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previously uncharacterized bli-5 locus in C. elegans. The mutant phenotype, spatial expression pattern and the over-expression phenotype of the BLI-5 protease inhibitor and their relevance to collagen biosynthesis are discussed.

Biosynthesis and Enzymology of the *Caenorhabditis elegans* Cuticle: Identification and Characterization of a Novel Serine Protease Inhibitor.

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Abstract

The nematode *Caenorhabditis elegans* represents an excellent model in which to examine nematode gene expression and function. A completed genome, straightforward transgenesis, available mutants and practical genome-wide RNAi approaches provide an invaluable toolkit in the characterization of nematode genes. We have performed a targeted RNAi screen in an attempt to identify components of the cuticle collagen biosynthetic pathway. Collagen biosynthesis and cuticle assembly are multi-step processes that involve numerous key enzymes involved in post-translational modification, trimer folding, procollagen processing and subsequent cross-linking stages. Many of these steps, the modifications and the enzymes are unique to nematodes and may represent attractive targets for the control of parasitic nematodes. A novel serine protease inhibitor was uncovered during our targeted screen, which is involved in collagen maturation, proper cuticle assembly and the moulting process. We have confirmed a link between this inhibitor and the previously uncharacterized *bli-5* locus in *C. elegans*. The mutant phenotype, spatial expression pattern and the over-expression phenotype of the BLI-5 protease inhibitor and their relevance to collagen biosynthesis are discussed. (171)

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1. Introduction

The nematode cuticle is a multilayered, flexible exoskeleton that affords protection from adverse environmental conditions including the host's immune response. This structure and the moulting process have been most extensively studied in the free-living model nematode *Caenorhabditis elegans* (Singh and Sulston, 1978); (Kramer, 1997); (Johnstone, 2000); (Page, 2001). The cuticle is synthesized during late embryogenesis, then shed and re-synthesized at each larval stage through a process known as moulting. This process involves extensive tissue remodelling and is assisted by numerous proteases (Lustigman, 1993); (Davis et al., 2004); (Brooks et al., 2003). The major component (80%) of this extracellular matrix (ECM) is collagen. Collagens are ubiquitous trimeric structural proteins with a characteristic Glycine-X-Y tripeptide repeat, where X is commonly proline and Y is hydroxyproline. In addition to collagens, a novel highly cross-linked insoluble class of protein called cuticlins are associated with the cuticle (Sapio et al., 2005). The outermost layer, the epicuticle is lipid-rich and this in turn is overlaid by the glycoprotein –rich surface coat (Page and Winter, 2003).

The cuticle collagen gene family in *C. elegans* encodes 154 members, 21 of these when mutated result in a range of informative body morphology defects Dumpy (Dpy), Roller (Rol), Blister (Bli), Squat (Sqt), Ray abnormal (Ram) and Long (lon). Likewise mutations in the structural cuticlin genes result in morphological defects, predominantly of the Dpy class (Sapio et al., 2005). In addition to the collagen and cuticlin-related mutants a smaller number of morphogenetic mutants are association with the collagen biosynthetic pathway. Collagen biosynthesis is a complex, multi-step process in both vertebrates and invertebrates (Figure 1). These modifications occur both intra- and extra-cellularly and involve chaperones and numerous modifying, folding and processing enzymes, and mutations in some members produce profound body morphology phenotypes.

64 The first important co-translational modification of procollagen is prolyl 4-
65 hydroxylation which allows the proper folding into a thermally-stable form (Figure 1).
66 The *C. elegans dpy-18* locus encodes a critical α subunit of the multi-component enzyme
67 prolyl 4-hydroxylase (Winter and Page, 2000). These enzymes associate with the β subunit
68 protein disulphide isomerase (PDI) to form active, soluble ER-resident enzymes. Null
69 mutants of *dpy-18* are medium Dpy and the cuticle collagens exhibit reduced
70 hydroxyproline content (Winter and Page, 2000). The combination of *dpy-18* and RNAi of a
71 second α subunit *phy-2* (Winter and Page, 2000) or by crossing with a *phy-2* deletion
72 mutant (Friedman et al., 2000), both result in embryonic lethality. Embryonic death was
73 also noted following RNAi of the *pdi-2* β subunit-encoding gene (Winter and Page, 2000).
74 Unique active complexes between these 3 subunits were detected, both *in vitro* and *in vivo*;
75 namely, mixed PHY-1/PHY-2/$(PDI-2)_2$ tetramers and PHY-1/PDI-2 and PHY-2/PDI-2
76 dimers (Myllyharju et al., 2002).
77 The next important step in vertebrate collagen folding that must precede trimer
78 formation is the correct registration of the monomers. Collagen trimerization has
79 principally been reported to be initiated from the C- terminus, and may be set up via PDI
80 catalysed disulfide bond formation (Figure 1). In support of this contention, the *C. elegans*
81 collagens have C-terminal conserved cysteine clusters. In addition to the role in proline
82 hydroxylation it is proposed that PDI-2 may be involved in this oxidative registration step.
83 The trimerization of imino-rich (~25 % proline and hydroxyproline) collagen is rate-
84 limited by the slow *cis-trans* proline isomerization, and requires the assistance of peptidyl
85 prolyl cis-trans isomerase (PPIase) enzymes, of the cyclophilin (CYP) and FK506 binding
86 protein (FKB) class (Figure 1). These genes constitute large redundant families in the
87 worm including several ER-resident isoforms that may perform this function (Page et al.,
88 1996); (Page, 1997); (Bell et al., in press).

Various chaperones, including SEC-23, have been implicated in the transit of procollagen from the ER into the secretory pathway (Roberts et al., 2003). The transport steps are thought to coincide with the pro-collagen N- and C –terminal processing events, that change the solubility properties and precede the mutlimerization and crosslinking of the mature insoluble collagens (Figure 1). The Kex2, furin-type serine protease encoding gene *bli-4* is essential for post-embryonic viability in *C. elegans* (Peters et al., 1991). All cuticle collagens possess a highly conserved N-terminal cleavage site for BLI-4, a site experimentally demonstrated to be essential for the proper processing of ROL-6 and SQT-1 collagens (Yang and Kramer, 1994). Null mutants of *bli-4* are embryonically lethal, whereas partial loss-of-function mutants were viable but Bli and Dpy (Peters et al., 1991) ;(Thacker et al., 1995), and recent RNAi screens targeting *bli-4* also describe a moult defect (Kamath et al., 2001). The C-terminal processing of vertebrate fibrillar collagens is an essential step, and is carried out by zinc metalloprotease of the astacin, BMP class (bone morphogenic protein) (Canty and Kadler, 2005); (Pappano et al., 2003). The majority of nematode collagens have very short C-terminal non Gly-X-Y domains and until recently this processing step was not considered to be important for their maturation. Isolation and characterization of mutations in the astacin-encoding gene *dpy-31* result in temperature sensitive severe Dpy, Emb and Let phenotypes in the worm (Novelli et al., 2004). This enzyme is hypodermally expressed and is required for normal cuticle collagen secretion. Suppressor screens identified the essential cuticle collagen SQT-3 to be a major substrate for this enzyme, and highlighted its cleavage site adjacent to the C-terminal tyrosine cross-linking site, demonstrating that *dpy-31* encodes an essential procollagen C-peptidase (Novelli et al., 2004), (Figure 1).

The final steps in collagen maturation and cuticle synthesis are the structural cross-linking events. The cuticle collagens and cuticlins are covalently cross-linked via unusual di- and tri- tryrosine cross-links. These non-reducible cross-links impart the characteristic

115 strength and integrity to the cuticle (Page, 2001) and differ from the hydroxylysine-
116 derived crosslinks of the vertebrate collagens (Myllyharju and Kivirikko, 2001). The major
117 cuticle cross-linking enzyme is a dual oxidase enzyme encoded by the *bli-3* locus (Edens et
118 al., 2001); (Simmer et al., 2003), (Figure 1). This large enzyme has a signal peptide, a
119 membrane-bound peroxide generating domain and a functionally-active peroxidase
120 domain (Edens et al., 2001). RNAi (Edens et al., 2001) or single point mutations (Simmer et
121 al., 2003) in this enzyme result in weakened cuticles that lack tyrosine cross-links, with
122 resulting adult worms devoid of cuticle struts and exhibiting Dpy and Bli phenotypes.
123 Characterization of this critical pathway is helping to uncover many nematode-
124 specific features that may prove to be fruitful in future nematode control strategies. In
125 addition to forward genetic screens to identify factors that affect body morphology and
126 cuticle synthesis, numerous genome-wide RNA interference studies have identified novel
127 genes and indeed have confirmed the significance of previously identified components of
128 the collagen biosynthetic machinery (Kamath et al., 2003); (Simmer et al., 2003); (Frand et
129 al., 2005). We have carried out a selective RNAi screen to identify cuticle collagen
130 biosynthetic pathway factors using a tagged adult specific collagen strain, COL-19::gfp
131 (Thein et al., 2003). This targeted approach focused on predicted collagen-associated
132 enzymes and their inhibitors and uncovered, amongst other targets, a serine protease
133 inhibitor F45G2.5 and linked it to the previously uncharacterized morphological defect *bli-*
134 *5*. The identification and characterization of the BLI-5 kunitz protease inhibitor and its
135 predicted role in the cuticle collagen biosynthesis pathway is described.
136
137 **2. Materials and Methods**
138 *2.1 Strains.* CB518 (*bli-5),* DR96 (*unc-76)* and N2 (wild type) *C. elegans* strains were obtained
139 from the *C. elegans* Genetics Center. Worms were cultured and manipulated following
140 standard *C. elegans* methods (Sulston and Hodgkin, 1988).

141

142 *2.2 COL-19::gfp RNAi screens*. The construction of the TP12 (COL-19::gfp) integrated strain
143 and TP18 (CB518/TP12) strains are described elsewhere (Thein et al., 2003). Briefly, the
144 annotated genome (www.wormbase.org) was screened for predicted collagen biosynthetic
145 enzymes, together with expected inhibitors. These primary hits were either retrieved from
146 the RNAi feeding library (Kamath et al., 2003) or cloned independently into the feeding
147 vector L4440. TP12 COL-19::gfp nematodes were then allowed to feed on the RNAi
148 bacteria and viewed at regular intervals under epifluorescence to observe the effect on the
149 tagged collagen. For F45G2.5, a 550bp cDNA fragment was generated by PCR (primers
150 bli5F, gcgggatccaaatgcctgaccaatgaag and bli5R, gcgctgcagtcagaaaagatgaaagttggac) and
151 cloned into the RNAi feeding vector L4440. Details of feeding experiments are described
152 in detail elsewhere (Thein et al., 2003).

153

154 *2.3 Microscopy*. Worms were mounted on agar pads viewed under DIC or epifluorescence
155 optics on a Zeiss Axioscope 2 and images were captured on an Axiocam MRm camera
156 using Axiovision software. TEM samples were fixed in 2.5% gluteraldehyde then 1%
157 osmium tetroxide in 0.1M phosphate buffers. Samples were dehydrated and infiltrated in
158 LR-White (Page et al., 1992) and viewed on a Zeiss 902 transmission electron microscope.

159

160 *2.4 Bli-5 gene cloning*. The *bli-5* genomic and cDNA transcripts were cloned into a Topo
161 vector (Invitrogen) prior to sequencing, using the above bli5F and R primers on genomic
162 and cDNA template derived from *bli-5 (e518)* mutant worms using standard methods
163 (Page et al., 1996).

164

165 *2.5 bli-5 rescue*. Two *bli-5* rescue constructs were employed in this study, the first was a
166 full-length copy of the cosmid clone F45G2 that was provided by Alan Coulson (Sanger

Centre, Cambridge). The cosmid was co-injected at 10ng/µl with a marker for transformation, *dpy-7*::gfp, at 5ng/µl. The second construct was generated by PCR using genomic DNA as a template with the following primers; RescueF, gcgctgcagctgtacctcgagacgtgggcg and RescueR, cgcggatcctgacgatgttagtttccttcac. The 4,184bp fragment comprised the putative promoter (1,776bp), genomic coding domain and 3'UTR and was cloned into Topo and co-injected at 2ng/µl with the *dpy*-7::gfp marker at 5ng/µl. Transgenic lines were selected based on fluorescence and examined for repair of phenotype of the CB518 mutant background.

2.6 bli-5 spatial expression. A 2,310bp genomic insert encompassing the putative promoter to the second exon of F45G2.5 was generated by PCR using the following primer pair; RescueF and PromoR, gcgggatccgtcaggcatttctggagttatg. The insert was digested with *Pst* I and *Bam* HI and cloned into the reporter vector pPD96:04 (Addgene), then injected into DR96 worms at 5ng/µl together with the *unc-76* rescue plasmid at 5ng/µl. Rescued transformants (3 independent lines) were stained for β-galactosidase activity following published methods (Winter and Page, 2000) and different lines and stages were examined microscopically.

184

185 *2.7 bli-5 Overexpression.* The following primers were used to clone a 609bp cDNA copy and
186 1,991bp genomic copy of the *bli-5* gene; HsF gcgggtaccatggtatctatccataattc and HsR,
187 gcggagctctcagaaaagatgaaagttg, using either cDNA or genomic DNA as a template. The
188 insert was digested with *Kpn* I and *Sac* I and ligated into similarly digested hypodermal
189 heat shock vector pPD49:78 (Addgene). The constructs were co-injected at 25ng/µl
190 together with the *unc-76* rescue plasmid (100ng/µl) into DR96 (*unc-76*) worms. Mixed
191 stage rescued transgenic lines (4 independent lines) were heat shocked at 33°C for 5 hours,
192 allowed to recover at 20°C overnight and examined for associated phenotypes.

193

194 **3. Results**

195

196 **3.1 RNAi of F45G2.5 leads to a COL-19 disruption pattern that is similar to that of *bli-5***
197 **mutants.**

198 The TP12 COL-19::*gfp* strain displays a uniform annular and alae distribution of
199 fluorescence in the wild type adult cuticle (Figure 2A) and has proved to be a powerful
200 tool in the identification of cuticle collagen biosynthetic pathway components (Thein et al.,
201 2003). Crossing with uncharacterized morphological mutants has also been highly
202 informative with respect to their characterization and potential identification (Thein et al.,
203 2003). Crossing this integrated marker strain with *bli-5 (e518)* mutant alleles demonstrates
204 COL-19 disruption in both the dorso/ ventral annulae and the lateral alae (Figure 2B).
205 RNAi screens of potential collagen biosynthetic pathway components identified a kunitz
206 type serine protease inhibitor encoded by F45G2.5 (Figure 2 C) to have a comparable COL-
207 19 disruption pattern to *bli-5* mutant alleles. In addition to the aberrant fluorescent
208 phenotype, F45G2.5 RNAi also produced blister and moult defective phenotypes (data not
209 shown). These observations and the close genetic map position of F45G2.5 and the *bli-5*

locus on the end of linkage group III (Figure 3) made the encoded protease inhibitor a good candidate for the *bli-5* locus. *bli-5* mutant alleles were originally isolated in early morphological screens (Brenner, 1974) but previously remained uncloned. One major phenotype of this mutation took the form of blisters in the adult cuticle particularly around the head region. One of the main distinguishing features that sets the adult cuticle apart from the larval counterparts is the presence of electron dense medial struts that separate the external (cortical) and internal (fibrous and basal) layers of the cuticle (Figure 2D). This medial layer is proposed to be fluid filled and the regular position of the struts corresponds exactly to the position of the annulae when viewed in freeze fractured cuticle preparations (Kramer, 1997). In contrast to wild type worms (Figure 2 D), the cuticle of *bli-5 (e518)* mutant nematodes is virtually devoid of struts, and those present were incomplete when viewed by TEM (Figure 2 E). The lack of struts often corresponded to the presence of blisters that are characterized by vesicle- filled swellings between the basal and cortical cuticle layers (Figure 2 F).

3.2 Cloning of the F45G2.5 gene from *bli-5 (e518)* reveals a single point mutation and phenotype rescue confirms their predicted association.

To determine if indeed the kunitz protease inhibitor corresponded to *bli-5*, the gene was cloned and sequenced from both cDNA and genomic DNA isolated from the *bli-5 (e518)* mutant worms (Figure 3), and the cosmid that contains F45G2.5 was tested in transformation rescue experiments with this mutant allele (Figure 4). The F45G2.5 open reading frame encodes a 202 amino acid protein with a signal peptide, followed by a nematode specific EB domain and a kunitz-type trypsin inhibitor domain (Figure 3). A homologue of this gene has been partially cloned from the parasitic nematode *Ostertagia ostertagi* (BQ457535) and shares 60% identity over 156 residues that include the EB and kunitz domains. Cloning of this gene from both genomic and cDNA templates revealed a

single point mutation TCA- TTA in the highly conserved EB domain, resulting in a single serine to leucine change (Figure 3). The association was further confirmed by transformation rescue of *bli-5 (e518)* mutant worms, with the cosmid F45G2 (Figure 4) and with a 4,184 bp genomic rescue construct comprising the putative promoter, coding sequence and putative 3'UTR of the F45G2.5 gene (data not shown). Co-expression of the *dpy-7*::gfp marker corresponded to F45G2.5 transformed worms that were no longer blistered, but wild type in appearance (Figure 4 A & B). These experiments validated the link between F45G2.5 and the *bli-5* mutant locus and this gene will subsequently be referred to as *bli-5*.

3.3 Localization of *bli-5* is consistent with its predicted role in cuticle collagen maturation.

The putative promoter region incorporating the first 2 exons of *bli-5* was "translationally" fused to *LacZ*, and was co-transformed with an *unc-76* rescue plasmid into DR96 nematodes. Rescued transformants were selected, maintained and stained with β-galacotosidase to examine the spatial expression of the *bli-5* transcript. *bli-5* is abundantly expressed in the larval (Figure 5A) and adult hypodermis, the hermaphrodite vulva (Figure 5B) and the excretory cell and duct (Figure 5C). These localizations are consistent with an enzyme inhibitor playing a role in cuticle collagen biosynthesis, cuticle remodelling and the moulting process.

3.4 Over-expression of BLI-5 results in moulting, cuticle and vulval defects.

The full length cDNA and genomic *bli-5* genes were cloned into a hypodermally expressed heat shock construct, co-injected into DR96 nematodes with a rescue plasmid and resulting rescue lines were maintained and analysed following heat shock (Figure 6). Heat shock of genomic and cDNA lines produced identical phenotypes, namely moult and

protruding vulva defects (Figure 6 A-D). Control heat shock of wild type nematodes did not confer a phenotype (data not shown). These results are consistent with the hypothesis that BLI-5 is regulating an enzyme that is involved in cuticle synthesis, collagen processing and the general moulting process.

4. Discussion

The free-living nematode *Caenorhabditis elegans* has long been proposed as a genetic and experimentally amenable model nematode system (Politz and Philipp, 1992); (Page, 2001); (Page and Winter, 2003); (Gilleard, 2004). The full genome sequence of *C. elegans* was deciphered in 1998 (Consortium, 1998) and numerous genome –wide RNAi screens (Kamath et al., 2003); (Simmer et al., 2003); (Frand et al., 2005) have since been performed to elucidate a function to the 20,000 encoded genes. We have exploited this system in an attempt to investigate further the enzymology of the nematode cuticle collagen assembly (Page and Winter, 2003); (Figure 1). In addition to examining known components of the collagen biosynthetic pathway (Page and Winter, 2003); (Winter and Page, 2000); (Eschenlauer and Page, 2003) we have set up RNAi-based screens with a collagen marker strain (Thein et al., 2003) to look for novel factors involved in this pathway. The identification and characterization of one such target, namely *bli-5* is described in this report.

The *bli-5* gene encodes an unusual serine protease inhibitor that has many features that link it to the collagen biosynthetic pathway. RNAi of F45G2.5 produces blister, moult (Kamath et al., 2003); (Frand et al., 2005) and COL-19 disruption defects (Figure 2), phenotypes that mirror the closely associated *bli-5* mutant locus (Figure 2). Through mutant allele cloning and mutant phenotype rescue experiments we successfully confirmed the link between F45G2.5 and *bli-5* (Figure 3 & 4). BLI-5 is a secreted serine protease inhibitor with an EB domain and a kunitz-type pancreatic protease inhibitor domain. A homologue of this unusual inhibitor is present in the veterinary parasite *Ostertagia ostertagi* (accession number BQ457535). Kunitz-type serine protease inhibitors have been described from many species, including *Drosophila melanogaster* (Kramerova et al., 2000); (Kress et al., 2004) and from parasitic nematodes including *Ancylostoma caninum*

293 (Hawdon et al., 2003) and *A. ceylanicum* (Milstone et al., 2000). Indeed, the *A. ceylanicum*
294 inhibitor was also localized to the sub-cuticle of the adult stages (Chu et al., 2004). In
295 addition, a class of inhibitors called the papilins that also contain a kunitz domain have
296 likewise been implicated in controlling the function of the invertebrate procollagen N-
297 peptidase ADAMTS (Kramerova et al., 2000); (Kramerova et al., 2003).
298 The presence of the EB domain (Pfam identifier PF01683) is intriguing since this
299 domain is completely nematode specific. EB domains characteristically have 8 conserved
300 cysteines that are predicted to form 4 disulphide bridges and are usually found in
301 association with kunitz domains. The functional significance of this domain was
302 confirmed during the sequencing of the *bli-5 (e518)* mutant allele, revealing the presence of
303 a point mutation in this domain that changed a hydrophilic serine to a hydrophobic
304 leucine residue (Figure 3). Such a change may be predicted to alter the binding properties
305 of this domain to its protein partner and hence lead to the resulting mutant phenotype.
306 This serine was also conserved in the *O. ostertagi* inhibitor (Figure 3).
307 Together with the BLI-5 cuticle-related phenotypes (Figure 2); the predicted role of
308 kunitz-type inhibitors in extracellular matrix remodelling and moulting in insects
309 (Kramerova et al., 2003), (Kress et al., 2004); the BLI-5 spatial expression pattern in the
310 hypodermal and excretory tissues (Figure 5); and the BLI-5 overexpression effects in the
311 cuticle (Figure 6), all point to a role for this inhibitor in the cuticle collagen biosynthetic
312 pathway.
313 What is the *specific* role of BLI-5 *in vivo*? This is currently unknown, but the following
314 model may be relevant. The inhibitor BLI-5 may be interacting with, and controlling the
315 protease activity of the BLI-4 blisterase kex2-like enzyme, or indeed a functionally related
316 regulatory enzyme. This is a plausible assumption, as both *bli-4 (e937)* and *bli-5 (e518)*
317 mutations and RNAi of their transcripts (www.wormbase.org) result in moulting defects
318 and blister phenotypes. Moulting and blister phenotype are also a feature of BLI-5 over-

319 expression. In support of this assertion, kunitz-type serine protease inhibitors also control
320 tissue remodelling enzymes that are involved in the moulting process of *Drosophila* (Kress
321 et al., 2004). Interestingly, BLAST analysis of BLI-5 in addition to hitting kunitz-type
322 inhibitors also hits papilins and more intriguingly, kex-like proprotein convertases (data
323 not shown) and may support the hypothesis that BLI-5 and BLI-4 can interact directly.
324 BLI-4 is a well-characterized kex2-like protease in *C. elegans* (Peters et al., 1991); (Thacker
325 et al., 1995); (Thacker et al., 2000) that has homologues in parasitic nematodes (Poole et al.,
326 2003). The *bli-4* gene generates 9 alternative protein isoforms of the blisterase enzyme that
327 are expressed in the hypodermis, vulva and the neurones (Thacker et al., 2000). The
328 majority of mutant alleles are embryonic lethal, however *bli-4 e937* affects a limited subset
329 of these forms and results in the blistering phenotype of the adult cuticle (Thacker et al.,
330 1995). The main function of BLI-4 is predicted to be as an N-terminal procollagen
331 processing enzyme (Yang and Kramer, 1994); (Kramer, 1997); (Thacker et al., 2000), and all
332 cuticle collagens possess the characteristic N-terminal basic RXXR recognition site of kex2-
333 like enzymes. The cuticle collagen disruption pattern of BLI-5, the shared phenotypes and
334 the shared tissue expression patterns between BLI-5 and BLI-4 are all consistent with an
335 association. In addition to procollagen, numerous other proteins possess the RXXR
336 processing site, including known moulting and collagen processing enzymes. The NAS-37
337 astacin metalloprotease (Davis et al., 2004) and the procollagen C-peptidase DPY-31
338 (Novelli et al., 2004), also an astacin metalloprotease, are both activated following
339 processing via an RXXR site. An additional, or indeed an alternative function BLI-5 may
340 therefore be in the fine control of BLI-4 and/or another subtilisin-like protease that is
341 performing this critical enzyme activation step.

342 In this paper we review some of the recent data regarding the nematode cuticle
343 collagen biosynthetic pathway. In addition, we highlight the relatively simple application
344 of the *C. elegans* model system, in combination with RNAi and the well-annotated genome

345 data (www.wormbase.org) to rapidly assign a function to a previously uncharacterized
346 gene and provide a link to a previously uncloned morphogenetic mutant. Similar reverse
347 genetic approaches will help identify additional nematode-specific genes that are involved
348 in nematode-specific processes, such as cuticle assembly and moulting, and may
349 ultimately help identify potential drug and/ or vaccine targets of relevance to the control
350 of parasitic nematodes.
351
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356
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Figure Legends

Figure 1

Schematic representation of the critical modification steps in the biosynthesis of nematode cuticle collagens and assembly of the nematode cuticle (see accompanying text for a full description of listed enzymes).

Figure 2

The COL-19 cuticle collagen and gross cuticle morphology are disrupted in a similar manner in *bli-5 (e518)* mutants and following F45G2.5 RNAi in a wild type background. A, Wild type expression pattern of COL-19::gfp in the wild type adult cuticle (TP12 strain). B, Aberrant COL-19::gfp expression in *bli-5 (e518)* mutant nematodes. C, Aberrant COL-19::gfp expression following F45G2.5 RNAi in TP12 wild type strain. A-C, arrows define annulae and arrowheads the lateral alae, scale bar represents 10μM. D, TEM of wild type cuticle, depicting regularly spaced medial struts (arrowed). E, TEM of *bli-5 (e518)* mutant nematodes revealing comparative lack of struts. F, TEM of *bli-5 (e518)* mutant nematodes depicting a surface blister separating the basal and cortical layers of the cuticle (arrowed). D-F scale bar represents 1 μM. All images depict adult nematodes.

Figure 3

Genomic and physical relationship between the *bli-5* locus and F45G2.5. The cosmid clone F45G2 maps at the end of linkage group III in the vicinity of the *bli-5* locus. The open reading frame F45G2.5 encodes a transcript with 4 exons that encodes a 202 amino acid protein with a signal peptide (Sp), and nematode specific EB domain and a kunitz-type serine protease inhibitor domain. Sequencing of the genomic and cDNA copies of F45G2.5 from *bli-5 (e518)* mutants identified a single TCA-TTA (serine to leucine) point mutation in

510 the conserved EB domain. The same regions from *C. elegans* (N2) and *Ostertagia ostertagi*
511 (*O. ost*) are shown for comparison.
512
513 **Figure 4** (Top panel)
514 Cosmid rescue of *bli-5 (e518)* mutant locus. The cosmid clone F45G2 was co-injected with
515 *dpy-7*::gfp marker and transgenic worms were assessed microscopically. A, DIC field of 2
516 rescued and 1 non-rescued worms (arrowhead). B, Fluorescence image of same field
517 revealing correlation of fluorescence with rescue of blister phenotype, in comparison to
518 the non–rescued (blistered) non-fluorescent worm (arrowhead).
519
520 **Figure 5** (Bottom panel)
521 Spatial expression of the *bli-5* transcript. Nematodes were transformed with the *bli-5*
522 promoter reporter construct, transgenic lines were selected and populations were fixed
523 and stained for β-galactosidase activity. A, L3 larvae with predominant hypodermal seam
524 cell expression (arrow). B, Adult hermaphrodite vulval expression (arrow). C, Adult
525 excretory cell and excretory duct expression (arrow).
526
527 **Figure 6**
528 Overexpression of BLI-5 under a hypodermal heat shock promoter results in moult and
529 vulval defects following heat shock. A, L2 larvae encased in unshed L1 cuticle, tip of head
530 (black arrow) and tip of tail (white arrow) positions are indicated. B, Unshed L3 cuticle
531 (black arrow) retained on head of L4 larvae. C, Unshed L4 cuticle on the head of and adult
532 worm (black arrow), blisters also observed on cuticle (white arrow). D, Protruding vulva
533 (arrow) on adult hermaphrodite.

Figure 1

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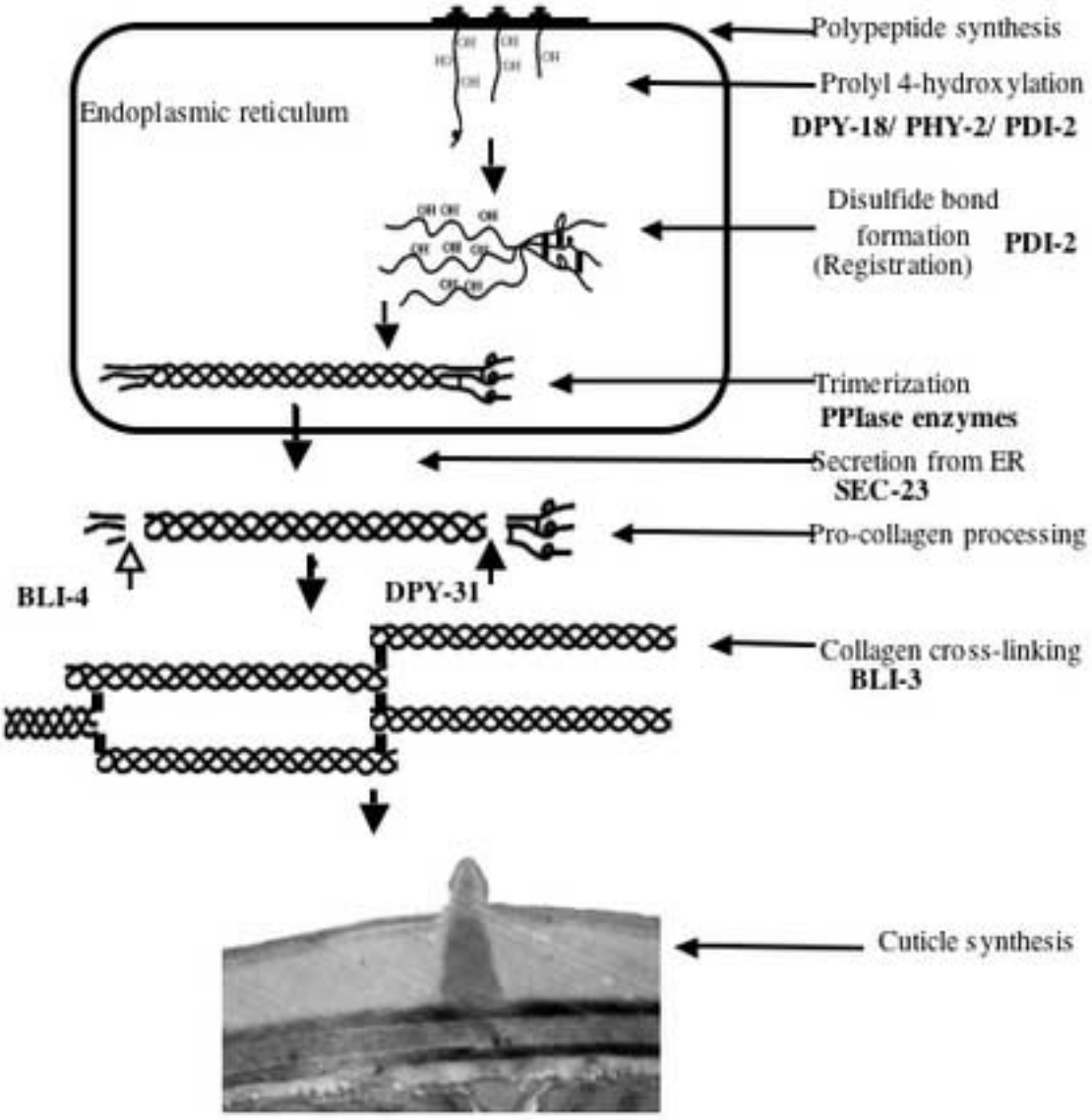

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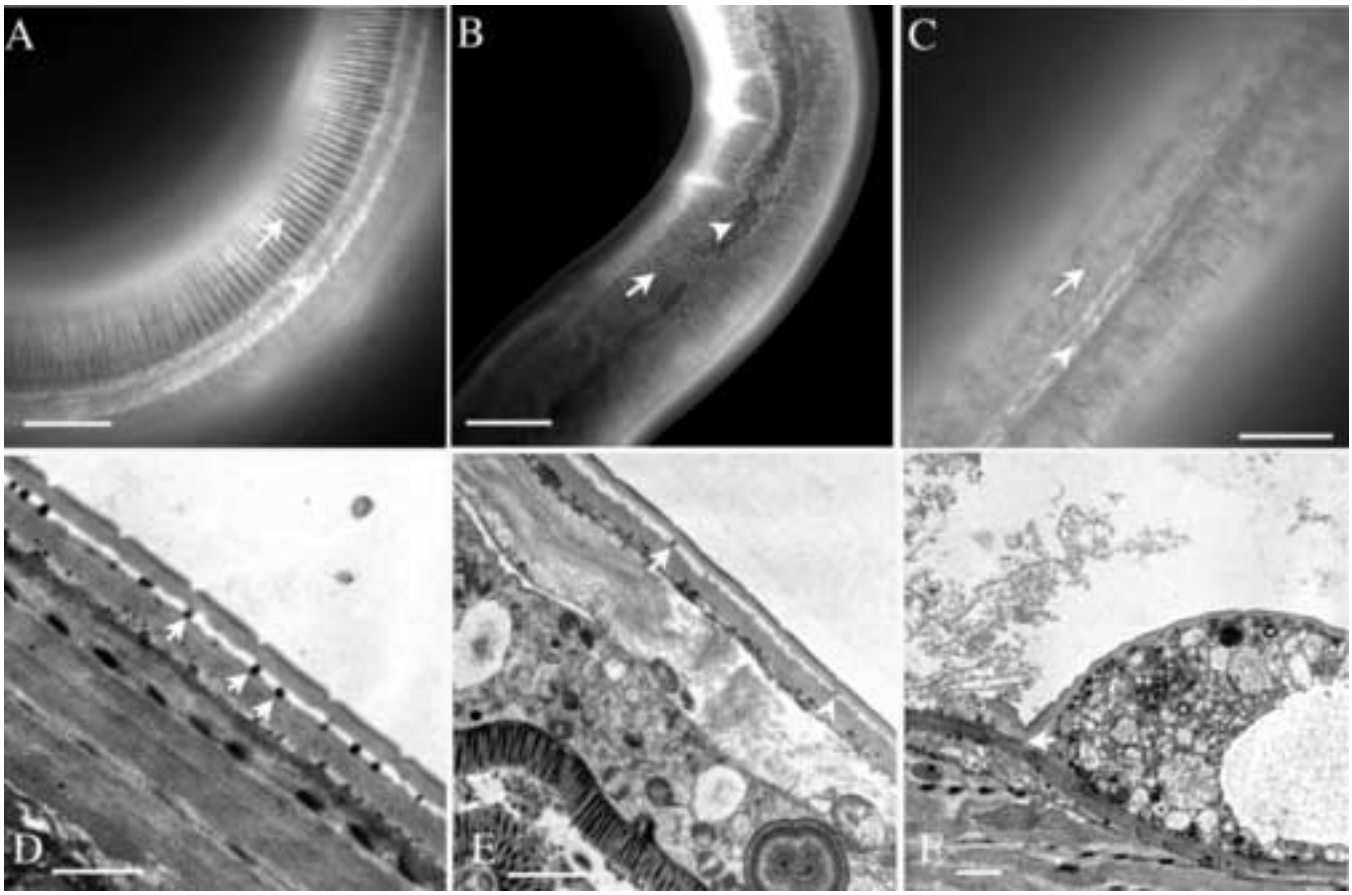

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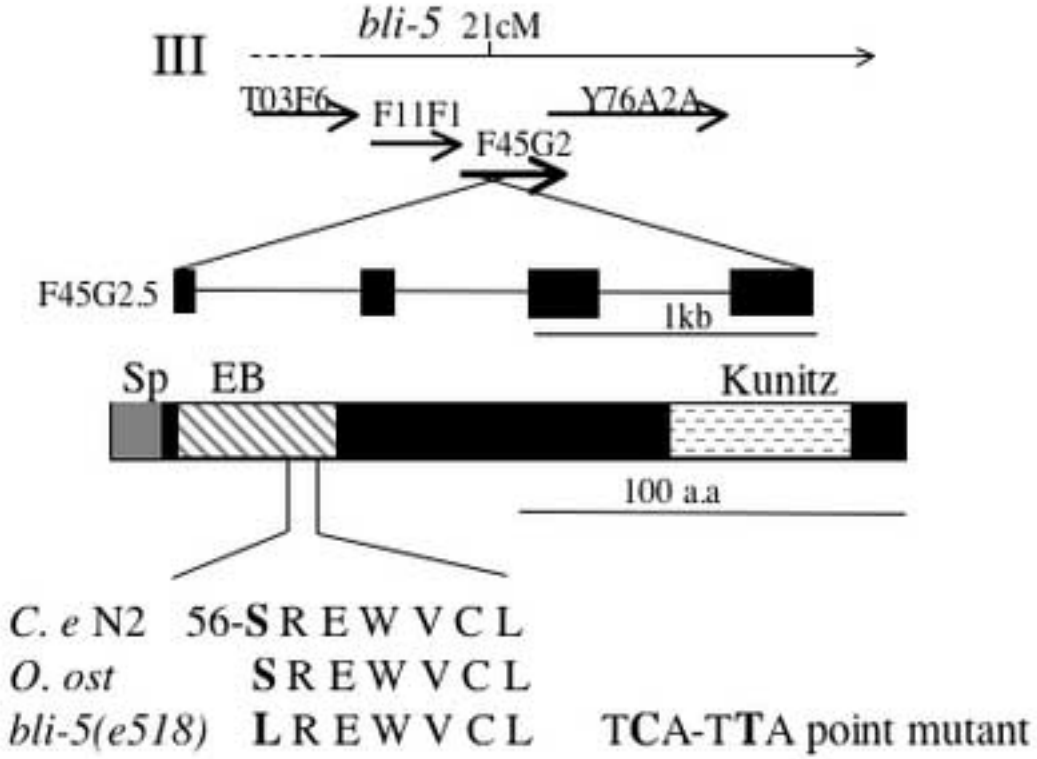

Figures 4 and 5

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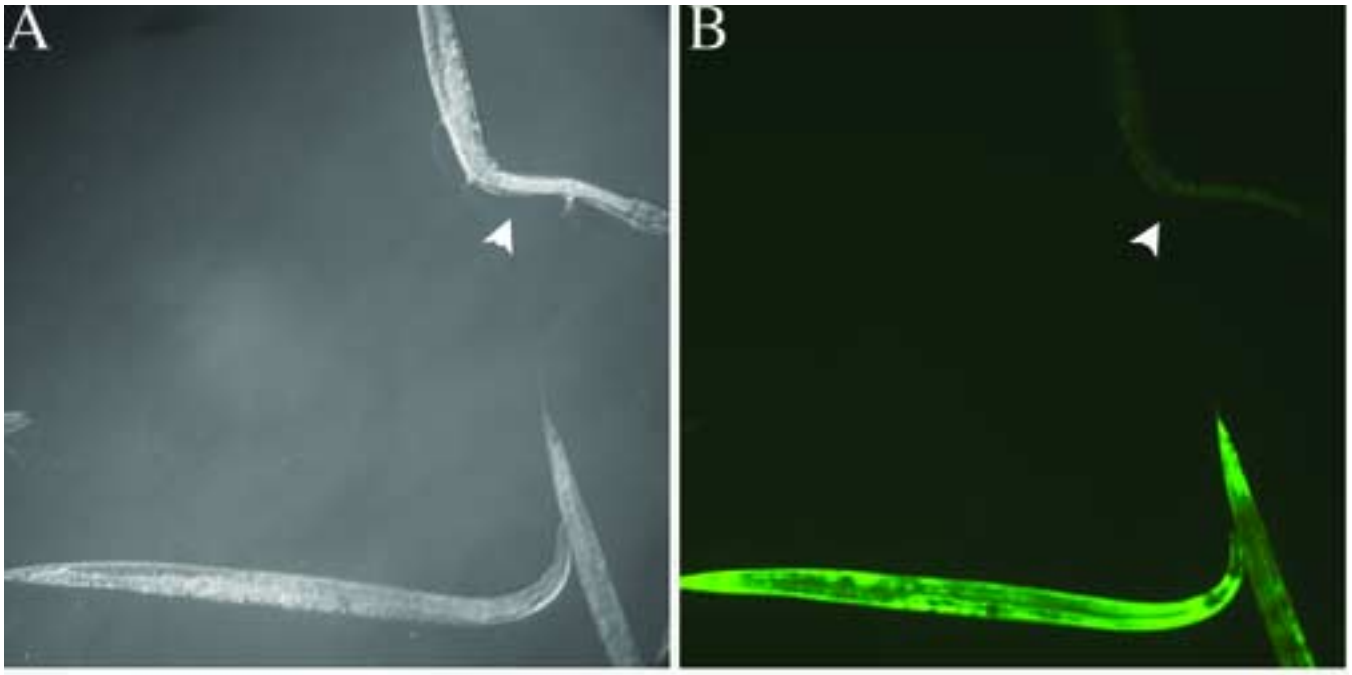

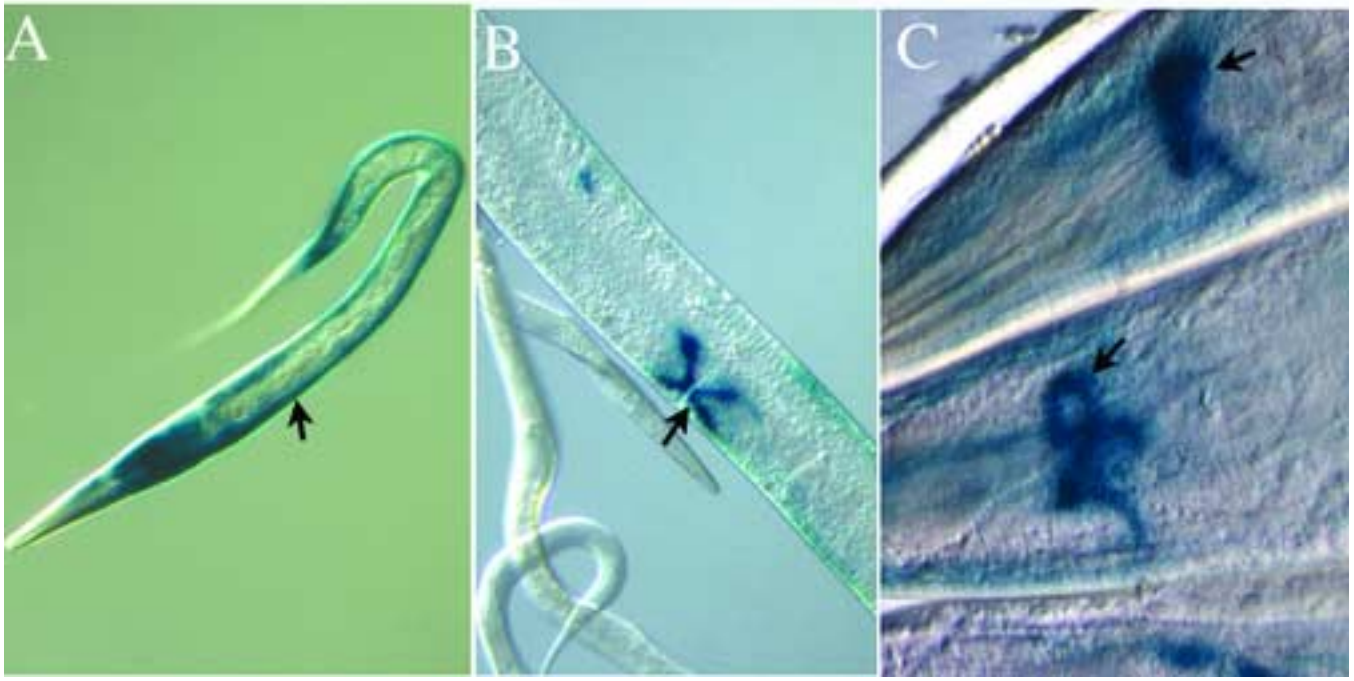

Figure 6

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