2* overexpression in day 1 adult worms (72 h after hatch). Yellow arrowheads indicate body wall muscle intracellular aggregates. Increased intracellular aggregation was seen in EMB-9::mNG (C) worms with *emb-9::mRuby2* overexpression in all body wall muscle segments ($n = 11/11$ animals observed). The scale bar is 10 μ m. (G) Representative sum projections of confocal z-stacks of LET-2::mNG (C) and EMB-9::mNG (C) wild-type animals, LET-2::mNG G1287D and EMB-9::mNG (C) G1173D mutant animals, and animals with a wild-type fluorescently tagged protein paired with an untagged mutant protein: EMB-9 G1173D; LET-2::mNG (C) and EMB-9::mNG (C); LET-2 G1173D. The imaged L1 larvae were grown at 25°C (restrictive temperature for *emb-9* and *let-2* mutants). Increased intracellular aggregation was seen in animals carrying *let-2* or *emb-9* glycine mutant alleles ($n = 20/20$ animals per condition). The scale bar is 10 μ m.

Provided online are Table S1, Table S2, Table S3, and Table S4. Table S1 shows quantification of intramuscular aggregates. Table S2 shows *C. elegans* strains used in this study. Table S3 shows oligonucleotide sequences used in this study. Table S4 shows chemicals and DNA reagents used in this study.