

1 **Title: The conserved, secreted protease inhibitor MLT-11 is necessary for *C.***  
2 ***elegans* molting and embryogenesis**

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## 21 **ABSTRACT**

22 Apical extracellular matrices (aECMs) are associated with all epithelia and form a  
23 protective layer against biotic and abiotic threats in the environment. *C. elegans* molting  
24 offers a powerful entry point to understanding developmentally programmed aECM  
25 remodeling. Several protease inhibitors are implicated in molting, but their functions  
26 remain poorly understood. Here we characterize *mlt-11*, an unusual protease inhibitor  
27 with 10 conserved Kunitz domains. MLT-11 oscillates and is localized in the cuticle and  
28 in lysosomes in larvae and in the embryonic sheath starting at the 3-fold embryo stage.  
29 *mlt-11* (RNAi) produced a developmental delay, motility defects, failed apolysis, and a  
30 defective cuticle barrier. *mlt-11* null and C-terminal Kunitz domain deletion mutants are  
31 embryonic lethal while N-terminal deletions cause a rolling phenotype indicative of cuticle  
32 structure abnormalities. *mlt-11* activity is primarily necessary in seam and hypodermal  
33 cells and accordingly *mlt-11* (RNAi) causes defects in localization of the collagens ROL-  
34 6 and BLI-1 over the cuticle. *mlt-11* (RNAi) molting phenotypes can be suppressed by  
35 genetically inhibiting endocytosis. Our model is that MLT-11 is acting in the aECM to  
36 coordinate remodeling and timely ecdysis.

## 37 **INTRODUCTION**

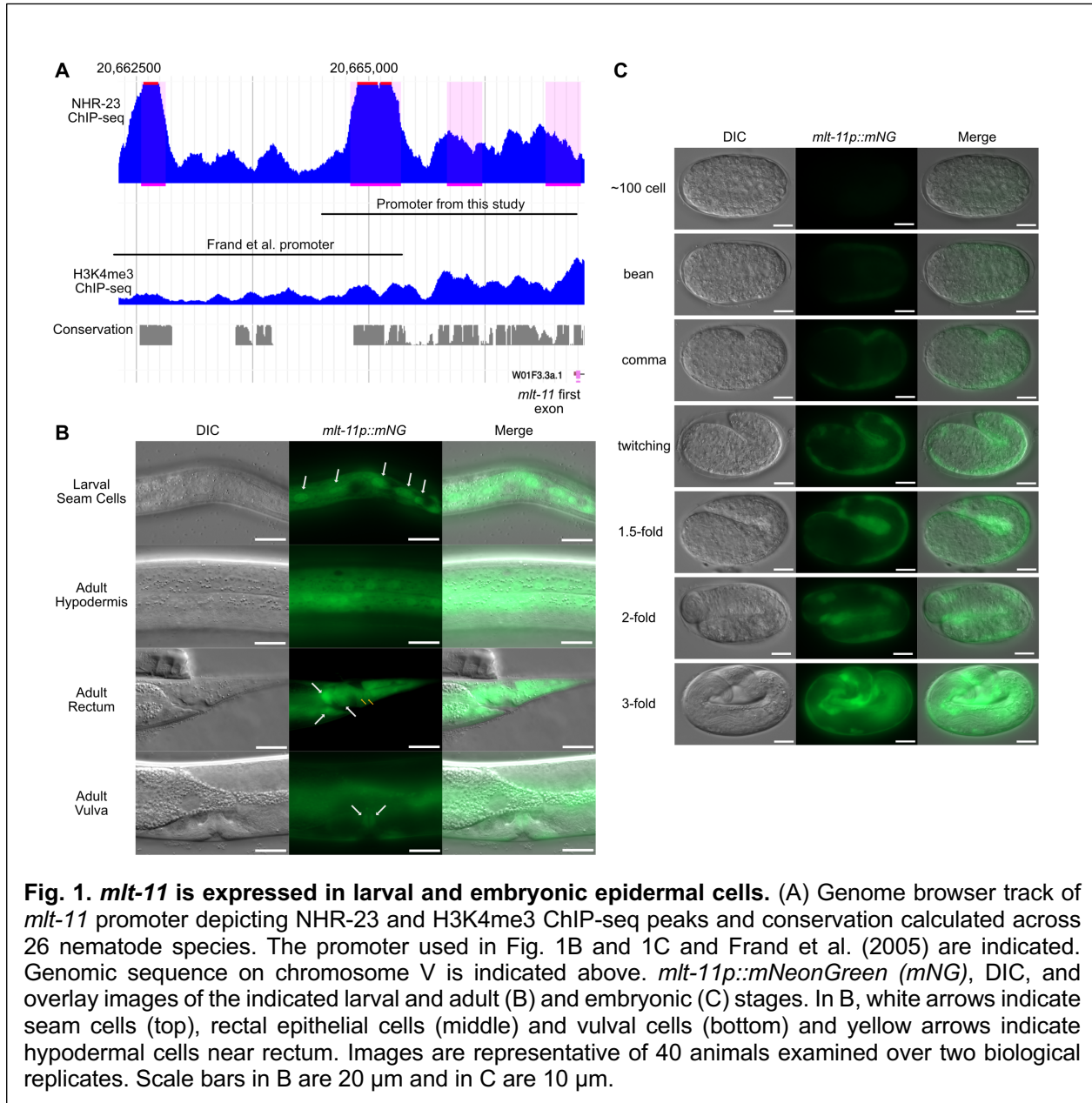
38 Specialized extracellular matrices cover the apical surface of all epithelial cells and form  
39 the skin in almost all animals (Li Zheng et al., 2020). These apical extracellular matrices  
40 (aECMs) also line the lumen of internal tubular epithelia to form a protective layer against  
41 biotic and abiotic threats (Li Zheng et al., 2020). Despite their importance, understanding  
42 the structure and dynamics of aECM components in development and disease remains  
43 challenging.

44  
45 *C. elegans* is emerging as a powerful model to study aECM structure and remodeling.  
46 They have a collagen-based ECM so understanding its assembly may provide insight into  
47 mammalian skin (Page and Johnstone, 2007). The components of the cuticle are  
48 secreted by hypodermal and seam cells and are assembled in distinct layers (Page and  
49 Johnstone, 2007). During each larval stage animals must build a new aECM underneath  
50 the old one, separate the old aECM (apolysis) and then shed it (ecdysis) (Lažetić and

51 Fay, 2017b). A specialized, transient structure known as the pre-cuticle is thought to  
52 pattern the new cuticle and is then shed during ecdysis (Cohen and Sundaram, 2020).  
53 The sheath is a similar structure in embryos which ensures embryonic integrity and directs  
54 force during elongation (Vuong-Brender et al., 2017). The vulval aECM has recently been  
55 shown to be highly dynamic, and specialized aECMs also line the rectum and excretory  
56 system (Cohen et al., 2019; Cohen et al., 2020; Gill et al., 2016).

57  
58 A major question is how is the aECM remodeled during molting? Proteases are required  
59 for ecdysis in both *C. elegans* and in parasitic nematodes, presumably by promoting  
60 apolysis, though some are thought to function in collagen processing (Davis et al., 2004;  
61 Hashmi et al., 2004; Kim et al., 2011; Stepek et al., 2011). Protease inhibitors have been  
62 implicated in molting through RNAi screening, and have been suggested to suppress  
63 ecdysis (Frاند et al., 2005; Lažetić and Fay, 2017b). BLI-5 has homology to the Kunitz  
64 domain family of protease inhibitors and mutations cause molting defects. However,  
65 recombinant BLI-5 enhanced the activity of two serine proteases from distinct classes  
66 (Page et al., 2006; Stepek et al., 2010). MLT-11 is another putative protease inhibitor in  
67 the Kunitz family and *mlt-11* (RNAi) causes molting defects (Frاند et al., 2005). *mlt-11*  
68 mRNA oscillates, peaking mid molt and its expression is regulated by NHR-23, a nuclear  
69 hormone receptor transcription factor necessary for molting (Frاند et al., 2005). However,  
70 *mlt-11* remains poorly characterized.

71  
72 Here we demonstrate that MLT-11 is localized to the cuticle, lysosomes, the rectal  
73 epithelium, excretory duct lumen, and vulval lumen. It is also secreted into the  
74 extracellular space in embryos before localizing to the cuticle prior to hatching. *mlt-11* null  
75 alleles cause embryonic lethality, characterized by disorganization of adherens junctions.  
76 RNAi in larvae causes developmental delay, apolysis, and ecdysis defects. *mlt-11* activity  
77 in seam cells is necessary for molting and a normal developmental rate. *mlt-11*  
78 inactivation causes a defective cuticle barrier and aberrant localization of the collagens  
79 ROL-6 and BLI-1. Genetic data suggests that MLT-11 acts in the aECM. This work  
80 provides the first insight into how MLT-11 functions to promote embryogenesis and aECM  
81 integrity during molting.



## 82 RESULTS

### 83 *mlt-11* is expressed in embryonic, larval, and adult epidermal cells

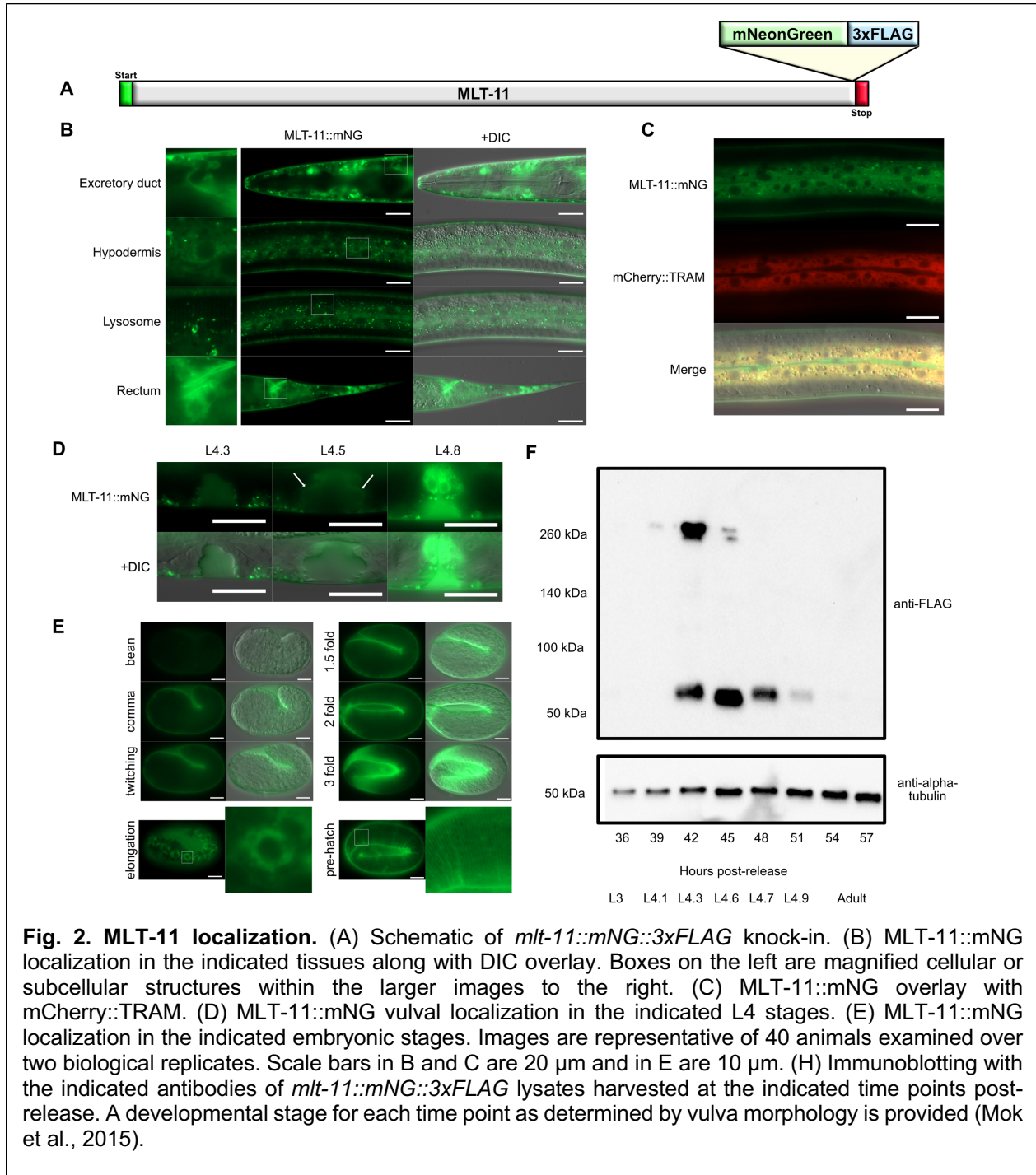
84 *mlt-11* has been reported to be an NHR-23 target gene (Frand et al., 2005). There are  
85 four NHR-23 ChIP-seq peaks in the *mlt-11* promoter (Johnson et al., 2022), and the  
86 sequences under these peaks are highly conserved in other nematodes (Fig. 1A; see  
87 Conservation track). There are additional areas of the promoter which display elevated

88 conserved sequence, which may indicate other regulatory elements (Fig. 1A). We used  
89 2.8 kilobases of upstream sequence to create a single copy *mlt-11p::NLS::mNeonGreen*  
90 promoter reporter. We note that this sequence differs in part from the promoter used by  
91 Frand et al., 2005 (Fig 1A). Expression in embryos was first detected at the bean stage  
92 in posterior epithelial cells and persisted through the 3-fold stage spreading more  
93 anteriorly (Fig. 1C). Expression was detected in hypodermal, rectal and vulval cells in  
94 both larvae and adults as well as seam cells in larvae (Fig. 1B).

95

### 96 **MLT-11 is an oscillating secreted protein that localizes to the aECM and lysosomes**

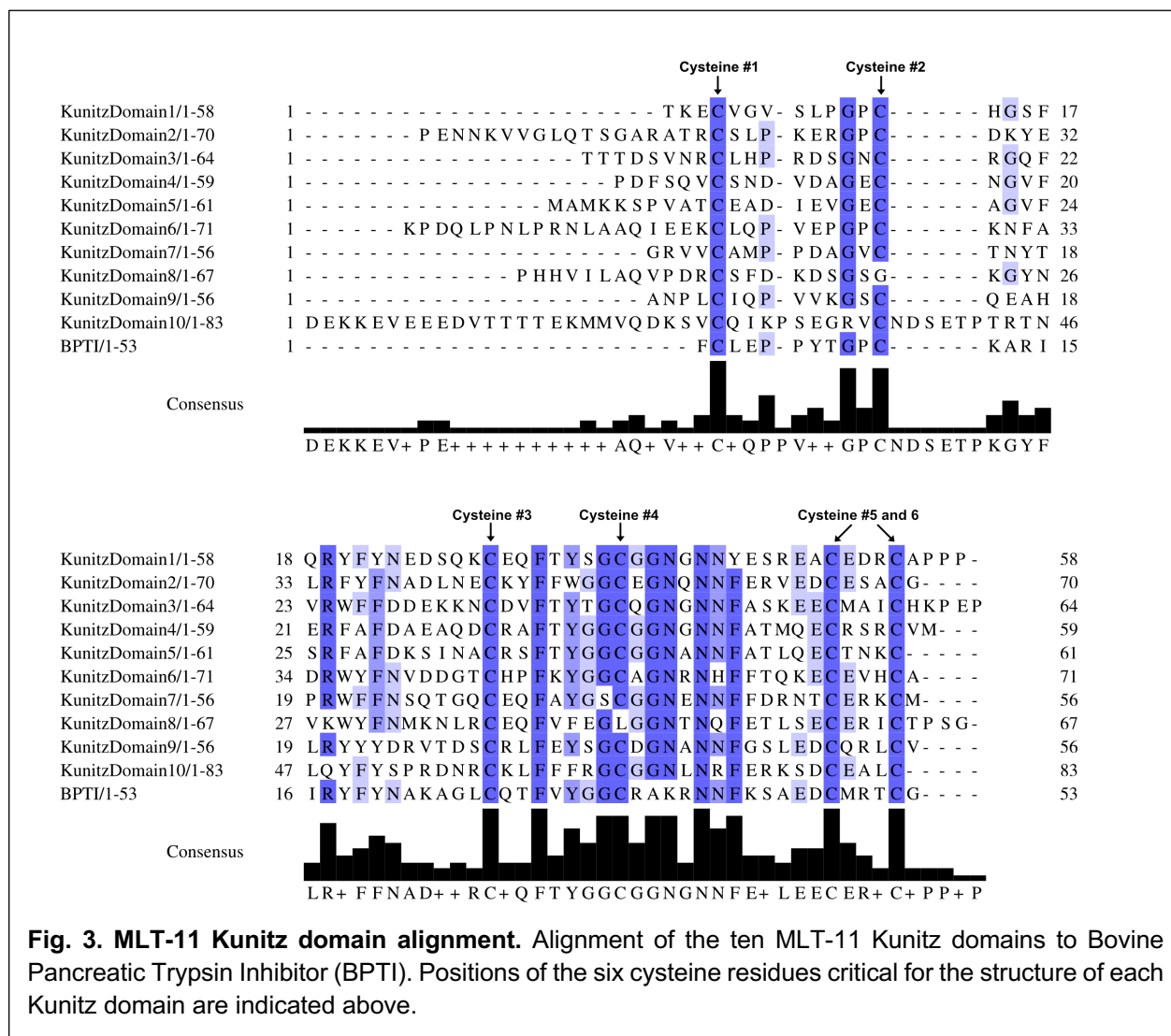
97 To determine where MLT-11 localized, we knocked an *mNeonGreen::3xFLAG* cassette  
98 into the 3' end of the gene producing a C-terminal translational fusion that labels all  
99 described *mlt-11* isoforms (Fig. 2A). MLT-11::mNeonGreen::3xFLAG (MLT-11::mNG)  
100 was detected in the excretory duct, hypodermal cells, seam cells and the rectum (Fig.  
101 2B). In the hypodermis MLT-11::mNG was non-nuclear and either diffuse through the  
102 cytoplasm or in bright punctae, lysosomal based on morphology (Miao et al., 2020). The  
103 cytoplasmic expression was reminiscent of secreted proteins. We confirmed that this  
104 pattern reflected localization to the endoplasmic reticulum through co-localization with an  
105 mCherry::TRAM marker (Fig. 2C)(Chen et al., 2012). MLT-11::mNG localization in the  
106 vulva was dynamic. In early L4 (stage 4.3 by vulva morphology) MLT-11::mNG was  
107 luminal and by mid-L4 (stage 4.5) we saw expression within the vulD cell (Fig. 2D). In  
108 late L4 (stage 4.8) MLT-11::mNG was robustly expressed in vulD and in the vulval lumen  
109 (Fig. 2D). In embryos, MLT-11::mNG was first observed at the bean stage (Fig. 2E). From  
110 this stage to the 3-fold stage MLT-11::mNG appeared to be secreted, localizing in the  
111 space between the embryo and the eggshell with enrichment at the embryo epidermis  
112 (Fig. 2E). During elongation, MLT-11::mNG perinuclear expression was observed,  
113 reminiscent of the collagen DPY-7 localization to embryonic endoplasmic reticulum  
114 (McMahon et al., 2003). Before hatching there was a striking shift in MLT-11::mNG  
115 localization where it labeled annuli in the aECM (Fig. 2E). Immunoblotting revealed that  
116 MLT-11 oscillates, and three isoforms were detected (Fig. 2F). Two isoforms are large  
117 (260 kDa) and align with the predicted size of full-length MLT-11 isoforms with an mNG  
118 tag (268.5-371.4 kDa); these isoforms peak in early L4 and rapidly disappear (Fig. 2H).



119 A ~50-70 kDa band smaller than any predicted isoform appears in early L4 and persists  
 120 until late L4 (Fig. 2H). Together, these data indicate that MLT-11 is an oscillating secreted  
 121 protein with dynamic localization to aECM in embryos and larvae.

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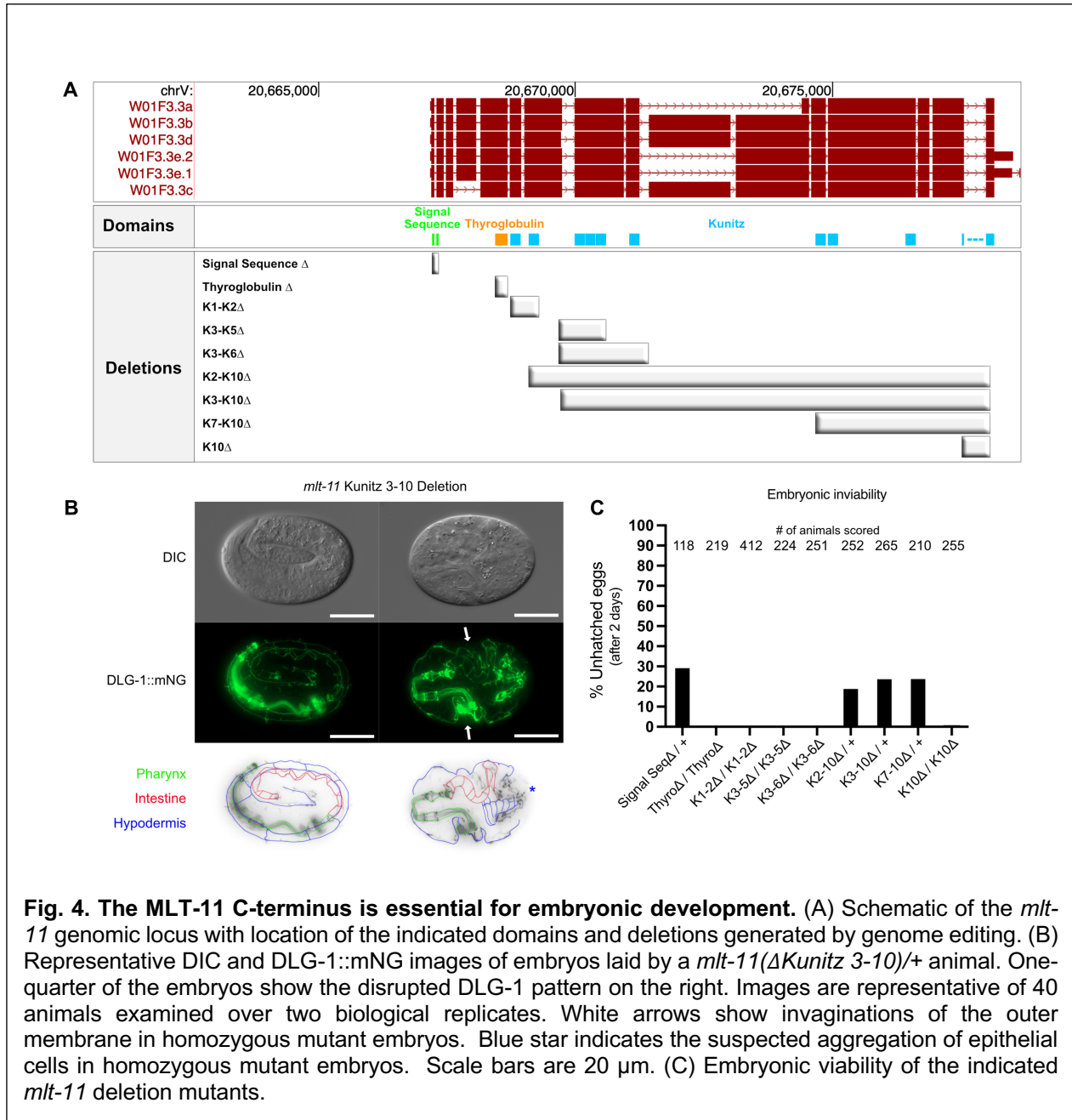


124

### 125 *mlt-11* is an essential gene required for embryogenesis and molting

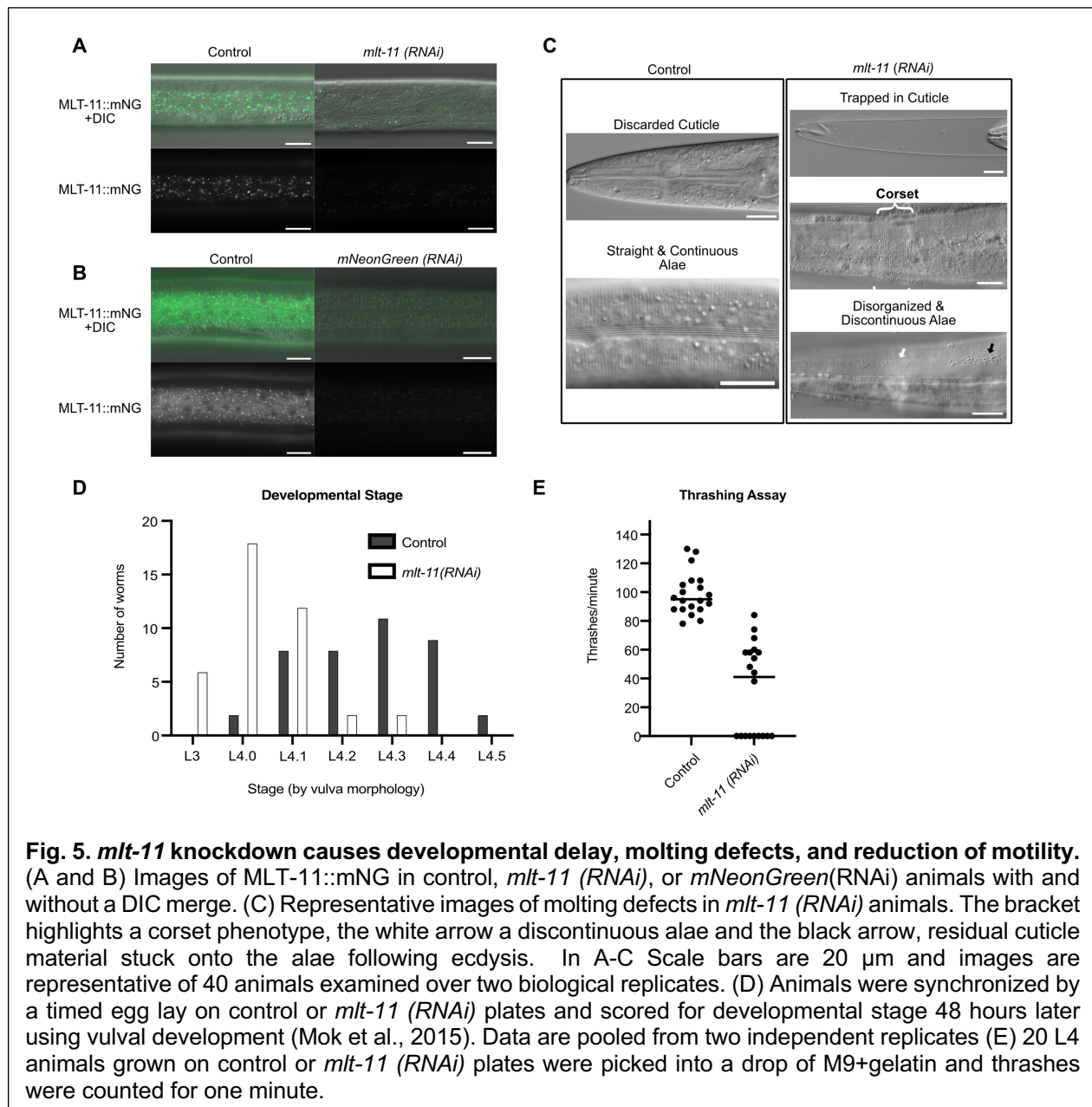
126 MLT-11 is predicted to be a large protein (234-341 kDA) with a signal sequence, a  
 127 thyroglobulin domain, and 10 Kunitz protease inhibitor domains (Fig. 3, 4A). A key feature  
 128 of Kunitz domains is the presence of 6 conserved cysteine residues which form three  
 129 disulfide bonds critical for stabilizing the domain (Ranasinghe and McManus, 2013; Fig.  
 130 3). While Kunitz domain 8 may not be active as it is missing cysteines in the second and  
 131 fourth position, the remaining Kunitz domains appear functional as they contain key  
 132 conserved residues (Fig. 3). To gain insight into MLT-11 structure and function, we  
 133 generated a deletion series to determine which domains were necessary for *mlt-11*  
 134 function. Homozygous deletion of the signal sequence, Kunitz domains 2-10, 3-10, or 7-

135 10 caused embryonic lethality. We balanced the mutations genetically by crossing to a  
 136 strain with a *myo-2p::GFP::unc-54 3'UTR* cassette inserted into F46B3.7, a gene roughly  
 137 40kb away. We never observed progeny from balanced mutant worms lacking GFP.  
 138 There was no evidence of haploinsufficiency as we could maintain balanced deletion  
 139 strains. Additionally, these balanced worms produced roughly 25% dead embryos, a rate  
 140 expected for a homozygous lethal mutation (Fig. 4C). In contrast, Kunitz domain 10  
 141 appeared dispensable for development as deletion animals were viable (Fig. 4C).





142 Deletion of Kunitz domains 1-2, 3-5 and 3-6 were completely viable, producing no dead  
 143 eggs as homozygotes, but instead had coordination defects rolling to the right during  
 144 forward movement. Given MLT-11::mNG expression in embryos (Fig. 2), we next  
 145 examined the nature of the embryonic lethality in *mlt-11* deletion mutants using a DLG-  
 146 1::mNG allele to mark adherens junctions (Heppert et al., 2018). In control embryos, DLG-  
 147 1::mNG labeled adherens junctions in the pharynx, intestine, and hypodermis (Fig 4B). In  
 148 contrast, Kunitz 3-10Δ embryos matched to the same stage displayed severe  
 149 disorganization (Fig. 4B). The pharynx and foregut adherens junctions appeared wild



150 type, but the remainder of the junctions were disorganized and there was evidence of  
151 invaginations in the hypodermis (Fig. 4B). These data implicate the signal sequence and  
152 Kunitz 7-10 region as being essential for embryonic development.

153

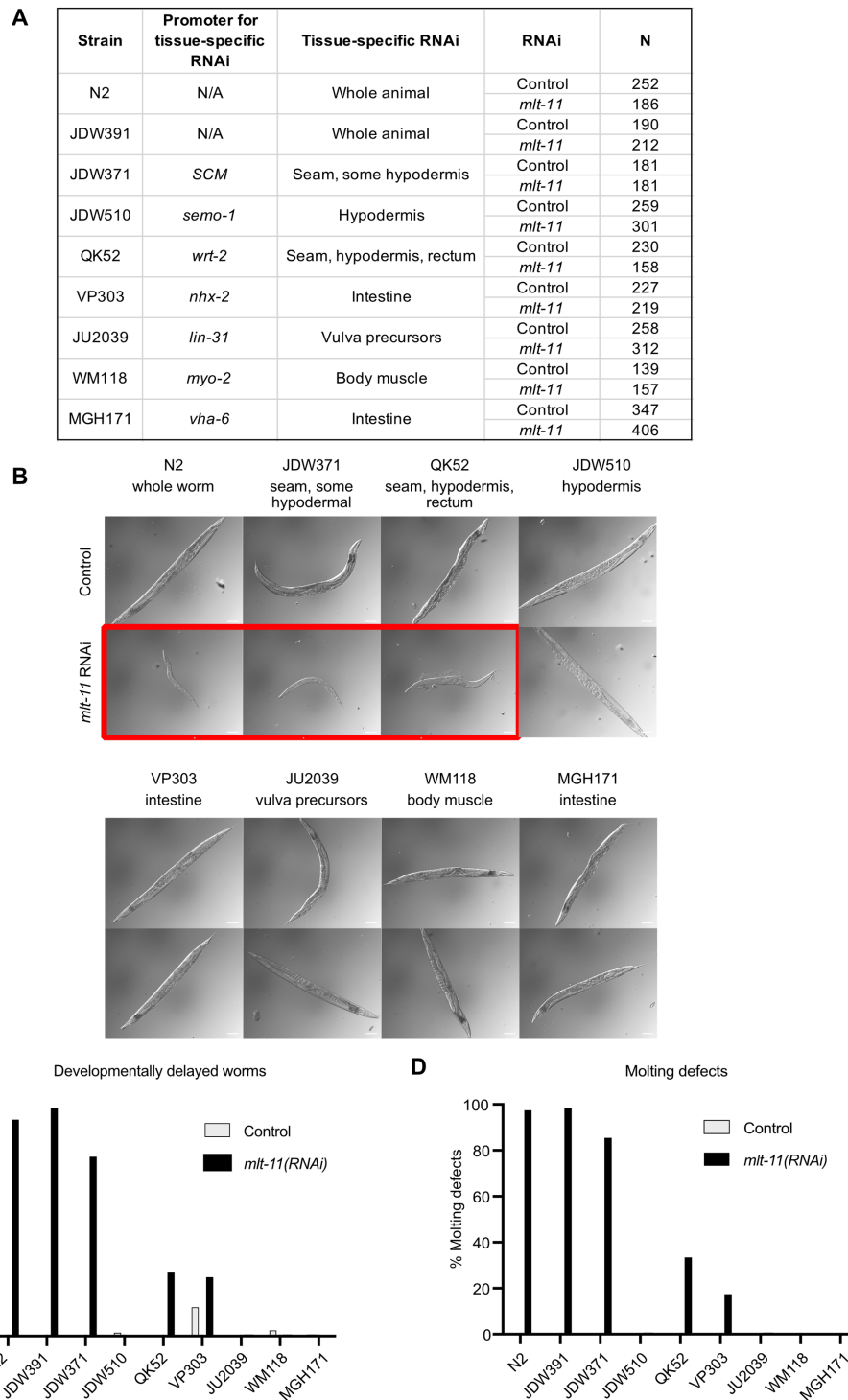
#### 154 ***mlt-11* knockdown causes defective cuticle structure and function**

155 As we were interested in the role of *mlt-11* in promoting molting, we turned to RNAi. Both  
156 *mlt-11* (RNAi) and *mNeonGreen* (RNAi) reduced levels of MLT-11::mNG (Fig. 5A,C), and  
157 resulting phenotypes included ecdysis defects where animals were trapped in the old  
158 cuticle or failed to shed the old cuticle producing a corset (Fig. 5B). We also observed  
159 disorganized and discontinuous alae (Fig. 5B). *mlt-11* (RNAi) animals developed more  
160 slowly than control animals (Fig. 5D) and appeared to move more slowly. To test whether  
161 *mlt-11* (RNAi) caused a locomotion defect, we performed a thrashing assay, scoring body  
162 bends/minute. *mlt-11* RNAi caused nearly a 2.5-fold decrease in thrashing compared to  
163 control animals (Fig. 5E). To determine in which tissue(s) *mlt-11* was necessary to  
164 promote molting we used a set of tissue-specific RNAi strains (Fig. 6A). *mlt-11* knockdown  
165 in JDW371, a tissue-specific RNAi strain that restricts knockdown to seam, hypodermal,  
166 and intestinal cells (Johnson et al., 2022), phenocopied *mlt-11* (RNAi) in wildtype or *mlt-*  
167 *11::mNG* (JDW391) animals with respect to developmental delay and molting defects  
168 (Fig. 6A-C). *mlt-11* (RNAi) in QK52, a hypodermal and seam cell-specific RNAi strain,  
169 produced less penetrant developmental delay and molting defects (Fig. 6A-C). Notably,  
170 *mlt-11* (RNAi) in a hypodermal-specific RNAi strain, JDW510, produced no  
171 developmental delay or molting defects, suggesting that *mlt-11* activity is necessary in  
172 seam cells.

173

#### 174 ***mlt-11* inactivation causes defects in aECM structure and function**

175 The developmental delay and molting defects caused by seam cell specific *mlt-11* (RNAi)  
176 were reminiscent of our recent work on *nhr-23* (Johnson et al., 2022). As NHR-23  
177 depletion causes a defect in the cuticle barrier, we tested whether *mlt-11* inactivation also  
178 compromises this barrier. We incubated control and *mlt-11* (RNAi) animals with the cuticle  
179 impermeable, cell membrane permeable Hoechst 33258 dye and scored animals with  
180 stained nuclei. In control animals we observed no Hoechst staining while in *mlt-11* (RNAi)



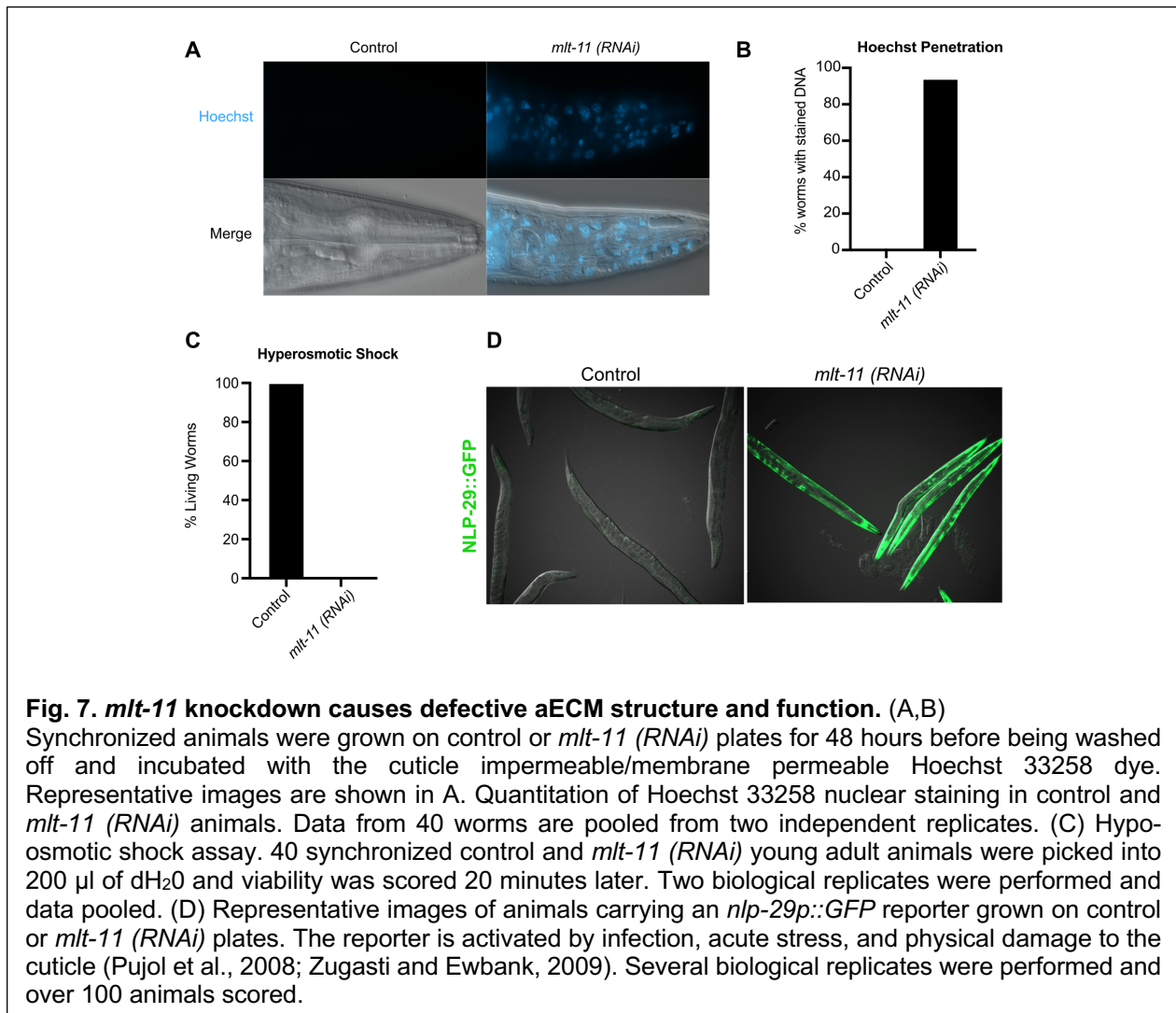
**Fig. 6. *mlt-11* is necessary in seam cells for molting and larval development.** (A) Tissue-specific RNAi strains used. A timed egg lay of animals of the indicated genotype were performed on control or *mlt-11* (RNAi) plates and phenotypes were scored three days later. (B) Representative images of control and *mlt-11* (RNAi) on the indicated strains. The red box highlights conditions that produced smaller larvae with molting defects. (C) Developmental delay was scored in the indicated strains grown on control or *mlt-11* (RNAi) plates and classified as a failure to reach adulthood after 72 hours of growth. (D) Molting defects were scored in the indicated strains on control or *mlt-11* (RNAi) plates. Scored defects included animals dragging cuticles, ecdysis failure and cuticle corsets. Tissue-specific RNAi data is from two independent replicates.

182 depleted animals (Johnson et al., 2022), *mlt-11* (RNAi) also caused sensitivity to hypo-  
183 osmotic shock (Fig. 7C) and activation of an *nlp-29::GFP* promoter reporter activated by  
184 infection, acute stress, and physical damage to the cuticle (Fig 7D; Pujol et al., 2008;  
185 Zugasti and Ewbank, 2009).

186

### 187 **Weak *nekl* alleles suppress *mlt-11* (RNAi) phenotypes**

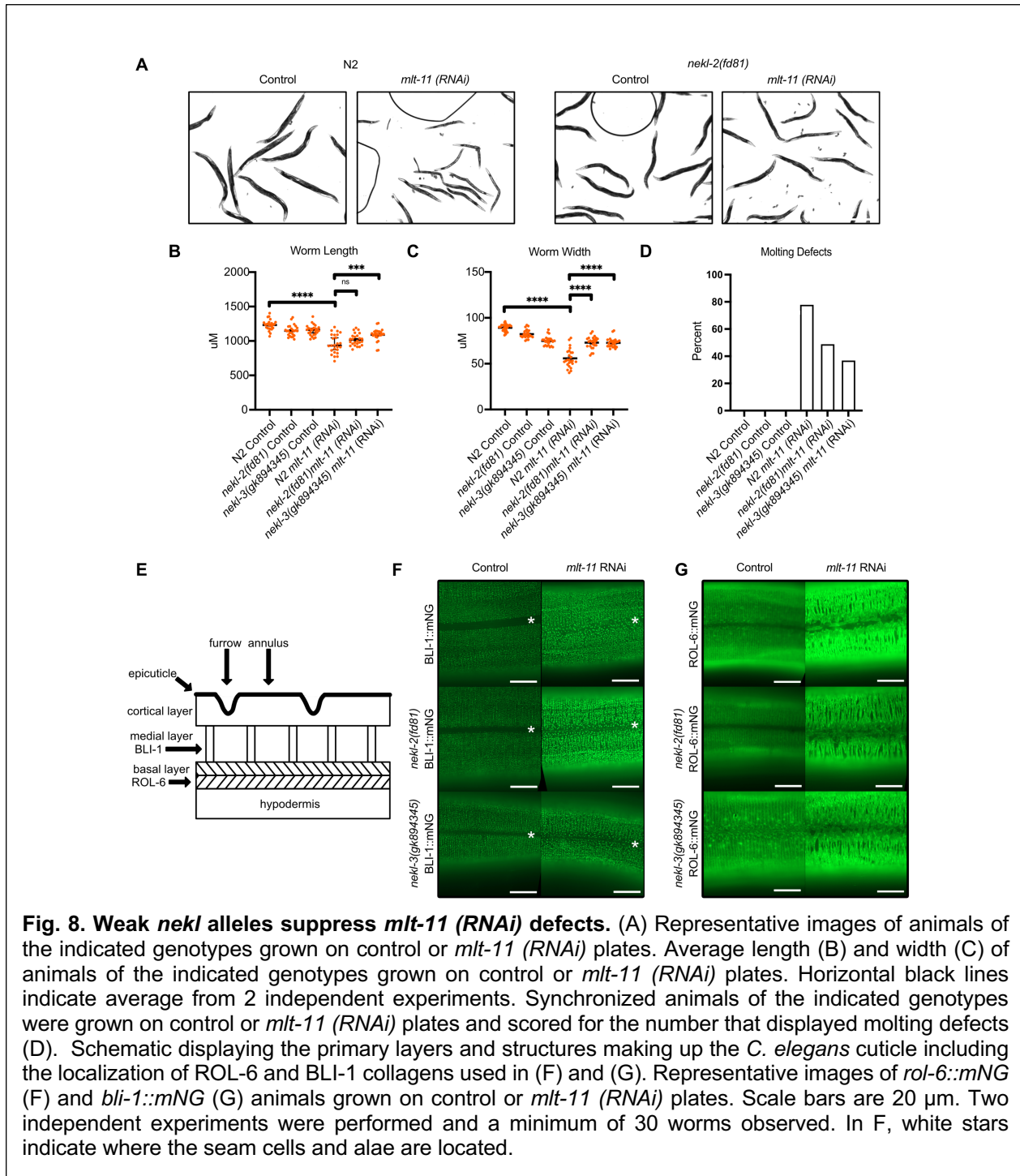
188 We observed MLT-11::mNG localization to lysosomes and the aECM in the cuticle, rectal  
189 epithelium, vulva, and excretory duct (Fig. 2) and *mlt-11* inactivation caused defects in  
190 the aECM barrier function and localization of select aECM components (Fig. 7). MLT-11  
191 could be acting directly in the aECM or could function in lysosomes, as this organelle has  
192 been shown to play an important role in aECM remodeling during molting (Miao et al.,



193 2020). To distinguish between these possibilities, we examined the genetic interaction  
194 between *mlt-11* (RNAi) and weak *nekl* alleles. *nekl-2* and *nekl-3* encode NIMA-related  
195 kinases that regulate endocytosis and are required for completion of molting (Joseph et  
196 al., 2020; Lažetić and Fay, 2017a; Yochem et al., 2015). Weak *nekl-2* and *nekl-3*  
197 hypomorphs are viable but display reduced clathrin-mediated endocytosis (Joseph et al.,  
198 2020). We reasoned that if MLT-11 acted in the aECM then weak *nekl* alleles might  
199 suppress the *mlt-11* (RNAi) defects by trapping more MLT-11 in the aECM. Conversely,  
200 if MLT-11 activity was necessary in lysosomes then weak *nekl* alleles might enhance the  
201 *mlt-11* (RNAi) defects by reducing the amount of MLT-11 that is trafficked to lysosomes.  
202 Weak *nekl-2(fd81)* and *nekl-3(gk894345)* alleles suppressed the small body size of *mlt-*  
203 *11* (RNAi) animals (Fig. 8A-C) and suppressed *mlt-11* (RNAi) molting defects (Fig 8D).

204  
205 NHR-23 depletion causes reduced levels and mis-localization of the medial cuticle layer  
206 strut collagen BLI-1 and defective localization of the basal layer collagen ROL-6 (Johnson  
207 et al., 2022), so we examined the effect of *mlt-11* (RNAi) on these markers. In control  
208 animals, BLI-1::mNG localized to regularly spaced punctae in rows and was excluded  
209 from the area of the aECM over seam cells (Fig. 8F). *mlt-11* (RNAi) caused BLI-1 to  
210 localize to larger, irregularly spaced punctae which were also found over the seam cells  
211 (Fig. 8F). This exclusion zone and BLI-1::mNG organization pattern were partially  
212 restored in weak *nekl* allele worms grown on *mlt-11* (RNAi). ROL-6::mNG, in control  
213 animals, localized to striped annuli, with an irregular but tight zipper-like pattern over  
214 seam cells (Fig. 8G). *mlt-11* (RNAi) animals displayed thick and aggregated ROL-6::mNG  
215 with a large gap over seam cells where left and right side extensions typically meet (Fig.  
216 8G). Weak *nekl* allele worms treated with *mlt-11* (RNAi) had a similar aggregation of  
217 ROL-6::mNG over hypodermal cells, but more frequent connections across seam cells to  
218 ROL-6::mNG in annuli on the opposite side. These data indicated that *mlt-11* is  
219 necessary for aECM structure and MLT-11 acts in the aECM to promote development  
220 and molting.

221



## 222 DISCUSSION

223 How aECMs are dynamically remodeled during development and disease remains poorly  
 224 understood. Using the *C. elegans* cuticle as a model aECM we demonstrate a role for the

225 protease inhibitor MLT-11 in promoting embryogenesis, molting, wild type developmental  
226 rate, and the aECM barrier. MLT-11::mNG oscillates and localizes to the aECM in the  
227 larval cuticle, vulva, rectum, and excretory pore, and is also in lysosomes. In embryos,  
228 MLT-11::mNG is secreted into the space between the eggshell and the embryo and then  
229 localizes to the cuticle prior to hatching. Tissue-specific RNAi data indicates that *mlt-11*  
230 primarily acts in seam cells. Depletion of *mlt-11* results in mislocalization of the collagens  
231 ROL-6 and BLI-1, and genetic data suggests that MLT-11 functions primarily in the aECM.

232  
233 We observed three distinct phenotypes depending upon the severity of *mlt-11* mutation  
234 or depletion: i) embryonic lethality; ii) larval molting defects and developmental delay; and  
235 iii) rolling. Any C-terminal deletions removing Kunitz domains 7-10, including Kunitz 2-10  
236 and Kunitz 3-10 deletions, produced embryonic lethality (Fig. 4). DLG-1::mNG revealed  
237 severe disorganization of adherens junctions in these mutants with defects being most  
238 pronounced in the hindgut and hypodermis (Fig. 4). One possibility is that MLT-11 is  
239 required for embryonic sheath function. The embryonic sheath is an aECM that preserves  
240 embryonic integrity and distributes force during embryo elongation (Kelley et al., 2015;  
241 Vuong-Brender et al., 2017). MLT-11 is secreted during the window of morphogenesis,  
242 when the embryo elongates. One model is that MLT-11 restrains protease activity to  
243 ensure sheath integrity during elongation and in its absence the sheath is compromised.  
244 Inactivation of sheath components has been shown to cause embryo arrest and rupturing  
245 (Vuong-Brender et al., 2017). It is unclear whether the molting defects and developmental  
246 delay incurred by *mlt-11* (RNAi) reflect a distinct molecular defect or arise from a similar  
247 role for MLT-11 during larval aECM remodeling. The collagens ROL-6 and BLI-1 exhibited  
248 aberrant localization in *mlt-11* (RNAi) treated larvae. A conditional deletion approach  
249 would be ideal to create a *mlt-11* null in larvae, bypassing the embryo phenotypes.

250  
251 Our Kunitz 1-2, 3-5 and 3-6 deletions all produced a weak right roller phenotype. The  
252 mapping locus *rol-9* was recently discovered to be encoded by a gain-of-function *mlt-11*  
253 allele (Rich et al., 2022). How does a protease inhibitor mutation cause a roller  
254 phenotype? Aside from *mlt-11*, the only non-collagen roller gene is *rol-3*, which encodes  
255 a predicted receptor tyrosine kinase (Jones et al., 2013). ROL-3 is hypodermally

256 expressed and necessary for ecdysis and cuticle formation (Jones et al., 2013). *rol-3*  
257 mutations cause defects in seam cell formation and *mlt-11* is necessary in seam cells for  
258 developmental progression and molting (Fig. 6). One possibility is that weak *mlt-11* alleles  
259 provide sufficient activity to promote ecdysis but elevated protease activity disrupts  
260 collagen processing, leading to a roller phenotype. Interestingly, *mlt-11* (RNAi) disrupts  
261 ROL-6::mNG localization and specific alleles of both *mlt-11* and *rol-6* cause a right roller  
262 phenotype. In the future it will be interesting to test whether *mlt-11* and *rol-3* genetically  
263 interact and whether *mlt-11* inactivation affects the localization of collagens that when  
264 mutated produce left roller phenotypes.

265

266 Transcription factors regulate complex networks of genes to control cellular and  
267 developmental processes. Assigning the regulation of a single regulated gene to a  
268 phenotype incurred by inactivation of a given transcription is challenging. NHR-23  
269 depletion causes developmental delay, molting defects, and defective aECM structure  
270 and barrier function (Johnson et al., 2022). Strikingly, *mlt-11* (RNAi) phenocopies NHR-  
271 23 depletion in many regards. Both cause developmental delays, apolysis defects, and a  
272 loss of the aECM barrier function (Fig. 5-7; Johnson et al., 2022). The ROL-6::mNG  
273 localization defects are highly similar, with annular disorganization and a gap over the  
274 seam cells (Fig. 8; Johnson et al., 2022). Tissue-specific RNAi indicates that the seam cells  
275 are a key site of action for both *nhr-23* and *mlt-11*, though *nhr-23* activity also appears  
276 necessary in hypodermal cells (Fig. 6; Johnson et al., 2022). NHR-23-regulated genes  
277 are enriched in protease inhibitors (Johnson et al., 2022), and *mlt-11* is a critical gene for  
278 promoting aECM remodeling during molting (Fig. 8). An open question is whether MLT-  
279 11 is unique in mediating the NHR-23-dependent molting program or whether these  
280 terminal phenotypes are a common feature of disrupting components in the NHR-23 gene  
281 regulatory network. Given that *mlt-11* is a protease inhibitor gene, the common  
282 phenotypes suggest that some aspects of the NHR-23 depletion phenotype may be due  
283 to unrestrained protease activity. Identifying which protease(s) that MLT-11 inhibits and  
284 the protease substrates is a critical future direction.

285



286 Why does MLT-11 have so many Kunitz domains? The extensively studied bovine  
287 pancreatic trypsin inhibitor has a single Kunitz domain (Ascenzi et al., 2003), as do other  
288 proteins such as Alzheimer Precursor Protein (Beckmann et al., 2016). Others such as  
289 Tissue Factor Pathway Inhibitor and *C. elegans* MEC-9 have multiple Kunitz domains  
290 (Broze and Girard, 2012; Du et al., 1996). One possibility was that the large number of  
291 Kunitz domains in *C. elegans* MLT-11 arose through recent duplication. Arguing against  
292 this possibility many *Caenorhabditis* species, as well as more distantly related nematodes  
293 (*P. pacificus*, *O. vivululus*, *B. malayi*) have large MLT-11 homologs with predicted signal  
294 sequences and 10 Kunitz domains (Fig. S1). In *C. elegans*, Kunitz domains 1-6 and 10  
295 appear dispensable whereas deletion of Kunitz domains 7-9 causes embryonic lethality  
296 (Fig. 4). Notably, there is additional sequence flanking Kunitz domain 9 that is conserved  
297 (Fig. S1). Interestingly, our immunoblotting experiments detect a smaller isoform of 50-  
298 70 kDa that could be produced by cleavage at or near the start of Kunitz domain 9. An  
299 interesting approach would be to exogenously express a C-terminal fragment of *mIt-11*  
300 containing Kunitz domains 7-10 in worms with an endogenous null allele of *mIt-11* to see  
301 if this region is sufficient to rescue embryonic inviability. We would reasonably expect  
302 these rescued worms to be right rollers as our deletion strains lacking Kunitz 1-2, 3-5 and  
303 3-6 are right rollers.

304  
305 Our data could suggest that the different Kunitz domains may play distinct roles, or that  
306 their location within the protein is important. Kunitz domains work as competitive protease  
307 inhibitors, which would suggest that MLT-11 could serve as a scaffold to bind to and  
308 inactivate proteases. Kunitz domains tend to inactivate serine proteases, yet there are no  
309 serine proteases implicated in molting (Frandsen et al., 2005). *nas-37*, an astacin  
310 metalloprotease, peaks in expression 30 minutes after *mIt-11* mRNA peaks in expression  
311 and genes expressed at similar points in development often function in common  
312 processes (Davis et al., 2004; Farrell et al., 2018; Hendriks et al., 2014; Meeuse et al.,  
313 2020). MLT-11 may regulate uncharacterized protease inhibitors or could inactivate  
314 different classes of protease inhibitors. An unusual family of Kunitz domain protease  
315 inhibitors from the parasitic nematode *Fasciola hepatica* was shown to inhibit cathepsin  
316 proteases, not serine proteases (Smith et al., 2020). Alternatively, MLT-11 may not

317 function as a protease inhibitor. The Kunitz domain containing molting factor BLI-5 was  
318 shown to enhance the activity of two serine proteases, rather than inhibit them (Steppek et  
319 al., 2010). Similarly, the ADM-2 protease regulates molting by modulating levels of the  
320 low-density lipoprotein receptor-related protein, LRP-1, through a mechanism  
321 independent of its protease activity (Joseph et al., 2022).

322

### 323 **Future perspective**

324 Our characterization of MLT-11 provides an entry point into understanding how proteases  
325 and protease inhibitors interact to promote aECM remodeling. Going forward, exploring  
326 whether MLT-11 plays roles in specialized aECM such as the vulval lumen, excretory  
327 duct, and glial socket cuticle will be important. As proteases are important targets to  
328 combat parasitic nematode infections, understanding how they are regulated during  
329 development by endogenous protease inhibitors will be critical to develop novel  
330 approaches to combat this group of devastating pathogens.

## 331 **MATERIALS AND METHODS**

### 332 **Strains and culture**

333 *C. elegans* were cultured as originally described (Brenner, 1974), except worms were  
334 grown on MYOB media instead of NGM. MYOB agar was made as previously described  
335 (Church et al., 1995).

### 336 **Strains created by injection in the Ward Lab and used in this study:**

Name	Genotype
JDW330	<i>rde-1(ne300) V.</i> ; <i>jsTi1493 [mosL loxP mex-5p FLP sl2 mNeonGreen rpl-28p FRT GFP-HIS58 FRT3 mosR] IV</i> (Johnson et al., 2022)
JDW371	<i>jsTi1493 {mosL loxP [wrdSi72(SCMp::pes-10delta::rde-1 CDS+3'UTR)] FRT3::mosR} IV</i> ; <i>rde-1(ne300) V</i> (Johnson et al., 2022)
JDW380	<i>jsTi1493 {mosL loxP [wrdSi72(mlt-11(-2.8kb)p::mNeonGreen(dpi)::tbb-2 3'UTR)] FRT3::mosR} IV</i>

JDW383	<i>mlt-11(wrd78[<b>mlt-11 Kunitz 2-10 deletion</b>]), oxTi633 [eft-3p::tdTomato::H2B::unc-54 3'UTR / F46B3.7(gk5359[loxP + myo-2p::GFP::unc-54 3' UTR + rps-27p::neoR::unc-54 3' UTR + loxP]) V.</i>
JDW385	<i>him-8(e1489) IV; mlt-11(wrd80[<b>mlt-11 Kunitz 7-10 deletion</b>]), oxTi633 [eft-3p::tdTomato::H2B::unc-54 3'UTR] / F46B3.7(gk5359[loxP + myo-2p::GFP::unc-54 3' UTR + rps-27p::neoR::unc-54 3' UTR + loxP]) V</i>
JDW386	<i>him-8(e1489) IV ; mlt-11(wrd81[<b>mlt-11 Kunitz 10 deletion</b>]), oxTi633 [eft-3p::tdTomato::H2B::unc-54 3'UTR] V</i>
JDW387	<i>mlt-11(wrd82[<b>mlt-11 Kunitz 3-10 deletion</b>]) , oxTi633 [eft-3p::tdTomato::H2B::unc-54 3'UTR] / F46B3.7(gk5359[loxP + myo-2p::GFP::unc-54 3' UTR + rps-27p::neoR::unc-54 3' UTR + loxP]) V</i>
JDW389	<i>bli-1(wrd84[linker::mNeonGreen::3xFLAG::linker])</i>
JDW391	<i>mlt-11(wrd86[C-terminal mNeonGreen::3xFLAG]) V</i>
JDW458	<i>rol-6(wrd117[rol-6::C-term mNeonGreen (dpi)::3xFLAG::linker]) (Johnson et al., 2022)</i>
JDW497	<i>mlt-11(wrd122[<b>mlt-11 Kunitz 3-6 deletion</b>]), oxTi633 [eft-3p::tdTomato::H2B::unc-54 3' UTR] V</i>
JDW503	<i>mlt-11(wrd124[<b>mlt-11 thyroglobulin domain deletion</b>]) , oxTi633 [eft-3p::tdTomato::H2B::unc-54 3'UTR + Cbr-unc-119(+)] V</i>
JDW504	<i>mlt-11(wrd125[<b>mlt-11 Kunitz 3-5 deletion</b>]) , oxTi633 [eft-3p::tdTomato::H2B::unc-54 3'UTR] + Cbr-unc-119(+)] V</i>
JDW510	<i>jsTi1493 {mosL loxP [ wrdSi97(<b>suro-1p::rde-1 CDS+3'UTR</b>) FRT3::mosR} IV ; rde-1 (ne300) V (Johnson et al., 2022)</i>
JDW512	<i>wrdEx40[dpy-7p::mCherry::TRAM::unc-54 3' UTR]; mlt-11(wrd86[C-terminal mNeonGreen::3xFLAG]) V</i>

337 **Strains created by crossing in the Ward Lab and used in this study:**

Strain	Genotype	Crossing strain 1	Crossing strain 2

JDW359	<i>ezIs2 III [fkh-6::GFP + unc-119(+)] ; him-8(e1489) IV ; F46B3.7(gk5359[loxP + myo-2p::GFP::unc-54 3' UTR + rps-27p::neoR::unc-54 3' UTR + loxP]) V.</i>	DZ325	VC4276
JDW401	<i>mlt-11(wrd82[mlt-11 Kunitz 3-10 deletion]) , oxTi633 [eft-3p::tdTomato::H2B::unc-54 3'UTR] / F46B3.7(gk5359[loxP + myo-2p::GFP::unc-54 3' UTR + rps-27p::neoR::unc-54 3' UTR + loxP]) V. dlg-1(cp301[dlg-1::mNG-C1^3xFlag]) X</i>	JDW387	LP598
JDW511	<i>ezIs2 [fkh-6::GFP + unc-119(+)] / + III ; him-8(e1489) / + IV ; mlt-11(wrd126[mlt-11 signal sequence deletion...mNG::3xFLAG]) / F46B3.7(gk5359[loxP + myo-2p::GFP::unc-54 3' UTR + rps-27p::neoR::unc-54 3' UTR + loxP]) V</i>	EG7968	JDW359
JDW516	<i>bli-1(wrd84[linker::mNeonGreen::3xFLAG::linker]) II ; nekl-3(gk894345) X</i>	WY1141	JDW389
JDW517	<i>rol-6(wrd117[rol-6::C-term-mNeonGreen (dpi)::3xFLAG::linker]) II ; nekl-3(gk894345) X</i>	WY1141	JDW458
JDW522	<i>nekl-2(fd81); rol-6(wrd117[rol-6::C-term-mNeonGreen (dpi)::3xFLAG::linker]) II</i>	WY1122	JDW458
JDW526	<i>nekl-2(fd81); bli-1(wrd84[linker::mNeonGreen::3xFLAG::linker]) II</i>	WY1122	JDW389

338 **Strains provided by the *Caenorhabditis* Genetics Center:**

N2	Wild-type
QK52	<i>rde-1(ne219) V ; xkIs99(wrt-2p::rde-1::unc-54 3'UTR)</i> (Melo and Ruvkun, 2012)
VP303	<i>rde-1(ne219) V ; kbIs7[nhx-2p::rde-1 + rol-6(su1006)]</i> (Espelt et al., 2005)
JU2039	<i>mfls70 [lin-31p::rde-1 + myo2p::GFP] IV ; rde-1(ne219) V</i> (Barkoulas et al., 2013)
WM118	<i>rde-1(ne300) V ; nels9(nels9 [myo-3::HA::RDE-1 + rol-6(su1006)]) X</i> (Watts et al., 2020)

MGH171	<i>alxIs9 [vha-6p::sid-1::SL2::GFP] sid-1(qt9) V ; alxIs9</i> (Melo and Ruvkun, 2012)
WY1122	<i>nekl-2(fd81[Y84L,G88A]) I</i> (Lažetić and Fay, 2017a)
WY1141	<i>nekl-3(gk894345) X</i> (Lažetić and Fay, 2017a)
DZ325	<i>ezIs2 III [fkh-6::GFP + unc-119(+)] ; him-8(e1489) IV</i> (Chang et al., 2004)
VC4276	<i>F46B3.7(gk5359[loxP + myo-2p::GFP::unc-54 3' UTR + rps-27p::neoR::unc-54 3' UTR + loxP]) V</i> (Au et al., 2019)
LP598	<i>dlg-1(cp301[dlg-1::mNG-C1^3xFlag]) X</i> (Heppert et al., 2018)
EG7968	<i>unc-119(ed3) III; oxTi633 [eft-3p::tdTomato::H2B::unc-54 3'UTR + Cbr-unc-119(+)] V</i> (Frøkjær-Jensen et al., 2014)

339

#### 340 **Other strains**

341 NM5548 *jsSi1579 jsSi1706 jsSi1726[loxP myo-2p FRT nlsCyOFP myo-2 3' mex-5p FLP*  
 342 *D5 glh-2 3' FRT3] II* was a gift from Dr. Michael Nonet and will be described elsewhere.  
 343 The sequence of this landing pad can be found on the Nonet lab website  
 344 (<https://sites.wustl.edu/nonetlab/rmce-insertion-strains/> -last edited 6-4-2022) and is  
 345 inserted at an sgRNA within 50 base pairs away from the ttTi5605 insertion site.

346

#### 347 **Genome Editing**

348 All plasmids used are listed in Table S1. Annotated plasmid sequence files are provided  
 349 in File S1. Specific cloning details and primers used are available upon request. JDW380  
 350 *jsTi1493 {mosL loxP [wrdSi72(mIt-11(-2.8kb)p::mNeonGreen(dpi)::tbb-2 3'UTR)]*  
 351 *FRT3::mosR} IV* was created by recombination-mediated cassette exchange  
 352 (RMCE)(Nonet, 2020). A 2.8 kb *mIt-11* promoter fragment was initially Gibson cloned into  
 353 the *NLS::mScarlet (dpi)::tbb-2 3'UTR* vector pJW1841 (Ashley et al., 2021) to generate  
 354 pJW1934. The mScarlet cassette was then replaced with mNeonGreen (dpi) to generate  
 355 pJW2229. The *mIt-11p (-2.8kb) mNeonGreen (dpi)-tbb-2 3'UTR* fragment was PCR  
 356 amplified from pJW2229 and Gibson cloned into *SphI*-HF+*SpeI*-HF double digested  
 357 RMCE integration vector pLF3FShC to produce pJW2337. This vector was integrated into  
 358 NM5179 and the SEC was excised as previously described (Nonet, 2020).

359

360 *mlt-11* deletion strains were created by injection of Cas9 ribonucleoprotein complexes  
361 (RNPs)(Paix et al., 2014; Paix et al., 2015) [700 ng/μl IDT Cas9, 115 ng/μl each crRNA  
362 and 250 ng/μl IDT tracrRNA], oligonucleotide repair template (110 ng/μl) and pSEM229  
363 co-injection marker (25 ng/μl)(El Mouridi et al., 2020) for screening into strain EG7968.  
364 Where possible, we selected “GGNGG” crRNA targets as these have been the most  
365 robust in our hand and support efficient editing (Farboud and Meyer, 2015). F1s  
366 expressing the co-injection marker were isolated to lay eggs and screened by PCR for  
367 the deletion. F2 progeny of a verified F1 deletion mutant were crossed to JDW359 males  
368 expressing *myo-2::GFP* to genetically balance the mutation. Genotyping primers are  
369 provided in Table S2. JDW389 and JDW391 and were created by injection of RNPs [700  
370 ng/μl IDT Cas9, 115 ng/μl crRNA and 250 ng/μl IDT tracrRNA] and a dsDNA repair  
371 template (25-50 ng/ul) created by PCR amplification of a plasmid template into N2  
372 animals (Paix et al., 2014; Paix et al., 2015)(Table S1). PCR products were melted to  
373 boost editing efficiency, as previously described (Ghanta and Mello, 2020). For the *mlt-*  
374 *11* C-terminal knock-in, the mNeonGreen::3xFLAG cassette was inserted right at the  
375 double-strand break and a stop codon followed the 3xFLAG sequence. We re-coded the  
376 sequence between the insert and native stop codon and placed it in 5' to the mNeonGreen  
377 3xFLAG insertion (File S2). Sequences of CRISPR/Cas9-mediated genome edits are  
378 provided in File S2. crRNAs used are provided in Table S3. F1 progeny were screened  
379 by mNeonGreen expression. JDW512 *wrdEx40[dpy-7p::mCherry::TRAM::unc-54 3'*  
380 *UTR]; mlt-11(wrd86[C-terminal mNeonGreen::3xFLAG]) V* was generated by injection of  
381 a *dpy-7p::mCherry::tram-1::unc-54 3'UTR* vector (25 ng/μl)(Chen et al., 2012) into  
382 JDW391. F1 progeny were screened by mCherry::TRAM expression.

383

## 384 **Imaging**

385 Synchronized animals were collected from MYOB, control, or auxin plates by either  
386 picking or washing off plates. For washing, 1000 μl of M9 + 2% gelatin was added to the  
387 plate or well, agitated to suspend animals in M9+gelatin, and then transferred to a 1.5 ml  
388 tube. Animals were spun at 700xg for 1 min. The media was then aspirated off and  
389 animals were resuspended in 500μl M9 + 2% gelatin with 5 mM levamisole. 12 μl of

390 animals in M9 +gel with levamisole solution were placed on slides with a 2% agarose pad  
391 and secured with a coverslip. For picking, animals were transferred to a 10  $\mu$ l drop of  
392 M9+5 mM levamisole on a 2% agarose pad on a slide and secured with a coverslip.  
393 Images were acquired using a Plan-Apochromat 40x/1.3 Oil DIC lens or a Plan-  
394 Apochromat 63x/1.4 Oil DIC lens on an Axiomager M2 microscope (Carl Zeiss  
395 Microscopy, LLC) equipped with a Colibri 7 LED light source and an Axiocam 506 mono  
396 camera. Acquired images were processed through Fiji software (version: 2.0.0-rc-  
397 69/1.52p). For direct comparisons within a figure, we set the exposure conditions to avoid  
398 pixel saturation of the brightest sample and kept equivalent exposure for imaging of the  
399 other samples.

400

#### 401 **Western Blot**

402 For the western blot in Fig. X JDW391 animals were synchronized by alkaline bleaching  
403 ([dx.doi.org/10.17504/protocols.io.j8nlkkyxdl5r/v1](https://doi.org/10.17504/protocols.io.j8nlkkyxdl5r/v1)) and released on MYOB plates.  
404 Animals were harvested at the indicated time points by picking thirty animals into 30  $\mu$ l of  
405 M9+0.05% gelatin. Laemmli sample buffer was added to 1X and then samples were  
406 immediately incubated for five minutes at 95°C. Lysates were stored at -80°C until  
407 resolution by SDS-PAGE. Lysates were resolved using precast 4-20% MiniProtean TGX  
408 Stain Free Gels (Bio-Rad) with a Spectra™ Multicolor Broad Range Protein Ladder  
409 (Thermo; # 26623) protein standard. For the anti-FLAG blots, proteins were transferred  
410 to a polyvinylidene difluoride membrane by wet transfer using Towbin buffer (25 mM Tris,  
411 192 mM glycine, 20% methanol, pH 8.3) supplemented with 0.1% SDS and 30V was  
412 applied for 16 hours in a cold room. The buffer was chilled prior to use and a freezer back  
413 was added to the transfer container. For the anti-Tubulin blots, a semi-dry transfer with a  
414 TransBlot Turbo (Bio-Rad) was performed. Blots and washes were performed as  
415 previously described (Johnson et al., 2022, 23). Anti-FLAG blots used horseradish  
416 peroxidase (HRP) conjugated anti-FLAG M2 (Sigma-Aldrich, A8592-5x1MG, Lot  
417 #SLCB9703) at a 1:2000 dilution. Mouse anti-alpha-Tubulin 12G10 (Developmental  
418 Studies Hybridoma Bank; “-c” concentrated supernatant) was used at 1:4000 and Digital  
419 anti-mouse (Kindle Biosciences LLC, R1005) diluted 1:20,000 was used as the 2°. Blots  
420 were incubated for 5 minutes with 1 ml of Supersignal West Femto Maximum Sensitivity

421 Substrate (Thermo Fisher Scientific, 34095) and the final blot were imaged using the  
422 'chemi high-resolution' setting on a Bio-Rad ChemiDoc MP System.

423

#### 424 **RNAi Knockdown**

425 RNA interference experiments were performed as in Johnson *et al.* (2022). Control RNAi  
426 used either an empty L4440 or high-efficiency T444T RNAi vector (Sturm *et al.*, 2018).  
427 The *mlt-11* (RNAi) vector was streaked from the Ahringer library (Kamath *et al.*, 2003).  
428 The *mNeonGreen*(RNAi) vector was generated by synthesizing a cDNA fragment and  
429 cloning it into T444T. Synthesis and cloning were performed by Twist Bioscience. Vector  
430 sequences are provided in File S1.

431

432

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441 University of Iowa, Department of Biology, Iowa City, IA 52242.

442

#### 443 **Competing interests**

444 The authors declare no competing or financial interests.

445

#### 446 **Author Contributions**

447 Conceptualization: J.M.R, J.D.W.

448 Methodology: J.M.R, J.D.W.

449 Validation: J.M.R, J.D.W.

450 Formal analysis: J.M.R, J.D.W.

451 Resources: J.M.R, J.D.W.



452 Data curation: J.M.R, J.D.W.  
453 Writing - original draft: J.M.R, J.D.W.  
454 Writing - review & editing: J.M.R, J.D.W.  
455 Supervision: J.M.R, J.D.W.  
456 Project administration: J.D.W.  
457 Funding acquisition: J.D.W.

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634

635 **Figure S1. Alignment of MLT-11 homologs.** MLT-11 homologs from the indicated  
636 nematode species were aligned using Clustal Omega. The length in amino acids of each  
637 homolog follows the species and homolog name. To the left and right of the alignment  
638 are amino acid positions of the end residues for each protein. Blue shading indicates  
639 conserved sequences and the histogram at the bottom depicts the degree of conservation  
640 with a consensus sequence listed below. The positions of the *C. elegans* MLT-11 signal  
641 sequence, thyroglobulin domain, and ten Kunitz domains are indicated. We note that we  
642 cut off the extended *P. pacificus* C-terminus (amino acids 2626-3742) since no sequence  
643 aligned to it as all proteins terminated at the *C. elegans* MLT-11 stop codon. No predicted  
644 motifs are found in the *P. pacificus* C-terminus.

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C.elegans\_MLT-11b/1-3000 419 CDKYELRFFYNADLNCKYFFFWGGCEGNHNNFERVEDECENSCGVQKSG-----VTNRFN-TEIRTTQGRITPNGGKLS-W-EETEE 497  
C.briggsae\_MLT-11/1-2812 431 CDKYELRFFYNADLNCKYFFFWGGCEGNHNNFERVEDECENSCGVQKSG-----VTNRFN-TEIRTTQGRITPNGGKLS-W-EETSE 409  
C.remanei\_MLT-11/1-3019 549 CDKYELRFFYNADLNCKYFFFWGGCEGNHNNFERVEDECENSCGVQKSG-----VTNRFN-TEIRTTQGRITPNGGKLS-W-EETSE 627  
C.brenneri\_MLT-11/1-1246 -----  
C.japonica\_MLT-11/1-2899 399 CDKYELRFFYNADLNCKYFFFWGGCEGNHNNFERVEDECENSCGVARHGEP-----RVTNKFN-TEIRTTQGRITPNAGKLN-W-EVDN 481  
P.pacificus\_MLT-11/1-3742 361 CDKYELRFFYNSEKCKYFFFWGGCEGNHNNFERVEDECENSCGVKKAAP-----LPVHTPPPTTTPPT----- 425  
B.malayi\_MLT-11/1-3040 381 CDQYELRFFYNNRNLGCKYFFFWGGCEGNHNNFERVEDECENSCQRGRGKIVATSVSAPQLITSGPQMRKLNKEFGTKNIKKTGEEKELVLELISN 474  
O.volvulus\_OVOC7267/1-2760 356 CDRYELRFFYNSRNLGCKYFFFWGGCEGNHNNFERVEDECENSCRRHGKVVVP-ALVSAPQLITSGPQMRKLTKEYETKSIKKISEMSELVLESTNT 448

Consensus

CDKYELRFFYNADLNCKYFFFWGGCEGN+NNFERVEDECEN+CGVQKSG+PT++VSAPQLVTNRFNMTETIRTTQGRITPNGGKLS-EWEEETSE

C.elegans\_MLT-11b/1-3000 498 DEEHAVP-----T-----T-----PLAPAPTAR---VS-TQ----- 521  
C.briggsae\_MLT-11/1-2812 410 ED--EP-----T-----T-----PLAPAPTAR---QTPRA----- 431  
C.remanei\_MLT-11/1-3019 628 EEE--LP-----T-----T-----PLAPAPTAR---QTPRA----- 650  
C.brenneri\_MLT-11/1-1246 -----  
C.japonica\_MLT-11/1-2899 482 ENGFESRGRDEQD-----EFSETVEAVRS-----T-----T-----PMSRPVQT---RV----- 518  
P.pacificus\_MLT-11/1-3742 -----  
B.malayi\_MLT-11/1-3040 475 DND--DNDDDDDDSAASTNISNDIKNINNDNTADTTTTTTDDNNEANLEITSSTTIFHDGSMINKNLSAKSSINQSKSHQLKIQDNIKKIK 566  
O.volvulus\_OVOC7267/1-2760 449 DAS--ATGENT-----SSISDAMANIDDDSTINSEIVLKT-----KQIDANLSANISHDQGTGSKDLF-AISTDQNKSHLSQKIQNIQKKII 528

Consensus

++E+++PG+D++DSAA++IS++++NI+T+++++++TTDNNNE+++++S+T+I+HD++++KPLAPAP+TAR+KSHQ++R++QNI+KKI+

C.elegans\_MLT-11b/1-3000 522 -----RAPVPTTPRPT---A-PAVQTTTT-RASRLETT-----RVPVKTVE--D-EEEEEEVVVEE-----QEDGK 575  
C.briggsae\_MLT-11/1-2812 432 -----SV-QTTQRVT---A-PPATTTT-RASRIQAT-----RGPASIVA--EE-SEEEEEVTVEP-----DDEEE 483  
C.remanei\_MLT-11/1-3019 651 -----PAPVHTTQRAT---A-PPPTTTTT-RASRVQAA-----RAPAPIVA--DEDESEEEVEVEP-----EELDD 706  
C.brenneri\_MLT-11/1-1246 -----  
C.japonica\_MLT-11/1-2899 519 -----TAPTTTTTTRO---R-VQPTAPTT-RAFRVQTT-----TARESDV-----TEEDEEEVVEE-----EEEKE 568  
P.pacificus\_MLT-11/1-3742 426 -----TTPQRT---AQTPVPTT-RATHVPTSGF-----SGRGPVVP--ARGGVEDSFETFE-P-----E- 471  
B.malayi\_MLT-11/1-3040 567 QKGAKLSLDSNHTDKITDFTT VKTKNTIATIDKSEKFTTNRNEQKLASESVAITTDQQLSQSDEEMKIIITSTTKT-----VESLSD 649  
O.volvulus\_OVOC7267/1-2760 529 QKETKTQLSADSTVKLTDDSTTIEPENTSATLDSNDSIDESNRSGQKLSKSIIVT-AGQLSSSEEQITTSPTATLETAMTDHADIRNHGKELSD 621

Consensus

QK++K+SAPV+TT+RRTD+TTA+P+PTTTTTDRASRVQT+NR++QKRAP+S+VAT+D+Q+EE+EEEVTEEP+T++T+MTDHADIRNHG+EELSD

Kunitz domain #3

C.elegans\_MLT-11b/1-3000 576 -----EEPPLHVQPPV+QNTVLLGGIEDTTDSVNRCLHPRDSGNCRGQFVRWFDDDEKKNCDVFTYTGCGGNGNFA-SKEEC 654  
C.briggsae\_MLT-11/1-2812 484 -----KEPLHVQPPV+QNTVLLGG-----VNRCLHQPDPGNCRGQFVRWFDDDKTCDVFTYTGCGGNGNFA-SKEEC 553  
C.remanei\_MLT-11/1-3019 707 -----KETPIHVQPPV+QNTVLLGGIDDMTTDSVNRCLHQPDAAGNCRGQFVRWFDDDKTKSCDVFTYTGCGGNGNFA-SKEEC 785  
C.brenneri\_MLT-11/1-1246 -----  
C.japonica\_MLT-11/1-2899 569 -----REQLHVQPPV+QNTVLLGGIDDMATDSVNRCLHPRDSGNCRGQFVRWFDDDEKKNCDVFTYTGCGGNGNFA-SKEEC 647  
P.pacificus\_MLT-11/1-3742 472 -----FTPETV--PLP--TTTETPRAGEKLAEGSRCHPLDAGNCGHFVRWFVWNEQKGNCEVFTYTGCGGNGNFA-SKEEC 544  
B.malayi\_MLT-11/1-3040 738 FTFMSTAL---ASSIALSPSTII---ITTSQSIILSTTAQPTANSNDRCFQKFDRTCTGQFIRWYWFDFEKSTCQVFTYSGGGGNGNFR-SREDC 737  
O.volvulus\_OVOC7267/1-2760 622 SLRSSSISTPPTPVNLLPNSTTNPSTISTTETLTQPVKIDERCFLSLDRCTCTGQFVRWHDWTDARTCQVFTYSGGGGNGNFR-SREDC 715

Consensus

+++S+++STPP+++KE+PLHVQPPV+QNTVLLGGIDD+TDSVNRCLHP+D+GNCRGQFVRWFDDDEKK+CDVFTYTGCGGNGNFA-SKEEC

Kunitz domain #3

Kunitz domain #4

Kunitz domain #5

C.elegans\_MLT-11b/1-3000 655 MAICHKPEPTPS---ATPDFSQVCSNDVDAGECNGVFERFAFDAEAQDCRAFTYGGCGGNGNFA-TMCECRSRVCMAMKSPVAT-CEADIEVG 744  
C.briggsae\_MLT-11/1-2812 554 MAICHKPEPTPS---ATPDFSQVCSNDVDAGECNGVFERFAFDSETOQDCRVFTYGGCGGNGNFA-TMCECRSRVCMAMKSPVAT-CEADIDVG 643  
C.remanei\_MLT-11/1-3019 786 MAICHKPEPTPS---ATPDFSQVCSNDVDAGECNGVFERFAFDAEATQDCRVFTYGGCGGNGNFA-TMCECRSRVCMAMKSPVAT-CEADIDVG 875  
C.brenneri\_MLT-11/1-1246 -----  
C.japonica\_MLT-11/1-2899 648 LAICHKPEPTPS---ATPDFSQVCSNDVDAGECNGVFERYAFDSEADCRVFTYGGCGGNGNFA-TMCECRARCVKS-----TSSCEADIEIG 732  
P.pacificus\_MLT-11/1-3742 545 LSCNGEALARGGILPTAAAVTNVCKHDVDAGECNGVQRFRAFNPAGECRPFTYGGCGGNGNFA-TMCECRSRVCMAMKSPVAT-CEADIDVG 636  
B.malayi\_MLT-11/1-3040 738 FAACHQPPQ-----PTPKLDNICEHSIHPDCTGIFQRFADFSTINDCRPFTYGGCGGNGNFGSSLECRNRCIVQKPTLSTDVCKHPIDVG 824  
O.volvulus\_OVOC7267/1-2760 716 FAACHRPVVPK-----SIPNMGNVCEHSIHPDCTGIFQRFADFSTINDCRPFTYGGCGGNGNFGSPLECRNRCIVQKQPVLPSTDVCKHPVEVG 804

Consensus

MAICHKPEPTPSGILP+ATPDFSQVCSNDVDAGECNGVFERFAFDSETOQDCRV+FTYGGCGGNGNFA-TM-CECRSRV+ A+KK+P+++CEADIEVG

Kunitz domain #5

C.elegans\_MLT-11b/1-3000 745 ECAGVFSRFAFDKSIACRSFTYGGCGGNGNFA-TLQECTNKCYNV---RGVCPPEPACDTRNRCQLVNDRSGCPFCSCPPVKQA-SPPGITS- 832  
C.briggsae\_MLT-11/1-2812 644 ECAGVFSRFAFDKSIACRSFTYGGCGGNGNFA-TLQECTNKCYNV---RPVCPPEPACDTRNRCQLVNDRSGCPFCSCPPVKQA-SPPGITS- 731  
C.remanei\_MLT-11/1-3019 876 ECAGVFSRFAFDKSIACRSFTYGGCGGNGNFA-TLQECTNKCYNV---RPVCPPEPACDTRNRCQLVNDRSGCPFCSCPPVKQA-SPPGITTP 964  
C.brenneri\_MLT-11/1-1246 -----  
C.japonica\_MLT-11/1-2899 733 DCSGVFRFAFDKSIACRSFTYGGCGGNGNFA-TLQECTNKCYNVSAARSQPEPACDTRNRCQLVNDRSGCPFCSCPPVKGA-SPPGTRTSS 826  
P.pacificus\_MLT-11/1-3742 637 ECGVFRFAFDKSIACRSFTYGGCGGNGNFA-TLQECTNKCYNVSAARSQPEPACDTRNRCQLVNDRSGCPFCSCPPVKGA-SPPGTRTSS 722  
B.malayi\_MLT-11/1-3040 825 ECGVFRFAFDKSIACRSFTYGGCGGNGNFA-TLQECTNKCYNVSAARSQPEPACDTRNRCQLVNDRSGCPFCSCPPVKGA-SPPGTRTSS 870  
O.volvulus\_OVOC7267/1-2760 805 ECGVFRFAFDKSIACRSFTYGGCGGNGNFA-TLQECTNKCYNVSAARSQPEPACDTRNRCQLVNDRSGCPFCSCPPVKGA-SPPGTRTSS 850

Consensus

ECSGVFSRFAFDKSIACRS+FTYGGCGGNGNFA-TLQECTNKCYNV+ AAR+VCPPEPACDTRNRCQLVNDRSGCPFCSCPPVKQA-SPPGITTS+

C.elegans\_MLT-11b/1-3000 833 -----IPKESLPCNPPVDRSACRDPCCMMFHNROGCEECVCPQTAPTPPHVPTGRP---TTVQG-AQPPASSSRRVT-EVGPAPR-----T 908  
C.briggsae\_MLT-11/1-2812 732 -----SPRDLPCNPPVDRSACRDPCCMMFHNROGCEECVCPQTAPTPPHVPTGRP---TTPGL-QPPASSSRR-V-EVGPAPR-----T 806  
C.remanei\_MLT-11/1-3019 965 -----GRDNLPCNPAFDRSACRDPCCMMFHNROGCEECVCPHTAPTTPPHVPTGRP---TTPGV-QPPS-P-SRR-F-EAGPPSPR-----T 1038  
C.brenneri\_MLT-11/1-1246 -----  
C.japonica\_MLT-11/1-2899 827 FSSSIATTAGLPCNPPVDRSACRDPCCMMFHNROGCEECVCPQTAPLPPVPTGRPT---TTFAQ-GLPPSALTTRRV-EVGPAPR-----N 907  
P.pacificus\_MLT-11/1-3742 723 -----VAQCPVDPVDRSACRDPCCMMFHNROGCEECVCHRGEEMVQPPSIPIPHPITSPSTSSPPTSATPHTSPRGSPPAPHHIHSPPSS 806  
B.malayi\_MLT-11/1-3040 871 -----SDELVKCPTIDASLVEPCILFTNRQGCHECPCPIAHSEIDNEPIEPS---VITSTL-----ISDKSS----- 930  
O.volvulus\_OVOC7267/1-2760 851 -----KS--PECTVDIISLVEPCILFENRHCHECPCPMVQSVAEENDLAET---TP-SAK-----ISDTSF----- 907

Consensus

FSSSI+P+D+LPCNPPVDRSACRDPCCMMFHNROGCEECVCPQTAPTPPHVPT+P+PHP+TPG+TPQP+ S+ SRS+PEVGPAPRHHIHSPPST

C.elegans\_MLT-11b/1-3000 909 T E - - - - - A A P P A P S S Q P R R Q F A V N T V E H - Q V V K P D Q F - - - - - P S L P R T I A A Q I E E K C L Q P V E P G P C K N F A D R W Y F N V D D G T C 979  
C.briggsae\_MLT-11/1-2812 807 T E - - - - - A A P P A P S A Q P P R Q F A V N T V E H - Q V V K P D Q F - - - - - P S L P R T I A A Q I E E K C L Q P V E P G P C K N F A D R W Y F N V D D G T C 877  
C.remanei\_MLT-11/1-3019 1039 T E - - - - - A A P P A P A T Q P A R Q F A V N T L Q H - Q V V K P D Q I - - - - - N S L P H S I A T Q V Q E K C L Q P V E P G P C K N F D D R W Y F N M D D G T C 1109  
C.brenneri\_MLT-11/1-1246  
C.japonica\_MLT-11/1-2899 908 T E - - - - - A A P P A S V - A P R Q F A V N V E Q - Q P R P - - - - - T - - - - - D Q L P R S L A A Q I E E K C L Q P V E P G P C K N F A D R W Y F N V D D G T C 975  
P.pacificus\_MLT-11/1-3742 807 H S A P S P P S A P S P P S P S R N H A R Q E A V T T H D T H D K P L P P I S S H P K I S H S P P P P S T T K S P L P D V L G E K C M Q P L D A G P C D R F I E R F Y F D S K D G R C 900  
B.malayi\_MLT-11/1-3040 931 D E - - - - - - - - - - - S K S L E A E - - - - - F I E N K V E - - - - - L K H G S M S H S G - - - - - R T S Q T N S V T E L G E K C Q P M D A G P C K N F I E R W F F N I N T S L C 997  
O.volvulus\_OVOCT267/1-2760 908 D E - - - - - - - - - - - S Q A I E D E Q L M L P V L E N N V E - - - - - K K H A S M S H S G - - - - - K T F Q T N S V T E L G E K C T Q P M D A G P C K N F I E R W F F I D I S S G L C 978

Consensus  
T E S A P S P P S A A P P A P S + Q P + R Q F A V N T L E H + Q Q + K P D Q I K H + S M S H S G P P P P T L P R N L A + Q + + E K C L Q P V E P G P C K N F + D R W Y F N V D D G T C

**Kunitz domain #6**  
C.elegans\_MLT-11b/1-3000 980 H P F K Y G G C A G N R N H F F T Q K E C E I H C A R F L S G S P T S S T S A L P K H A K F S P T T S E T E S T S A E D V E S S K N E E L E A F P A P K L H - R Q F - - - - - A S 1064  
C.briggsae\_MLT-11/1-2812 878 H P F K Y G G C A G N R N H F F T Q K E C E I H C A R F L S G S A T S - S A L P P H A N F S P T S S E S S E A P E - - - - - D V V T T T E T L E A F P A P K L H R R Q F - - - - - A S 958  
C.remanei\_MLT-11/1-3019 1110 H P F K Y G G C A G N R N H F F T Q K E C E I H C A R F L S G S A T S - T A L P P H V N F S P T S S S - E S E A P E - - - - - A P T P T T E T L E A F P A P K L H R R Q F - - - - - A S 1190  
C.brenneri\_MLT-11/1-1246  
C.japonica\_MLT-11/1-2899 976 H P F K Y G G C A G N R N H F F T Q K E C E I H C A R F L S S S A Q T S H - - - - - A N N S H T S S S T T - - - - - S S E A P S P S P S L E A F P A P K L S N R Q F - - - - - A A 1051  
P.pacificus\_MLT-11/1-3742 901 H S F R Y G G C A G N R N H F F S Q K E C E I H C A R F S N F L S E Q D P T L - - - - - A M I S R R L S E - N S D A S - - - - - L P F M E S P D S S P S T L - - - - - L L 969  
B.malayi\_MLT-11/1-3040 998 Q S F Q Y G G C A G N R N H F F S K H E C E I H C A R F F N G R T G R R R T A Y E S V T H - - - - - T S Q F - N T L - - - - - T G S Q S T K I L N E H D V I D N K Q N I L Q Q S S S N V T 1082  
O.volvulus\_OVOCT267/1-2760 979 Q S F Q Y G G C A G N R N H F F S E H E C E I H C A R F E N G R T G R R R T A Y Q N A S - - - - - R T Q H - N T L - - - - - K W S Q S T E T L N E H E H E D I - T Q S I V L D S S N D V N 1060

Consensus  
H P F K Y G G C A G N R N H F F T Q K E C E I H C A R F L S G S A T S S R T A L P P H A N F S P T S S E S S + A + E + + + + E + S + + T E T L E A F P A P K L H R R Q F I + + + S S + + A S

C.elegans\_MLT-11b/1-3000 1065 P I F K P V G P G P Q A Y D S E K I D N Y S P P T P N V N L V L S P P V H P T Y - - - - - F T Y N G Q D Q G R R A F S A G Q Q G A E N R I E V 1133  
C.briggsae\_MLT-11/1-2812 959 P I Y K P T G G Q Q N Y G S D K I D S Y S P P T P N S N L V G L S P P V H P T Y - - - - - F T Y N G Q D Q G R R A F S A G N Q - N T E N R I E V 1026  
C.remanei\_MLT-11/1-3019 1191 P I F K P I V - P Q N Y G S D K I D S Y S P P T P N S N L V G L S P P V H P T Y - - - - - F T Y N G Q D Q G R R A F S A G N Q - N T E N R I E V 1256  
C.brenneri\_MLT-11/1-1246  
C.japonica\_MLT-11/1-2899 1052 P N Y A P N S - - - - - P I G Y G S E K I D S F V P P T P S Q N L V G L S P P V H P T Y - - - - - F T Y N G Q D Q G R R L F S V P N F V Q N G P Q P N V 1118  
P.pacificus\_MLT-11/1-3742 970 P T - - - - - - - - - - - T T P L P N R V E T G S S P G T V V R T - - - - - F P K I S I D E I M Q W A L E H E G P E A - - - - - T A G P K - - - - - A 1018  
B.malayi\_MLT-11/1-3040 1083 P S F I T N N S - - - - - - - - - - - Q N L S K S N L E D G K I I K V K L D D K L A N N A L N R M T A I E N L A K S S I R R - - - - - H Q Q O O H Q Q Y Q Q K Q Q Q P L S I S S 1158  
O.volvulus\_OVOCT267/1-2760 1061 N Q I I N A S - - - - - - - - - - - K P D - - - - - I - - - - - I - - - - - I - - - - - I 1072

Consensus  
P I F K P N + + + P Q N Y G S + K I D S Y S P P T P N S N L V G L S P P V H P T Y K L D D K L A N N A L N R M T A + + + + + + + I F T Y N G Q D Q G R R A F S A G N Q + T E N R I E V

C.elegans\_MLT-11b/1-3000 1134 G Q Q L E A F G - - - - - S Q R P Q L P T H L Q G N N F V Q G R G R F D G R Q R F E V P Q G S T S P S Q P L - - - - - E E I P V T Q S R E R F D N R Q R A E A P Q G S Q R Q 1209  
C.briggsae\_MLT-11/1-2812 1027 G Q Q L E A F G - - - - - G Q A - - - - - F A R G R F D S R Q R F E V P T G S P Q I H Q - - - - - V - - - - - V - - - - - V - - - - - V - - - - - V - - - - - V 1062  
C.remanei\_MLT-11/1-3019 1257 G Q Q L E A F G - - - - - G Q A - - - - - F A R G R F D G R Q R F E V P T G G I H Q A P E T - - - - - V - - - - - V - - - - - V - - - - - V - - - - - V 1293  
C.brenneri\_MLT-11/1-1246  
C.japonica\_MLT-11/1-2899 1119 G Q N F E T F - - - - - - - - - - - T Q S R G R F D N R Q R E V V S P T Q I Q T S T S S - - - - - W P T P S G G N P E I Q V - R Q - F A A P N V P R R H 1176  
P.pacificus\_MLT-11/1-3742 1019 G N K S G T K A - - - - - G T T R T V P V T T T K - - - - - - - - - - - A A P T T T R T V P T T V - - - - - R P T P T K - - - - - P T T V - - - - - K V T P R V V E T H 1071  
B.malayi\_MLT-11/1-3040 1159 G H K I D S S V T N S N I P E K S I I P D D D K L I P M K V T K - Q Y G I N D K R I D N N W K A V I S N T Q N N S T Q T I N A L R S E I S T P K D S S S M M N S - - - - - F D W N N D D L A - 1247  
O.volvulus\_OVOCT267/1-2760 1073 - - - - - - - - - - - S A Q Q G D N R W T D N R L V S - - - - - L D E M T D T - - - - - E T - - - - - E T - - - - - D H K G D L I - 1105

Consensus  
G Q Q L E A F G T N S N I P E K S I I P G Q A + + + P + + + T K N + + + + + R G R F D N R Q R F E V P T + + I Q + S Q T + + + + + V P T P T + + S P E V + + R Q R F + A P N + D L R H

C.elegans\_MLT-11b/1-3000 1210 E H Q T S T A - - - - - P S E P L E H - I P V V Q S R G R F D S R Q R V E V P Q E S I R P A E A - - - - - T T I P R - - - - - H L G D V T I S T S S S R S S 1272  
C.briggsae\_MLT-11/1-2812 1063 - - - - - T P S - - - - - V P T P E L S - I P L I Q R G R F D G R E R L A V P E G N - - - - - - - - - - - Q P E - - - - - - - - - - - Q P E - - - - - V 1097  
C.remanei\_MLT-11/1-3019 1294 - - - - - T S - - - - - S V P P - V P P V Q R R G F D G R E R L A A P E N N - - - - - - - - - - - S H R - - - - - H L G D V T I S R A P - - - - - 1335  
C.brenneri\_MLT-11/1-1246  
C.japonica\_MLT-11/1-2899 1177 - - - - - - - - - - - E N F I - E E N T V P Q A R V H P K T F A - - - - - S E V P A E R - - - - - H L E G F F E R R P V - H E G A S T P P T E T - - - - - 1226  
P.pacificus\_MLT-11/1-3742 1072 R H E G K T K V E E S V D E P V K I V T P V E G V I R V P N P R V I V P Q D O T L P K E V F - - - - - D R F I H - - - - - D V D S F H S F V K E M G E I P P F P I H - H - 1149  
B.malayi\_MLT-11/1-3040 1248 - H Q Q K Q - - - - - S - - - - - Q V M T - - - - - V Q G I D I S Q I Q N E L S E Q Q K H N A Q M S Q H E M V E D I T S L K S D L F D R H L A K Q M L T D F A T I Q N S K - - - - - 1316  
O.volvulus\_OVOCT267/1-2760 1106 - D Q Q K Q - - - - - S - - - - - R I V I Q K I N N S Q A R Q K N E L I Q E K S T K K F L G H D T Q L F Q H E N I E D I T S L T S N I F D S Q I A K Q M L T D L A I V Q N S Q - - - - - 1182

Consensus  
+ H Q Q K + + V E E S V S E P V + + I + P + V Q + R G R F D G R + R + + V P Q E + + + P K E F + + H + Q + F Q H E + + E D I T S L + S D + F D R + P + K Q H L G D V T T + N S + S + S

C.elegans\_MLT-11b/1-3000 1273 Q V S R A S E P E I V R P R A P E P Q S R F H P K T V T R T - - - - - E E V P T P R G A E T F G R - 1317  
C.briggsae\_MLT-11/1-2812 1098 - - - - - T R V P E E P R A P E P Q S R F H P R T H T V Q - - - - - V P V Q E E N F G R S R - - - - - V E Q T E T S R V S P G G P - R A S - - - - - G V I S R V P E P E T R Q E V N - - - - - N E 1169  
C.remanei\_MLT-11/1-3019 1336 - - - - - E H A P E D I S A P E V P Q S R F H Q K T R I V D - - - - - V P I E E R Q F G R E P - - - - - E V E P E S A R P Q - - - - - E T V - - - - - 1385  
C.brenneri\_MLT-11/1-1246  
C.japonica\_MLT-11/1-2899 1227 - - - - - - - - - - - T E P A R T A S Q S I I H Q Q N R R V - - - - - E N V E N E N F G R R Q - - - - - E A T R - - - - - - - - - - - 1261  
P.pacificus\_MLT-11/1-3742 1150 - - - - - - - - - - - P S M P A G T L H L F H V P D G N S G I P M H P L A P Q I Q H M M R - - - - - V G E Q I M R V N E G N A - R M H H Q R V V D L T P L T D L H E A P M R E T K R V 1228  
B.malayi\_MLT-11/1-3040 1317 - - - - - L - - - - - S S - - - - - D E Q L I N L P Q A E M S G M Q I D - - - - - Q T V M I D - H E N K F E K R K Q N I A T N E S L I N N G A F N R S V N D D Y R I - - - - - F D W N N D D L A - 1377  
O.volvulus\_OVOCT267/1-2760 1183 - - - - - T - - - - - S N - - - - - D E Q L I K L P Q A E I S E S Q R T - - - - - S M M A N D - G K N Q L K T G I E K T A T K K T L T S N S A S D N L M H H N Q K I - - - - - 1243

Consensus  
Q + S R A S E + P E D E + A P E + P Q S R F H + K T R T V + I P + M V P V P V + E E + F G R R + + + E A T + E + L R V N S + + + + R M H H + + V I + + + P + + + + + E + + M R E T K + +

C.elegans\_MLT-11b/1-3000 1318 - - - - - - - - - - - S T Q Q V T V Q P R V A G V T S T - - - - - - - - - - - R D Q E A T R R S Q G P A E S V R - - - - - V P - - - - - S R A P E T S I I G 1364  
C.briggsae\_MLT-11/1-2812 1170 H S R R P E Q V G R P E A Q P P S R R P D S S F G R P P Q L P T A R P E S F G R E S - - - - - L G R S Q G G E N S I R V T S Q Q - - - - - A R A P E S T I I G 1239  
C.remanei\_MLT-11/1-3019 1386 - - - - - N R R P E A A - - - - - R A S G V P L R Q E S F G R P P L P T I R P E I R Q E S R P T - - - - - - - - - - - Q - - - - - K P F L P Q D H T N L I R V I S Q Q - - - - - S R A P E S T I I G 1454  
C.brenneri\_MLT-11/1-1246  
C.japonica\_MLT-11/1-2899 1262 - - - - - - - - - - - P A P E T F G R H Q E V P R V H G G V R N Q E V E N F E R R P L S T - Q T P R V Q E H V Q N H E A P S R N I Q V S Q Q N A E N F D R R P L P T Q T T G 1335  
P.pacificus\_MLT-11/1-3742 1229 S A S R T K V L A H P T S - - - - - E R V S P L V P L P G R E E E Q N S P G V A V K R T S - - - - - E E F I P L R H R P A P L R V R V P I P K - - - - - D M K E 1294  
B.malayi\_MLT-11/1-3040 1378 S T D R N - - - - - - - - - - - D G R L I L D E V - - - - - I R S S N F I S P K F E A K N E I H M P L N S V N D H W M K S Q F T - - - - - D T I T N D S I V G 1436  
O.volvulus\_OVOCT267/1-2760 1244 S I D R N - - - - - - - - - - - G G R L I S K E I - S M G S - - - - - T S P G F G S K N - A M Q L N S I D E L Y E K H K Q L T - - - - - D K V T N H M L L G 1298

Consensus  
S + + R + E + + + + P + + + + + + + R P + E S F G R + Q P L P T V R P R + + + Q + S P + F E + K N + S + M Q L N R V Q E H P + R + Q + P T N S I R V T S Q + + E N F D + R A P E + + I I G





C.elegans\_MLT-11b/1-3000 2339 P Q P V V V Q V S I V T P Q A P Q P T A A A A A H A A K T - - - - - V I P T Y K A E P V L G R H I P T 2419  
 C.briggsae\_MLT-11/1-2812 2159 - E P A V G Q Q A V G Q Q P V V Q Q - - - - - P A T Q P V P P P T T - - - - - V Q T S A P T T A S T P L P P S L E P R P P A A H A A K T V - - - - - V K A P T Y K A E P V L G R H I P T 2235  
 C.remanei\_MLT-11/1-3019 2380 - - - - - T P S E P A A P Q P P T V Q T S A P T A P T T T P L P P S L E P R P P A A H G V K N V E - - - - - K A T S T Y K A E P V L G R H I P T 2443  
 C.brenneri\_MLT-11/1-1246 589 P G A S V N H V S S V A P V V P T - - - - - A P V A P V A P Q P T V T Q G V S S V S P V S T P L P P S L E P R P P A A - F A A K T V - - - - - V Q T P T Y K A D P V V G R H I P T 668  
 C.japonica\_MLT-11/1-2899 2246 - - - - - P S A T G P I A P Q V K - - - - - P A P E I V D S D E S E M P P S L E P R P P A I H A S K T V I P T Q E V S L E K K I Y K A E P V L G R H I P T 2313  
 P.pacificus\_MLT-11/1-3742 2140 - - - - - D L T E K T A E I A P A V H G S K T - - - - - V Q K N V H T D P I R S - - - - - V V D V 2173  
 B.malayi\_MLT-11/1-3040 2374 - - - - - T Q T I - - - - - - - - - - - - - - - T A E L I P Q - - - - - N L D R S A S E T I N A R E S S - - - - - 2401  
 O.volvulus\_OVOC7267/1-2760 2103 - - - - - T L P F - - - - - - - - - - - - - - - S P V Q E I S H - - - - - I L D V T K N E T S T A Q E S S - - - - - 2130

Consensus

P+P+V+QVS+V+PVVQPQTAAAT+A+T+PIAQPPT++Q++AS+++T+STPLPPLSLEPRPPAAH++KT+VPTQEVSVV+PTYKAEPVLGREHIPT

C.elegans\_MLT-11b/1-3000 2420 A N D G Q P I V G A S P K E T V N Y Q T G A - - - - - K A S G I R S F D G S Q D - - - - - K I S V D I F N K G A D G P T K S I N G M P A C A N G R T E V R Y S D G R P V M C L P G K N Q C 2503  
 C.briggsae\_MLT-11/1-2812 2236 A N D G Q P L V A P S P K E T V N Y Q T G E V K T P G V P K A A G I K S F D E S Q G - - - - - G K L A V D L F N N - - - - - A G P T K S I N G M P A C A N G R T E V R Y S D G R P V M C L P G K N Q C 2324  
 C.remanei\_MLT-11/1-3019 2444 A N D G Q P L V A P S P K E T V N Y Q T G E V K S - - - - - K A A G I K S F D E S Q G - - - - - G K L S V D I F N K - - - - - A G P T K S I N G M P A C A N G R T E V R Y S D G R P V M C L P G K N Q C 2528  
 C.brenneri\_MLT-11/1-1246 669 A N D G Q P L V A P S P K E T I N Y Q T Q V N T P - - - - - T P R O A G I K S F D G S Q D - - - - - A K L P V D I F N N - - - - - V G P T K S I N G M P A C A N G R T E K R Y S D G R P V M C L P G K N Q C 756  
 C.japonica\_MLT-11/1-2899 2314 A N D G Q P L V G T S P K E T V N Y Q T G E V K T P A - - - - - K A S G I R N F N G S Q D - - - - - S K L S V D L F N K - - - - - A G P T K S I N G M P A C A N G R T E V R Y S D G R P V M C L P G K N Q C 2400  
 P.pacificus\_MLT-11/1-3742 2174 N G Q E L P I V A P S P E T V N Y Q T G H - - - - - R A A G I R T F A E S N G - - - - - R V S I E N A A D V D E E D A P T H P A L P S C P N S A P E L K Y A D G R P V M C L P G K N Q C 2257  
 B.malayi\_MLT-11/1-3040 2402 - - - - - K Q Q L L - - - - - S S F N D Q N S K - - - - - Q I S G K E I N N A K T F G A T I V N K N N D A I V D L P N - - - - - I F D D I S H T P S C P N G H K P I Q H A D G R P V M C L P G R N Q C 2480  
 O.volvulus\_OVOC7267/1-2760 2131 - - - - - E S L Q L - - - - - T N I M N E S G T - - - - - H V A E K K I K A K I F T E K I K S K D N D A I T D L S N - - - - - I L E D V G H A P S C P N G L K P I Q H A D G R P V M C L P G R N Q C 2209

Consensus

A N D G Q P L V A P S P K E T V N Y Q T G E V K T P A + + K A A G I K S F D E S Q + + K + + K L + V D L F N K + + A G P T K S I N G M P A C A N G R T E V R Y S D G R P V M C L P G K N Q C

C.elegans\_MLT-11b/1-3000 2504 P D G S S C Y F N G I D F F C C P E E E D P Y D K H A F G G Y G G D E T K N G Y K V F G A L N I R R L M D - - - - - E V P L R Q K R Q T F G N S N S F N I D S V V A P L R F D A E K P R Q V S R A L 2596  
 C.briggsae\_MLT-11/1-2812 2325 P D G S S C Y F N G I D F F C C P E E E D P Y D K H A F G G Y G G E E T K N G Y K V F G A L N I R R L M D - - - - - E V P L R Q K R Q A N - - - - - V N F N I D S V V A P L R F D A E K P R Q V S R A L 2414  
 C.remanei\_MLT-11/1-3019 2529 P D G S S C Y F N G I D F F C C P E E E D P Y D K H A F G G Y G G D E T K N G Y K V F G A L N I R R L M D - - - - - E V P L R Q K R Q A Q - - - - - V N F N I D S V V A P L R F D A E K P R Q V S R A L 2618  
 C.brenneri\_MLT-11/1-1246 757 P D G S S C Y F N G I D F F C C P E E E D P Y D K H A F G G Y G G E E T K N G Y K V F G A L N I R R L M D - - - - - E V P L R L K R Q V N - - - - - M N F N I D S V V A P L R F D A E K P R Q V S R A L 2460  
 C.japonica\_MLT-11/1-2899 2401 P E N S S C Y F N G I D F F C C P E E E D P Y D K H A F G G Y G G D E T K N G Y K V F G A L N I R R L M D - - - - - E V P L R Q K R Q A D - - - - - V N F N I D S V V A P L R F D A E K P R T V S R A L 846  
 P.pacificus\_MLT-11/1-3742 2258 P D K S V C Y F N G L D I F C C P E E E D P Y D K H A F G G Y G E E T K G Y K R F G H L N I R R L R D - - - - - S V I D H S R R K R E T - - - - - G L F - - - - - S I D P L R F D G A P I R O I S A R 2344  
 B.malayi\_MLT-11/1-3040 2481 A G N S L C Y F N G V D F F C C P N A E D P Y D E H V F G G Y G G E E V K R G Y K N V K K T P I N G N E L I V R K L L R R E Q M S S S R P L A M N V A A R I D S K V P K Y S L A R A S F 2574  
 O.volvulus\_OVOC7267/1-2760 2210 S G N S L C Y F N G I D F F C C P N A E D P Y D E H I F G G Y G G E E V K R G Y K N V K K T P I N A N E L I V R K L R L K R Q A Q V S S S P L T I N K A A R I D F K V S K N S L A S A S F 2303

Consensus

P D G S S C Y F N G I D F F C C P E E E D P Y D K H A F G G Y G G E E T K N G Y K V F G A L N I R R L M D I E V P L R Q K R Q A Q + S V N F N I D S V V A P L R F D A E K P R Q V S R A L

Kunitz domain #9

C.elegans\_MLT-11b/1-3000 2597 R M K S S A A V P R H G A N P L C I Q P V V K G S C Q E A H L R Y Y D R V T D S C R L F E Y S G C D G N A N N F G S L E D C Q R L C V L N I Q S I K N G K V A T T T A A P O I T P E E E 2690  
 C.briggsae\_MLT-11/1-2812 2415 R M K S S P A V P R H G A N P L C I Q P V V K G S C Q E A H L R Y Y D R V T D S C R L F E Y S G C D G N A N N F G S L V D C Q R L C V L N V Q H I S N G Q L A T T T A A P M I T P E E E 2508  
 C.remanei\_MLT-11/1-3019 2619 R M K S S P A V P R H G A N P L C I Q P V V K G S C Q E A H L R Y Y D R V T D S C R L F E Y S G C D G N A N N F G S L E D C Q R L C V L N I Q S I K N G Q A T T T A A P M I T P E E E 2712  
 C.brenneri\_MLT-11/1-1246 847 R M K S S P A V P R H G A N P L C I Q P V V K G S C Q E A H L R Y Y D R V T D S C R L F E Y S G C D G N A N N F G S L E D C Q R L C V L N I Q N I K N G O I A T T T A A P M I T P E E E 940  
 C.japonica\_MLT-11/1-2899 2491 R M N - V P A V P R H G A N P L C I Q P V V K G S C A A H L R Y Y D R V T D S C R L F E Y S G C D G N A N N F G S L E D C Q R L C V L N V K N I Q N G R I A T T T L A P - I T P E E E 2582  
 P.pacificus\_MLT-11/1-3742 2345 P A K A I R R H P G R - R K T P D Y P L E K G E C A E H L R Y F Y D K S S D S C R L F H Y S G C G N T N N F G S I R D C Q E M C V K T V K D T A A Q Q - - - - - K M A S 2426  
 B.malayi\_MLT-11/1-3040 2575 A V N N S - - S D T R Q I T N I C M D V N T G K C T E A H L R F F Y D R R V N T C R L F Y S G C G N E N N F A T E E E C Q Q C K S D K - - - - - A Y D E 2647  
 O.volvulus\_OVOC7267/1-2760 2304 P T V D S - D D D V D K V N D S C M H D L D K G T C S E A H L R F F Y D H K V G F C R L F Y Y T G C G G N E N N F V T E E E C R Q K C K - D K - - - - - I Y S E 2376

Consensus

R M K S S P A V P R H G A N P L C I Q P V V K G S C Q E A H L R Y Y D R V T D S C R L F E Y S G C D G N A N N F G S L E D C Q R L C V L N + Q + I K N G O I A T T T A A P M I T P E E + E

C.elegans\_MLT-11b/1-3000 2691 K L A P G Q C P G G R A P L G G S P V L C G N S A E S I G C P T S Y Y C R R G P P D V C C P G V D P K L M Q P E E I V K D V S - - - - - R G V V K N E S H M P R G F N 2769  
 C.briggsae\_MLT-11/1-2812 2509 K L A P G Q C P G G R S P L G G S P V L C G N S T E S I G C P T S Y Y C R R G P P D V C C P G T D P K L M Q P E E I V K D V G - - - - - R G V V K N E S H M P R G F N 2587  
 C.remanei\_MLT-11/1-3019 2713 K L A P G Q C P G G R A P L G G S P V L C G N S T E S I G C P T S Y Y C R R G P P D V C C P G T D P K L M Q P E E I V K D V G - - - - - R G V V K N E S H M P R G F N 2791  
 C.brenneri\_MLT-11/1-1246 941 K L A P G Q C P G G R S P L G G S P V L C G N S T E S I G C P T S Y Y C R R G P P D V C C P G T D P K L M Q P E E I V K D A G - - - - - R G V V K N E S H M P R G F N 1019  
 C.japonica\_MLT-11/1-2899 2583 K L A P G Q C P G G R A P L G G A S P V L C G N S T E S I G C P T S Y Y C R R G P P D V C C P G P D P K L Q P E E I V K D A G - - - - - R G V V K N E S H M P R G F N 2661  
 P.pacificus\_MLT-11/1-3742 2427 S L P P G T C P S G - E P L G G S A P V L C G N T T E S I G C P K G Y F R Q G P P D V C C P N L S L E I L S A - - - - - A S T - - - - - E G E V K E G V Q T - V P P T 2499  
 B.malayi\_MLT-11/1-3040 2648 D Y S P G S C P Y G E P P L G D N A P V I C G K D A G S F E C R K G Y Y C R M G P P N V C C L E K L L P V S E K I L V T K K H H E N I R F A Q P K V K T S E N V G Y Q F D E K Q K E N D A E 2741  
 O.volvulus\_OVOC7267/1-2760 2377 N A P P G S C P Y G E P P F G D N A P V I C G K D A G S F E C P N G Y Y C R M G P P N V C C L E K H L P A L E K I S V T R E S Q K N I R F S P E H - P G G N P S C Y Q G N K Y Q K E E - - - - - 2467

Consensus

K L A P G Q C P G G R A P L G G S P V L C G N S T E S I G C P T S Y Y C R R G P P D V C C P G T D P K L M Q P E E I V K D V G + N I R F + + + + V + + + + + R G V V K N E S H M P R G F N

C.elegans\_MLT-11b/1-3000 2770 R Q I F L S T P K Y M C P D A A D P L M L E - N G E P M L C G S G F D G V K M C P K G Y Y C A I D S A R N S R L C C P L Y G D A Q R I A S E E I F A P R L A S N T E T T - - - - T E A K V - 2857  
 C.briggsae\_MLT-11/1-2812 2588 R Q I F L S T P K Y M C P D A A D P L M L E - N G E P M L C G S G F D G V K M C P K G Y Y C A I D S A R N S R L C C P L Y G D A H R I A P E E V F A P R L A S N T E T T - - - - T E K L V - 2675  
 C.remanei\_MLT-11/1-3019 2792 R Q I F L S T P K Y M C P D A A D P L M L E - N G E P M L C G S G F D G V K M C P K G Y Y C A I D S A R N S R L C C P L Y G D A Q R I A Q E V F A P R M A S N T E T T - - - - T V R L V - 2879  
 C.brenneri\_MLT-11/1-1246 1020 R Q I F L S T P K Y M C P D A A D P L M L E - N G E P M L C G S G F D G V K M C P K G Y Y C A I D S A R N S R L C C P L Y G D A Q R I A P E E V F A P R L A S N T E T T - - - - T L K L T - 1107  
 C.japonica\_MLT-11/1-2899 2662 R Q I F L S T P K Y M C P D A A D P L M L E - N G E P M L C G S G F D G V K M C P K G Y Y C A I D S A R N S R L C C P L Y G D S Q R I H S E E I F A P R I A S N T E T T - - - - T R G I R I 2750  
 P.pacificus\_MLT-11/1-3742 2500 T T T T T K A P Q H Y C P D A S D P L L T K - A G K I R T C G S G F D G L K M C P K G Y Y C A I N A D Q G T R L C C P I S G S S R I P Y Q H G V I P Y F G K R N P S G E V I E R G S L 2592  
 B.malayi\_MLT-11/1-3040 2742 N S P L A I T S T N I C P D G T D A L L D E S T Q Q P L K C G S Y G D G S F C P V G Y Y C S I D S E K N G R L C C L G V V G V K I P P P P - K I P P Y F G L R P S N P G E I I P R G S L 2834  
 O.volvulus\_OVOC7267/1-2760 2468 V N S A L V V P K D I C P D G S N A L L D E D T G Q P L K C G S Y G D G - S F C P I D Y Y C S I N S E N E R L C C E L G V L G V K I P P L P - T I P P Y F G L R R S N P G E I I L R G S L 2559

Consensus

R Q I F L S T P K Y M C P D A A D P L M L E + N G E P M L C G S G F D G V K M C P K G Y Y C A I D S A R N S R L C C P L Y G D A Q R I A P E E V F A P R + A S N T E T T P G E I T E R + + L

C.elegans\_MLT-11b/1-3000 2858 - - - - - E N I D V E - - - - - - - - - - - - - - - E S - E - - - - - D D E E E D G E D F V A H L Q M K P - N 2885  
 C.briggsae\_MLT-11/1-2812 2676 - - - - - E E V E V G - - - - - - - - - - - - - - - E T - E - - - - - E E D D E E G E D F V A H L K V K P - D 2703  
 C.remanei\_MLT-11/1-3019 2880 - - - - - E D V E V G - - - - - - - - - - - - - - - T E E D - - - - - E E E E E E G E F V A H L K K P P E D 2909  
 C.brenneri\_MLT-11/1-1246 1108 - - - - - E D I N V E - - - - - - - - - - - - - - - N E D E - - - - - D E E E E G E D F V A H L K V K P - D 1136  
 C.japonica\_MLT-11/1-2899 2751 P N E D A A V I V E V E - - - - - - - - - - - - - - - D D E N - - - - - D E D D D D E E G E V A H M K M K P - D 2785  
 P.pacificus\_MLT-11/1-3742 2593 P D D H Q K T I I A D E - - - - - E Q V E M D K K A M D - - - - - D F I V P K S P K G S - - - - - L E R F I D E A E - - - - - V K S E E E D E D E L A R M M I K P T R 2655  
 B.malayi\_MLT-11/1-3040 2835 P S D Y V S L K Q Q A T G I S N T A E P S E Q Y Q S H L D L S G K K L T M N V R P I S D S N R A E E D A I E P S E N L I P S D E Q T T D S S G N V V P S D S K L Q D D I Y G R M M I K S N D 2928  
 O.volvulus\_OVOC7267/1-2760 2560 P S D D V P E K E S - G I I S D V A E P S E R H R S G F A L F G T K L A T D V R P A S D S H A S E E E - - - - - E N S E N L S Q S E S R F R D E V Y G R M M I K S N A 2637

Consensus

P S D D V + E + + E V E + I S + + A E P S E + + + S + + D L + G + K L + + + + V + P + S D S + + + E E + A I E P + E + + I + E + E Q T + + S S + N + + D E E E E E G E D F V A H + K + K P N D

