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The Zona Pellucida domain containing proteins, CUT-1, CUT-3 and CUT-5, play essential roles in the development of the larval alae in *Caenorhabditis elegans*

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Abstract

The alae, longitudinal ridges of the lateral cuticle, are the most visible specialization of the *Caenorhabditis elegans* surface. They are present only in L1 and dauer larvae and in adults. Little is known about the mechanisms through which at the appropriate stages secretion of cuticle components by the seam cells results in the formation of the alae. Here we show that three proteins, each containing a Zona Pellucida domain (ZP), are components of the cuticle necessary for larval alae development: CUT-1 and CUT-5 in dauer larvae and CUT-3 and CUT-5 in L1s. Transcriptional regulation of the corresponding genes contributes to the stage-specific role of these proteins. Larvae with reduced *cut-1*, *cut-3* or *cut-5* function not only lack alae but are also larger in diameter due to an increase in the width of the lateral cuticle. We propose a model in which reduction of the body diameter, which occurs in normal L1 and dauer larvae, is the result of a dorso-ventral shrinking of the internal layer of the lateral cuticle and formation of the alae results from the folding of the external layer of the lateral cuticle over the reduced, internal one. Alae of adults appear to form through a different mechanism.

Keywords: Nematode; Cuticle; Alae; Dauer larvae; L1 larvae; CUT-1-like proteins; ZP domain; Extracellular matrix

Introduction

Cell biology and developmental biology have, in the last several years, made important progress in elucidating the mechanisms through which biological shapes are attained both at the sub-cellular and at the multicellular level. In comparison, the mechanisms underlying the morphogenesis of extra-cellular structures have been poorly studied. The cuticle of *Caenorhabditis elegans* is a useful model to study these mechanisms (Johnstone, 2000; Kramer, 1994, 1997). The cuticle is a tough, elastic, extracellular layer, which surrounds the worm and is secreted by the underlying epithelia. It functions as an exoskeleton and determines the shape of the animal and its capacity to move. In addition to these mechanical functions the cuticle also mediates the interactions of the animal with its environment. The most visible surface specializations of the *C. elegans* cuticle are the annuli and the alae. The annuli are narrow, circumferential indentations, spaced about 1 μm apart that are present on the surface of all stages. The cellular and molecular mechanisms underlying their formation have been addressed and largely elucidated (Costa et al., 1997; McMahon et al., 2003).

The alae are lateral, symmetric, longitudinal ridges that run the entire length of the animal. They are present only in L1 and dauer larvae and in adults (Cox et al., 1981b). Their morphology is different and characteristic for each of these stages. Microscopic observations, laser ablation studies and phenotypes of various mutants indicate that presence and proper differentiation of the lateral epidermal cells, also referred to as seam cells, are necessary for the formation of the alae (Antebi et al., 2000; George et al., 1998; Koh and

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Rothman, 2001; Petalcorin et al., 1999; Singh and Sulston, 1978; Sulston and Horvitz, 1977). Many questions regarding the formation of the alae remain unanswered. The seam cells also secrete the lateral cuticle of the stages L2, L3 and L4 in which alae are not present. What happens in the lateral cuticle of L1 and dauer larvae and of adults that modifies it so that alae are eventually formed? Are there cuticle proteins specific to the alae and necessary for their formation? What is the mechanism by which the different stage-specific shapes of the alae are generated?

The major components of the cuticle are collagen-like proteins which can be extracted by strong ionic detergents and disulfide reducing agents (Cox et al., 1981a; Johnstone, 2000; Page and Winter, 2003). The insoluble residue left after extraction is composed of various heavily cross-linked proteins and is named cuticlin (Fujimoto and Kanaya, 1973; Lassandro et al., 1994; Sebastiano et al., 1991). CUT-1(C47G2.1), a ZP (Zona Pellucida) domain containing protein, was the first non-collagenous component of cuticlin to be identified molecularly and was shown to be localized in the internal layer of the cuticle of dauer larvae in a ribbon, running the whole length of the animal, underneath the alae (Ristoratore et al., 1994; Sebastiano et al., 1991). Recently, another ZP domain containing protein, CUT-6 (M142.2), was shown to be a component of the matrix involved in the determination of the dauer larva body shape (Muriel et al., 2003). Here we analyze the role in alae formation of *cut-1*, of *cut-6* and of three additional genes: *cut-3* (*f22b5.3*), *cut-4* (*e04d5.3*) and *cut-5* (*r07e3.3*) which code for proteins with sequence and domain organization remarkably similar to those of CUT-1 (Fig. 2A, http://www.wormbase.org/ and M. R. Sapio and P. Bazzicalupo, unpublished). We show that *cut-1* is necessary for the formation of the alae of dauer larvae and to produce a radial shrinking of the body such that dauer larvae with reduced CUT-1 function are dumpyish and lack alae. *cut-3* is necessary to accomplish in L1 larvae a function analogous to that of *cut-1* in dauer larvae: alae formation and correct body shape. We determined that CUT-3, expressed under the *cut-1* regulatory sequences, can substitute for CUT-1 to rescue the formation of dauer alae and the dumpyish body shape of *cut-1(RNAi)* dauer larvae. CUT-5 is instead necessary for the formation of the alae of both L1 and of dauer larvae, while CUT-4 contributes to the assembly of the adult cuticle but is not necessary for alae formation. Reduction of *cut-6* function by RNA interference also results in dumpyish dauer larvae (Muriel et al., 2003) but we show that the alae, albeit somewhat flattened, are in fact present indicating that the specific role of CUT-6 in the organization of the lateral cuticle is different from that of CUT-1 and CUT-5.

Analysis of the cuticle in EM transverse sections indicates that a reduction in the width of the lateral cuticle underlies both the development of the alae and the radial shrinking of the body in L1 and dauer larvae. Based on these observations we propose a model for larval alae formation.

Materials and methods

Strains

C. elegans was cultured as described previously (Brenner, 1974). The wild-type Bristol strain N2 and the following mutant strains were used: CB1370 *daf-2(e1370)* III; CB1372 *daf-7(e1372)* III; CB1393 *daf-8(e1393)* I; NA098 *daf-7(e1372)* III; *him-8(e1489) IV*; *cut-1(tm1126)* II. *cut-1(tm1126)* was isolated after chemical mutagenesis and PCR screening by the Mitani Consortium (Department of Physiology, Tokyo Women's Medical University School of Medicine, Japan) and kindly given to us. The *cut-1* locus of *tm1126* contains a complete deletion of exon 2. The deletion is 611 bp long and begins in the first and ends in the second intron. Because exon 1 and exon 3 are read in different frames the putative protein that the locus can code for contains only the first 39 residues of CUT-1 fused to 117 new amino acids and thus the allele is most likely a null.

RNA interference

cut-1, *cut-3*, *cut-4*, *cut-5* and *cut-6* RNA interference was performed by microinjection of dsRNA (Fire et al., 1998) into young adult hermaphrodites. Phenotypes were scored in the F1 progeny. Double-stranded RNA was generated from segments of these genes presenting reduced or no sequence similarity with the others (sequences of the primers available on request). The stage-specific effects and the specific phenotypes obtained by interference with the different genes (see Results) confirm that cross interference between genes was largely avoided. To control the formation of dauer larvae, RNA interference was performed in strains carrying *daf-7(e1372)*, a very tight temperature sensitive, reversible, dauer constitutive mutation. If grown at 25°, 100% of the larvae carrying *daf-7(e1372)* will develop into dauer larvae, independently of the amount of food and of crowding. Control experiments with the wild-type strain N2 and with another dauer constitutive mutant, *daf-2(e1370)*, gave similar results.

Temporal expression of cut-1-like genes

Time of expression of *cut-1*-like genes was determined by RT-PCR using total RNA from *daf-7(e1372)* synchronized animals. RNA was prepared from three life cycle stages. For late embryos, eggs were collected by hypochlorite treatment of gravid hermaphrodites, incubated at 25° for 7 h and then collected and lysed to prepare RNA. For L2d predauers, L1s were synchronized by allowing eggs to hatch in the absence of food. After food addition and incubation for 16 h at 25° the larvae were collected and lysed to prepare RNA. Synchronized young adults were obtained from *daf-7(e1372)* dauer larvae 24 h after lowering their growth temperature to 15°. Total RNA was prepared using TRI REAGENT (Sigma) and following the manufacturer's

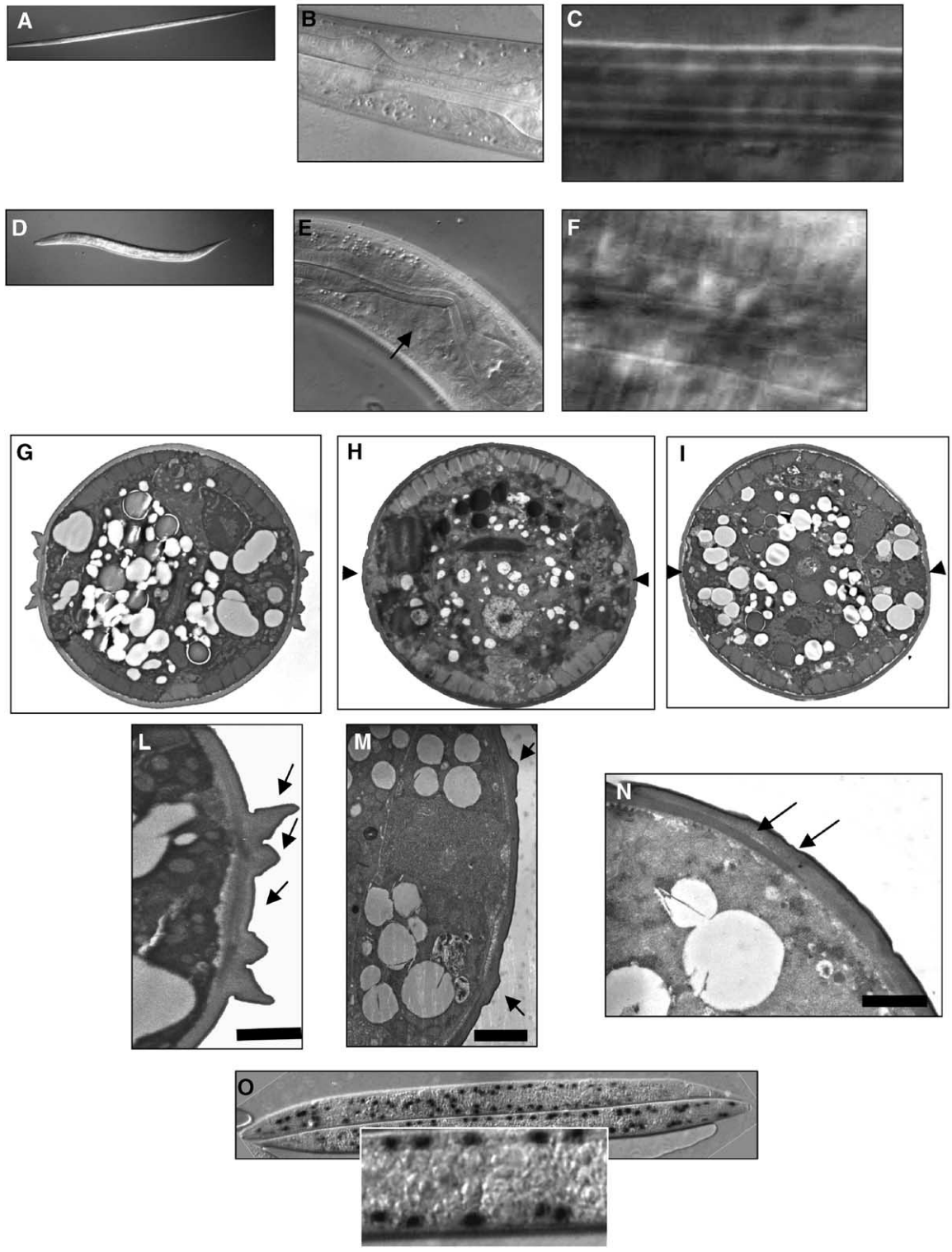


Fig. 1. Phenotype of *cut-1* mutants and *cut-1* expression. All animals are at the dauer stage and carry the *daf-7(e1372)* mutation. (A–C) Animals, wild type for *cut-1*, showing normal morphology (A); normal pharynx (B); and wild-type ala (C). (D–F) *cut-1(tm1126)* dauer larvae are dumpyish (D), the pharynx is bent at the hysthmus (E) and no alae are detectable at high magnification (F). (G–I) Transmission electron micrographs of transverse sections of dauer larvae: (G) wild type, (H) *cut-1(tm1126)* and (I) *cut-1(RNAi)*. In panels H and I dauer larvae completely lack alae; the arrowheads point to where the alae would normally be. (L–N) Enlargements of the alae region: (L) wild type, (M and N) *cut-1(RNAi)*. In panel L the completely extended ridges of the wild-type ala are visible. In panel M, only the incompletely extended two most external ridges of the ala (arrows) can be recognized. In panel N, the striated layer (arrows), typical of the dauer larva cuticle, is present in this *cut-1(RNAi)* larva that lacks alae. (O) The 5′ regulatory sequences of *cut-1* (see Materials and methods) drive expression of beta-galactosidase in the seam cells of the body and of the head and tail, during dauer larva formation. No expression was detectable in other stages. Scale bar is 3 μm.

procedure. Synthesis of the three cDNAs was performed with 1 μg of total RNA using SuperScript III reverse transcriptase (Life Technology). 5% of the cDNAs obtained was used as templates for amplification with the four gene-specific primer sets and with a set of actin primers. Primers were chosen in such a way that the amplified fragments would include one or more splicing junctions to avoid confusion with PCR products amplified from contaminating genomic DNA.

Transgenes

Germline transformation was performed by microinjection into the syncytial gonad of adult nematodes (Mello and Fire, 1995). For *cut-1* expression various fragments upstream of and including the ATG of *cut-1* were cloned in vector pPD21.28 (a gift from Andrew Fire, Carnegie Institute of Washington, USA) in frame with the *Escherichia coli lacZ* gene which carries a nuclear localization signal (NLS). Despite the different length of the fragments used for the constructs (400–7000 bp upstream of the ATG), the expression pattern observed, and reported in Fig. 1O (1622 bp fragment), was invariant with respect to the cells and the timing of expression; some differences could instead be detected with regard to the intensity of the staining (not shown). For *cut-5* expression 1100 bp upstream of and including the ATG of *cut-5* were fused in frame by amplification with the *gfp* gene from plasmid pPD95.75 from A. Fire. Constructs or amplification products were injected in *daf-7(e1372)* or *daf-8(e1393)* hermaphrodites at 100 μg/ml in a 1/1 ratio with plasmid pRF4, which carries the *rol-6(su1006)* gene, as a coinjection marker. The sequences of the primers for all the amplifications are available upon request.

For the rescue experiments of Fig. 4, the 1622-bp fragment of *cut-1* regulatory sequences, used to drive *lacZ*, was fused, by amplification, with the *cut-3* genomic coding sequence. The amplification product was injected in *daf-7(e1372)* hermaphrodites together with the *elt-2*::GFP marker, which is expressed in intestinal cells (a gift from Jim McGhee, University of Calgary). Several transforming lines were obtained. As a comparison we also obtained lines overexpressing CUT-1 by injecting a full-length genomic copy of *cut-1* together with the *elt-2*::*GFP* marker. *cut-1*::CUT-3 transformed dauer larvae and those that overexpress CUT-1 are apparently normal with alae that appear well formed under high resolution optical microscopy. Electron microscopy examination, however, showed that the ridges of the alae are often somewhat flattened and irregular (not shown).

Microscopy

Animals were mounted on 5% agar pads containing 5 or 10 mM sodium azide and observed with a Zeiss Axioskop using DIC (Differential Interference Contrast) optics or epifluorescence. Images were collected using a Zeiss Axiocam digital camera.

Anti-cuticlin antiserum

daf-7(e1372) dauer larvae were cleaned on a sucrose step gradient and cuticlin was prepared by digestion in 2% SDS and 5% beta-mercaptoethanol for several days as previously described (Sebastiano et al., 1991). The residue obtained was washed and used to immunize rabbits (Biopat, Piedimonte Matese, Caserta, Italy). For the experiment in Fig. 7 populations of normal, *cut-1(RNAi)* and *cut-6(RNAi)* dauer larvae from a *daf-7(e1372)* strain were digested o/n with SDS and beta-mercaptoethanol. The resulting cuticles were washed and incubated with the anti-cuticlin serum at a 1:50 dilution. After washing and blocking with 1% BSA the cuticles were incubated with goat anti-rabbit immunoglobulin conjugated with Texas Red (Molecular Probes) diluted 1:1000.

Transmission electron microscopy

For EM of RNA interfered animals, dumpyish larvae (L1 or dauers) were selected under the stereoscope and processed further. Larvae were collected, washed, resuspended in fixative (2.5% glutaraldehyde, 2% formaldehyde

Table 1
Phenotypes observed in L1 and dauer larvae

	L1		dauer	
	dumpyish shape	alae[§]	dumpyish shape	alae[§]
daf-7(e1372)	0%, *n* = 182	99%, *n* = 84	1.5%, *n* = 199	99%, *n* = 125
daf-7(e1372);him-8(e1489); cut-1(tm1126)	0%, *n* = 130	99%, *n* = 124	98%, *n* = 152	5%, *n* = 117
daf-7(e1372);cut-1(RNAi)	0%, *n* = 101	99%, *n* = 88	97%, *n* = 134	4%, *n* = 99
daf-7(e1372);cut-3 (RNAi)	95%, *n* = 156	6%, *n* = 91	1%, *n* = 150	99%, *n* = 107
daf-7(e1372);cut-5(RNAi)	97%*, *n* = 103	3%**, *n* = 98	97%*, *n* = 137	4%**, *n* = 111
daf-7(e1372);cut-6(RNAi)	1%, *n* = 148	98%, *n* = 80	98%, *n* = 144	n.d.

Animals were mounted 10 per slide.
* Larvae presenting at least one clear enlargement along the length of the body were counted as dumpyish.
** Only larvae presenting the alae along the entire length of the body were counted.
n.d. not detected; the presence or absence of alae in *cut-6(RNAi)* dauer larvae was difficult to detect unambigously by optical microscopy and is dicussed in the text.
§ The presence of the alae was scored only in animals laying on their side.

in cacodylate 0.1 M, pH 7.4) and transferred into a glass depression slide. They were cut in half with a razor blade and transferred and aligned on a thin layer of 1% agarose, 0.1 M cacodylate on a slide. After adding a small drop of agarose, 1 mm^3 blocks containing the worms were cut and transferred into fresh fixative overnight. After washing in cacodylate buffer, the nematodes were fixed in osmium 1% in cacodylate, washed and dehydrated in graded alcohol and propylene oxide, infiltrated and embedded in polybed (Polyscience, Warrington). Ultrathin sections were collected on nickel slots coated with formvar-carbon stabilized film. Observations were made on a transmission electron microscope Philips EM 208. Measurements on EM micrographs (Table 3) were made on highly enlarged prints using a measuring wheel. To minimize factors unduly influencing measurements we chose sections to be measured carefully checking that they were at the same level along the antero-posterior axis of the worms and that they were as close to an orthogonal cut as possible.

Scanning electron microscopy

Wild-type and RNAi nematodes fixed in 2.5% glutaraldehyde and 2% paraformaldehyde were dehydrated in glycerol 1.5% for several days. They were gold-sputtered after transfer onto metallic support and complete removal of glycerol. Observations were made at 0.5 kV on a Stereoscan Cambridge SEM.

Results

CUT-1 plays an essential function in the formation of the alae of dauer larvae

The restricted expression of CUT-1 only during dauer formation and its specific localization within the cuticle, in a ribbon under the alae (Sebastiano et al., 1991; Timinouni and Bazzicalupo, 1997), suggest that it might serve an essential role in alae formation. To test this hypothesis we analyzed a putative *cut-1* null mutant, *tm1126* (see Materials and methods), and also carried out RNA interference to determine the phenotypic consequences of abolishing or strongly reducing the function of *cut-1*.

Animals lacking CUT-1 function appeared normal at all stages except for dauer larvae which instead completely lacked the alae and were dumpyish with an increased diameter and a reduced body length (Figs. 1D–F). The pharynx was bent to fit the reduced length of the animal (Fig. 1E). *cut-1* mutant dauers were also analyzed by electron microscopy. In transverse sections the alae were completely absent and the external layer of the cuticle was smooth and no ridges were visible (Figs. 1H and I). In a few cases the two most external ridges of each ala could still be recognized, although these were not completely extended as they are in normal dauer larva alae (Figs. 1L and M).

The penetrance of all the phenotypes described was very high (Table 1) for both *cut-1(tm1126)* and *cut-1(RNAi)* worms which are in fact indistinguishable. Thus, complete loss of CUT-1 function does not result in a phenotype more severe than the one caused by RNA interference. *cut-1* mutant larvae completely lack alae, as do normal larvae at the L2, L3 and L4 stages. Several experimental observations indicate that these *daf-7(e1372);cut-1(RNAi)* or *daf-7(e1372);cut-1(tm1126)* larvae are indeed dauers: (i) their development is interrupted but can resume when they are shifted again to the permissive temperature; (ii) their pharynx does not pump; and (iii) the radially striated internal layer, which is typical of the dauer cuticle, is present (Fig. 1N).

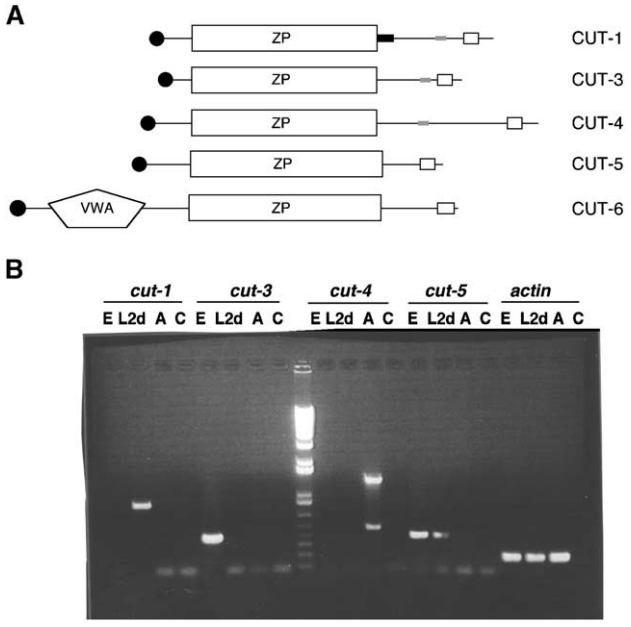


Fig. 2. Domain organization of CUT-1-like proteins and expression of *cut-1-like* genes. (A) The domain topology of CUT proteins. CUT-1-like proteins have a similar structure: all begin with a cleavable signal peptide (black circle) followed by a ZP domain (~250 aa), except for CUT-6 in which a VWFA domain is located N-terminal to the ZP. C-terminal to the ZP domain only in CUT-1 there is a stretch of 30–35 aa containing short alanine and proline rich repeats (black box) not found in either CUT-3, CUT-4, CUT-5 or CUT-6 but present in another component of the cuticlin residue, CUT-2 (Lassandro et al., 1994), which however lacks the ZP domain. (Grey box) Low complexity region. In the C-terminal portion the homology between the proteins is much lower than in the ZP domain region. However, all the proteins terminate with a hydrophobic stretch of 15–20 residues that could function as a transmembrane segment (white box) followed, in turn, by a short cytoplasmic tail. (B) Stage-specific transcription of *cut-1-like* genes. RT-PCR: total RNA from the three stages was prepared as described in Materials and methods, reverse transcribed and the cDNAs amplified separately with primers specific for each of the genes indicated at the top. E = late embryos; L2d = L2 predauer larva stage; A = adults; C = negative control (no cDNA). *cut-1* mRNA is present only during dauer larva formation (L2d), *cut-3* mRNA only during late embryogenesis and *cut-5* mRNA in both these stages, but not in adults. High levels of *cut-4* mRNA were detected only in adults. Amplification with actin primers was used as a control of the amount of the cDNAs. *cut-1* and *cut-5* mRNA could not be amplified when RNA was prepared from L2 larvae not undergoing dauer formation (not shown).

RT-PCR experiments on RNA prepared from late embryos, predauer larvae and adults confirmed previous results (Sebastiano et al., 1991) and showed that *cut-1* is expressed only during dauer formation but not in late embryogenesis and in adults (Fig. 2B). The seam cells have been shown to be responsible for the secretion of the lateral cuticle which forms the alae (Koh and Rothman, 2001; Singh and Sulston, 1978). A reasonable prediction would be that *cut-1* is expressed by these cells. To address this question we used reporter constructs, where sequences upstream of the initiator methionine (ATG) of *cut-1* were fused to *E. coli* beta-galactosidase with a nuclear localization signal (NLS) (see Materials and methods). In transgenic animals beta-galactosidase staining was observed only in larvae that were completing or had completed the transformation into dauer larvae. In these animals the reporter was expressed only by the seam cells, including most of those of the head and of the tail (Fig. 1O). The dauer larva-specific phenotype of *cut-1* mutants, the expression by the seam cells which are responsible for alae secretion and the localization of CUT-1 in a ribbon underneath the alae only in the dauer larva stage (Sebastiano et al., 1991) indicate that CUT-1 is a component of the cuticle that is secreted during dauer larva formation and that it plays an essential and specific role in the development of the alae of dauer larvae.

cut-3 is necessary for the formation of the alae of L1 larvae

The *C. elegans* genome codes for about 40 proteins containing a ZP domain and several of them have a domain organization similar to that of CUT-1 (M.R. Sapio and P. Bazzicalupo, unpublished and http://www.wormbase.org/). The dauer larva stage-specific effect of RNA interference with *cut-1*, and with *cut-6* (Muriel et al., 2003), prompted us to ask whether other proteins with a domain organization similar to that of CUT-1 (CUT-1-like proteins) might contribute to the formation of the alae of L1 larvae or of adults. We began our analysis with three genes, *cut-3* (*f22b5.3*), *cut-4* (*e04d5.3*) and *cut-5* (*r07e3.3*), that most closely resemble *cut-1*. The domain organization of CUT-1, CUT-6 and of the three proteins coded for by these genes is shown in Fig. 2A. RNA interference with *cut-3* resulted in defects of the newly hatched L1 larvae which are reminiscent of those described for *cut-1(RNAi)* dauer larvae: (i) no visible alae either with high resolution optical microscopy or with the electron microscope; (ii) dumpyish body with reduced length; and (iii) pharynx, and in some cases also intestine, bent to fit the reduced body length (Table 1 and Figs. 3C–E). In transverse sections at the EM, the circumference of the larvae appeared as a smooth circle without the typical mushroom shaped extroflection of the L1 alae. None of the other stages were affected, including dauer larvae and adults, which appeared normal, as did their alae. Interestingly, RT-PCR experiments showed that *cut-3* mRNA is present only in RNA from late embryos when the L1 cuticle is synthesized (Fig. 2B).

CUT-3 can replace CUT-1 to rescue alae and body shape of dauer larvae

The effect of RNA interference and the stage-specific expression and phenotypes suggest that CUT-1 and CUT-3 might exert the same function in two different stages, CUT-1 in dauer larvae and CUT-3 in L1s. CUT-3 is the closest paralog of CUT-1 (Timinouni and Bazzicalupo, 1997) and we asked if it could replace CUT-1 to restore the normal body shape and the formation of the alae of *cut-1* interfered dauer larvae. The approach used consisted in expressing the CUT-3 coding sequence under the *cut-1* promoter in a *cut-*

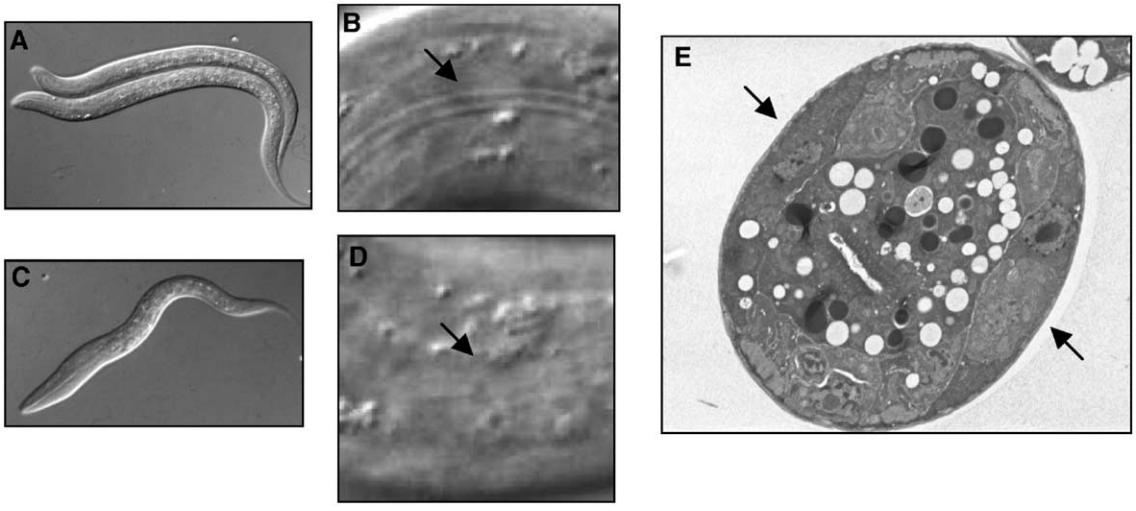


Fig. 3. Phenotypes associated with *cut-3* RNA interference. All animals are at the L1 larval stage. *daf-7(e1372)* L1 larvae showing normal morphology (A), and a normal, visible ala along the lateral line of the body (B, arrow). *daf-7(e1372);cut-3(RNAi)* L1s are dumpyish (C) and the ala is missing (D); the arrow indicates the position where it would normally be found. (E) Transmission electron micrograph of a *daf-7(e1372);cut-3(RNAi)* L1 larva: the alae are absent and the external layer of the cuticle appears completely smooth; the arrows indicate the positions where the alae would normally be found.

1(RNAi) background. To drive the CUT-3 coding sequence we used a 1622-bp fragment upstream of the *cut-1* ATG, which we had shown could drive a reporter in the seam cells at the appropriate time (see above and Fig. 1O). The *cut-1*::CUT-3 construct was injected, together with *elt-2*::GFP as a selection marker, in *daf-7(e1372)* animals. Several independents, non-integrated lines harboring the transgene, were obtained. We performed *cut-1* RNA interference on *cut-1*::CUT-3;*elt-2*::GFP transformed animals from three such lines.

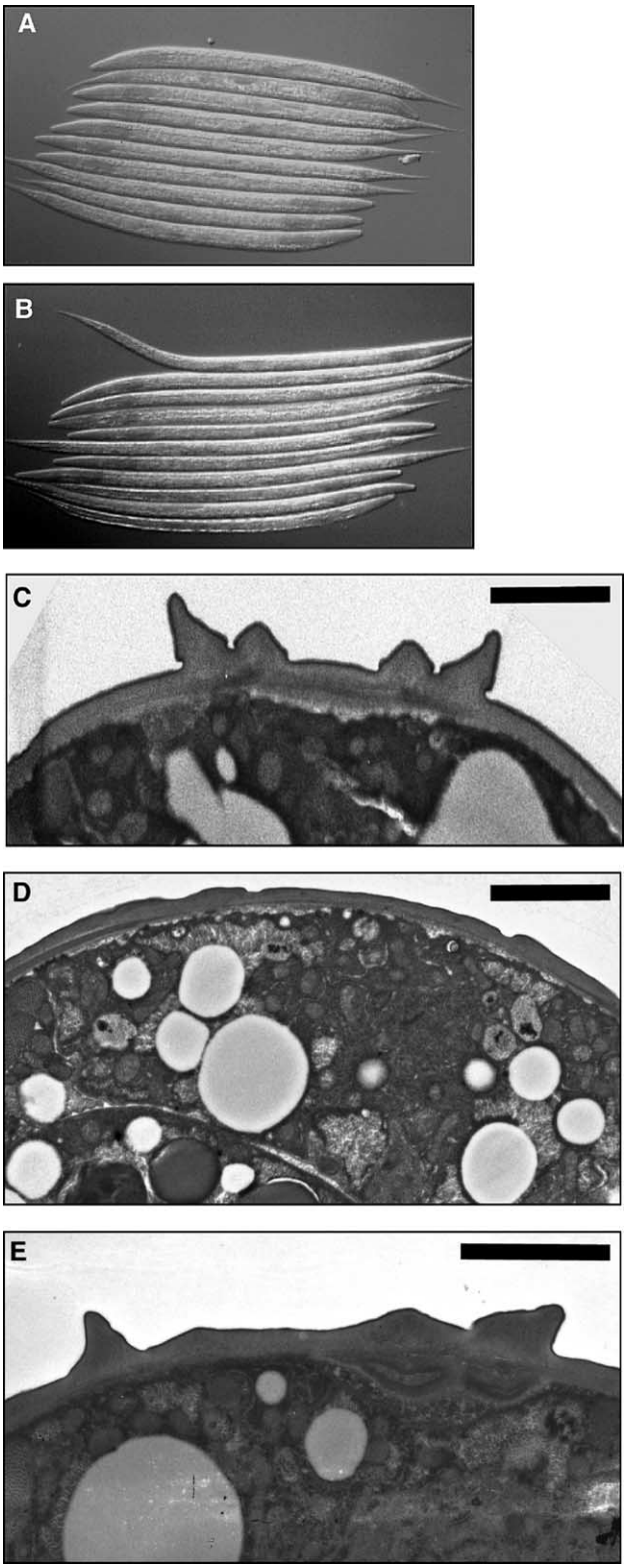


Table 2
cut-1(RNAi) phenotypes rescued by CUT-3

	wt pharynx	alae present
daf-7(e1372)	99%, *n* = 113	99%, *n* = 75
daf-7(e1372);cut-1(RNAi)	4%, *n* = 108	5%, *n* = 105
daf-7(e1372);cut-1::CUT-3;cut-1(RNAi)	92%, *n* = 117	34%*, *n* = 111

Animals were mounted 5 per slide and phenotypes were scored blindly.
* Only completely rescued alae in which ridges were clearly recognizable were counted.

All the dsRNA-injected hermaphrodites segregated both transformant and non-transformant animals (presence or absence of the *elt-2::GFP* marker) and, due to the RNA interference, all of them had reduced *cut-1* function. Our results show that *cut-3* is able to rescue the dumpyish body shape (Figs. 4A and B), the bent pharynx and the absence of alae of the *cut-1(RNAi)* dauer larvae (Table 2). Electron microscopic examination of these rescued dauer larvae showed that the alae were indeed formed although often the central ridges were shallower than normal (Fig. 4E). Interestingly the alae of these animals appeared as partially formed dauer-type alae rather than partially formed L1-type alae. This suggests that the different shapes of the alae in L1 and dauer larvae probably depend on other factors beside the difference between CUT-1 and CUT-3.

cut-5 is necessary for larval alae formation

RNA interference with *cut-5* affected the alae of both L1 and dauer larvae. In the majority of worms the alae were interrupted several times along the length of the animal (Figs. 5A and C). In EM transverse sections the alae were often present only on one side of the larvae (Figs. 5B and D). The diameter of the body correlated with the presence or absence of alae, with enlargements of the segments of the worm (dumpyish shape) where alae were absent (Table 1). This correlation is immediately evident in the scanning EM picture of a *cut-5(RNAi)* dauer larva in Fig. 5E. Although *cut-5* is expressed in all the seam cells (see below, Figs. 5F and G), in different worms different sections of the alae were affected by interference. At present we do not know the reason for the apparent mosaicism of *cut-5* RNA interference but some form of threshold effect of the reduction of *cut-5* expression in each seam cell may be

Fig. 4. CUT-3 rescues the phenotypes of *cut-1(RNAi)* dauer larvae. All animals are at the dauer larva stage. The dumpyish body shape of *daf-7(e1372);cut-1(RNAi)* dauer larvae (A) is rescued in *daf-7(e1372);cut-1(RNAi)* dauer larvae carrying the *cut-1*::CUT-3*;elt-2*::*GFP* transgene (B). (C–E) Transmission electron micrographs of transverse sections of dauer larvae. (C) *daf-7(e1372)*; (D) *daf-7(e1372);cut-1(RNAi);* (E) *daf-7(e1372);cut-1(RNAi)* dauer larvae carrying the *cut-1*::CUT-3*;elt-2*::*GFP* transgene. Note the dauer-type shape of the CUT-3 rescued ala. Scale bars are 3 μm.

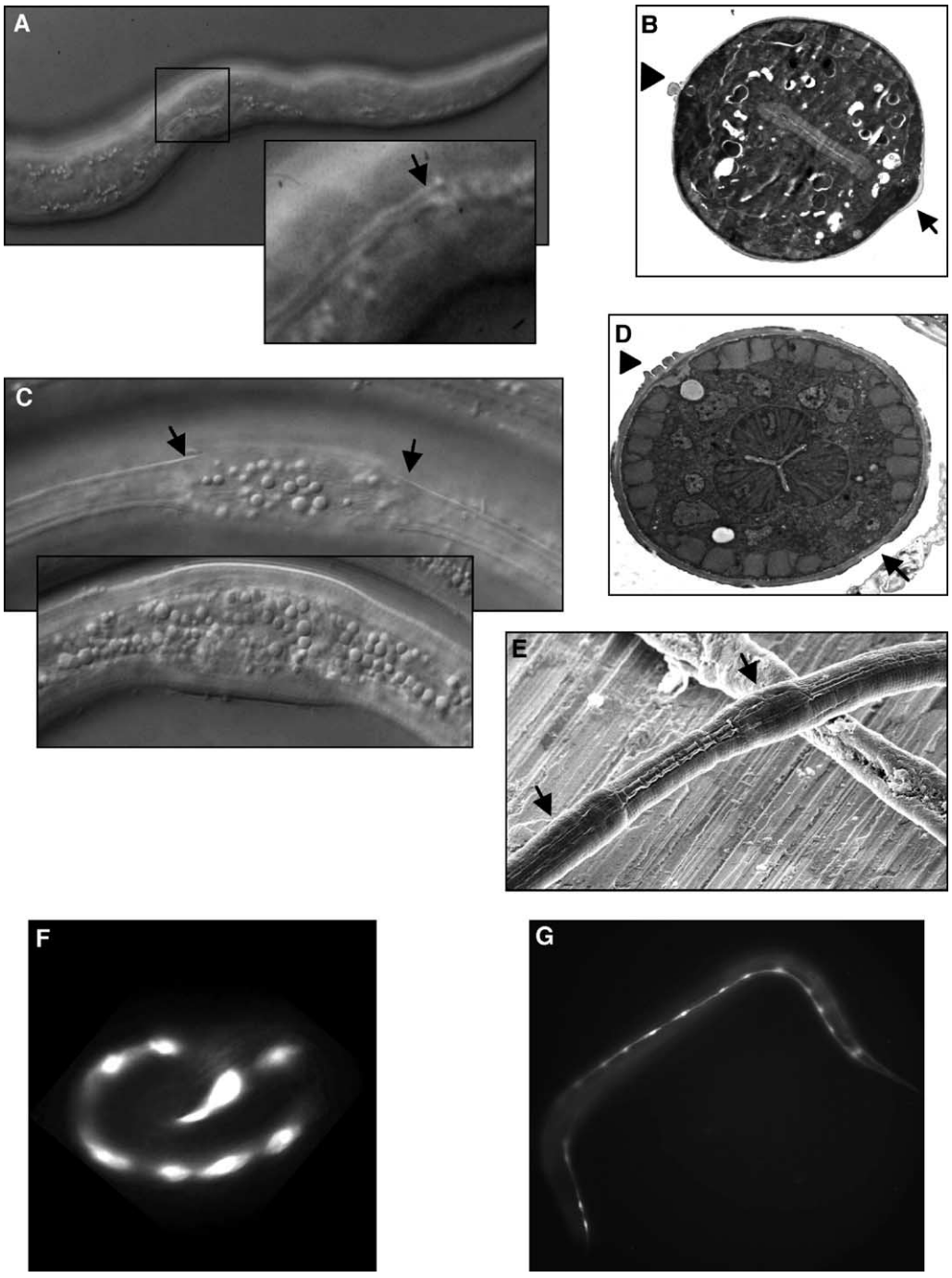


Fig. 5. *cut-5(RNAi)* phenotypes and *cut-5* expression. (A and B) *daf-7(e1372);cut-5(RNAi)* L1 larvae: in panel A, DIC images showing that the diameter of the body is irregular and that the ala (insert at higher magnification) is interrupted (arrow). In panel B, transmission electron micrograph showing that the ala is present only on one side of the larva, arrowhead; the arrow on the opposite side points to where the missing ala would normally be. (C–E) *daf-7(e1372);cut-5(RNAi)* dauer larvae: in panel C, DIC images, the ala is interrupted and the diameter of the body is larger where the ala is absent, arrows; the box shows the same animal on a different focal plane. In panel D, transmission electron micrograph, the ala is present only on one side of the larva (arrowhead); the arrow points to where the missing ala would normally be. (E) Scanning EM, the alae are interrupted several times along the length of the animal (arrows) and the diameter of the body correlates with the presence of the alae. (F and G) Epifluorescence images of GFP expressing animals. The 5′ regulatory sequences of *cut-5* (see Materials and methods) drive expression of GFP in the seam cells of a threefold stage, late embryo (F), and of a dauer larva (G). No expression was detectable in other stages.

responsible for it. Similarly to *cut-1* and *cut-3*, also in the case of *cut-5* RNA interference the alae of adults were normal (not shown). In agreement with these RNA interference results, RT-PCR showed that *cut-5* is expressed both in late embryogenesis and during dauer larva formation but not in adults (Fig. 2B). Expression of *cut-5* was also studied with the use of reporters. We prepared a fusion construct containing a 1112-bp fragment upstream of and

including the ATG of *cut-5*, fused in frame with the GFP coding gene. Animals transformed with this construct showed GFP expression only in seam cells in embryos at the threefold stage and in dauer larvae (Figs. 5F and G).

Interference with *cut-4* had no apparent effect on either L1 or dauer larvae or on their alae. Instead, the cuticle of adults appeared affected. The alae were not missing but were slightly abnormal. They appeared shallower than in wild-type adults and often had two or four ridges, instead of the normal three ridges of adult alae (Figs. 6A, C and D). We also observed a slight increase in the permeability of the adult cuticle to fixatives (formaldehyde and glutaraldehyde) and to stains (DAPI), not shown. Electron microscopic examination confirmed that the cuticle of *cut-4(RNAi)* adults was defective: The external and internal layers of the cuticle under the alae were detached and the alae, although present, often showed a rudimentary fourth ridge in addition to the three normal ones (Figs. 6B and E). In agreement with these results, RT-PCR showed that high levels of *cut-4* mRNA were present only in RNA from adults (Fig. 2B).

The temporal expression of *cut-1*, *cut-3*, *cut-4* and *cut-5* (Fig. 2B) indicates that transcription regulation is one of the factors underlying the stage specificity of the function of the proteins they encode.

CUT-1 and CUT-6 play different roles in the organization of the lateral cuticle

Recently RNA interference with *cut-6*, a gene that codes for a different ZP containing protein, was shown to result in dumpyish dauer larvae apparently similar to the *cut-1* mutant dauers described here. Although expression of *cut-6* was reported also in the late stages of embryogenesis no effect of RNA interference could be seen in L1 (Muriel et al., 2003). We confirmed that the effect of RNA interference with *cut-6* is dauer larva specific and that the resulting animals are dumpyish (Fig. 7A and Table 1). However, under high-resolution bright-field optical microscopy it was not clear if the alae are present or not since in *cut-6(RNAi)* dauer larvae, differently from what happens in wild-type or in *cut-1* mutant dauer larvae, the lateral cuticle is partially covered with the circumferential annuli (Fig. 7B). To better visualize the surface structures of the cuticle we then used an anticuticlin antiserum and immunofluorescence and compared *cut-6(RNAi)* dauer larvae to untreat and *cut-1(RNAi)* mature dauer larvae. In the absence/reduction of CUT-6, the annuli were incompletely interrupted and appeared to run over partially formed alae (Fig. 7D). In contrast in *cut-1(RNAi)* dauer larvae, interruption of the circumferential annuli at the border with the lateral cuticle occurred normally while compacting of the ridges to form the mature alae failed, resulting in a wider lateral cuticle (Fig. 7E). Finally we turned to EM analysis. We chose, among a population of *cut-6(RNAi)* dauer larvae, the most severely affected animals based on the dumpyish phenotype and analyzed them by EM. Observation of transverse sections confirmed that, differently from what happens in *cut-1* mutants (Figs. 1H and I), in *cut-6(RNAi)* dauer larvae the alae are present although their shape is not completely normal (Figs. 7F and G). We believe that the difference

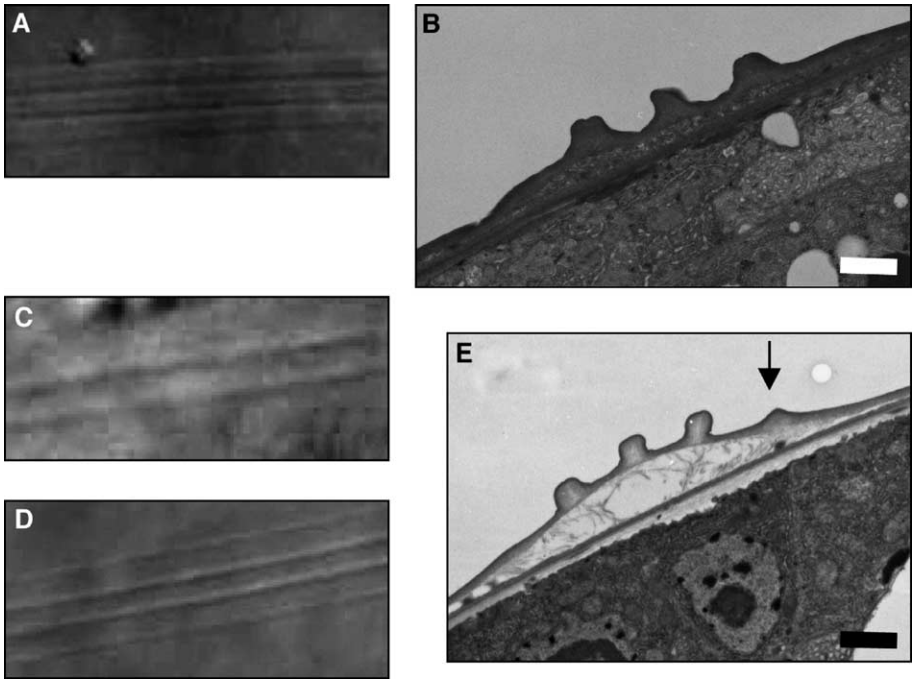


Fig. 6. Effect of *cut-4* RNA interference on adult worms. DIC images of adult alae show, in panel A, the three ridges of a wild-type adult ala, while in *cut-4(RNAi)* adults the alae are present but occasionally show two (C) or four (D) ridges. Transmission electron micrographs of transverse sections of adult worms: in panel B, a wild-type adult ala; in panel E, a *cut-4(RNAi)* adult shows the external layer of the cuticle detached from the internal layer which is also detached from the underlying epithelial cell. The ala is present but shows a rudimentary fourth ridge, arrow. Scale bars are 3 μm.

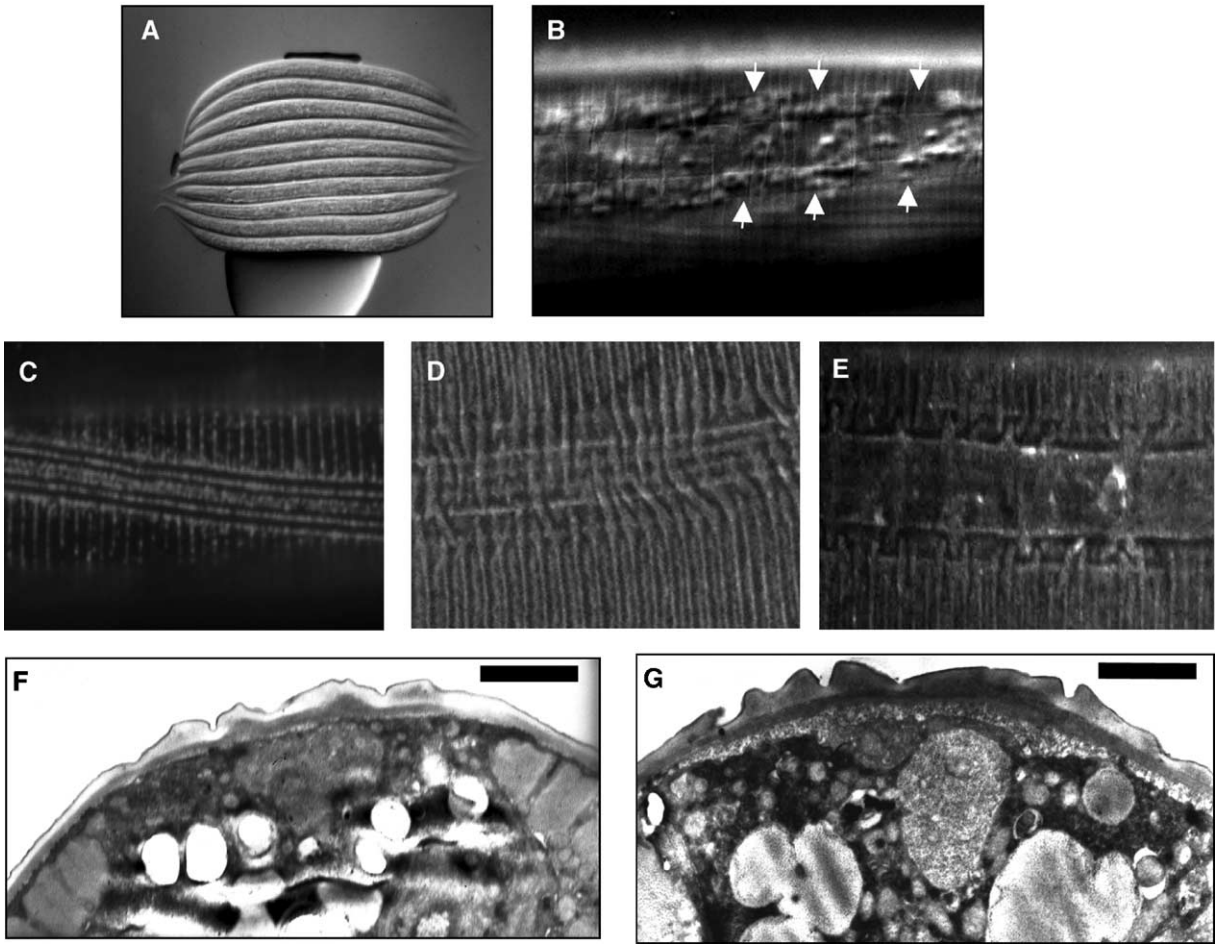


Fig. 7. Effect of *cut-6* RNA interference on dauer larvae. All animals are at the dauer larva stage and carry the *daf-7(e1372)* mutation. (A) *cut-6(RNAi)* dauer larvae are dumpyish. (B) At higher magnification, the strip of lateral cuticle of a *cut-6(RNAi)* dauer larva can be seen (between arrows). The alae are not easily recognizable. (C–E) Epifluorescence images of purified cuticles reacted with an anti-cuticlin antiserum. (C) A wild-type dauer larva shows, along the lateral lines, a well-formed ala with parallel, longitudinal ridges close to each other and interrupting the annuli. (D) A *cut-6(RNAi)* dauer larva shows that the annuli are not interrupted and many run over the lateral cuticle. Underneath the annuli, incompletely formed longitudinal ridges are visible. (E) A *cut-1 (RNAi)* dauer larva shows that the annuli are interrupted but the lateral cuticle is wider and without ridges and the ala has not formed. (F and G) Two examples of EM transverse sections of *cut-6(RNAi)* dauer larvae. The alae are present although their shape is not completely normal. Scale bars are 3 μm.

between our observations and those of Muriel et al. (2003) on *cut-6(RNAi)* dauer larvae is due to the different resolution of the microscopy techniques used. Thus, different alterations of the lateral cuticle underlie the similar phenotypes (enlarged body diameter and apparent lack of the alae) resulting from reduction of *cut-1* and *cut-6* function. A different role for CUT-1 and CUT-6 in alae formation is in agreement also with the different localization of the two proteins: CUT-1 is in a ribbon underneath the alae (Sebastiano et al., 1991) while CUT-6 is localized along two thin lines corresponding to the border between the seam cells and the hypodermal cells (Muriel et al., 2003). In addition, while *cut-1* is expressed by the seam cells and not by the hypodermal cells, the opposite is true for *cut-6* (Muriel et al., 2003). Finally the domain structure itself of CUT-6 is different from that of CUT-1, CUT-3, CUT-4 and CUT-5 since it contains, at its *N*-terminus, a von Willebrand factor type-A domain (VWFA-domain) which is absent in the other proteins (Fig. 2A).

cut-1 mutant dauer larvae have a larger body diameter

A common feature of the phenotypes of worms with reduced *cut-1*, *cut-3* or *cut-5* function is the correlation between the absence of the alae and the diameter of L1 and of dauer larvae. Larvae or sections of larvae in which the alae are not present have a larger diameter. To understand the cuticle alterations that must underlie the enlarged diameter of the RNA interfered larvae we took advantage of the resolution power of EM and focused on *cut-1(RNAi)* dauer larvae to determine which portions of the cuticle were indeed enlarged. The cuticle of *C. elegans* is made of a dorsal and a ventral portion which are synthesized by the dorsal and ventral hypodermis, respectively. In dauer larvae, dorsal and ventral cuticle are joined together by two lateral strips (lateral cuticle) which contain the alae and are secreted by the left and right rows of epithelial cells, the seam cells (see scheme in Fig. 8). In TEM transverse sections of dauer larvae it is possible to distinguish between these different

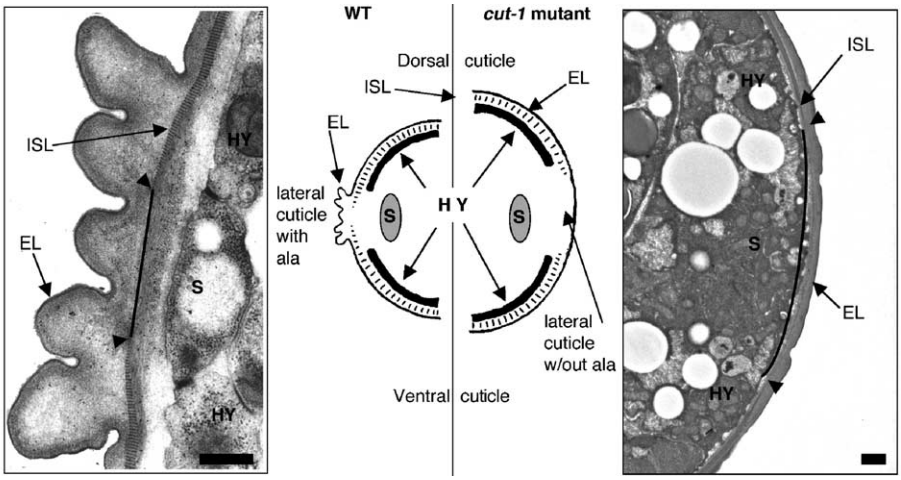


Fig. 8. *cut-1* mutant dauer larvae have a larger body diameter. Cross sections of a normal dauer larva (left side, WT) and of a *cut-1(RNAi)* dauer larva (right side, *cut-1* mutant) are represented schematically together with relative EM pictures of the region of the ala. The dorsal and ventral cuticles are secreted by the body hypodermis (HY). The lateral cuticle is secreted by the seam cells (S). External layer (EL) and internal striated layer (ISL) of the cuticle are indicated. The width of the lateral cuticle was measured (values reported in Table 3) as the distance (black lines) between the dorsal and ventral margins (black arrowheads) of the internal striated layer. Compared to wild type in *cut-1* mutant dauer larvae, which lack alae, the width of the lateral cuticle increases. Instead, the dorsal and ventral sections of the cuticle do not change. The thick black bars in the EM pictures are 1 μm.

portions of the cuticle because the dorsal and ventral halves have a characteristic, radially striated, internal layer which is interrupted in the lateral portions of the cuticle secreted by the seam cells (Fig. 8). We measured the length of the different sections of the circumference of the cuticle in normal and in *cut-1(RNAi)* dauer larvae (Fig. 8 and Table 3). The dorsal and ventral portions of the circumference (length of dorsal and ventral striated layers) of the cuticle were the same in normal and in *cut-1* interfered dauer larvae (Table 3). Instead, the lateral cuticle, measured as the distance between the tapered ends of the dorsal and ventral striated layers (Fig. 8) was more than three times larger in interfered dauer larvae than in normal ones (Table 3). Thus, in *cut-1(RNAi)* dauer larvae the larger diameter of the body is due to a wider lateral cuticle.

We also measured the length of the external layer of the lateral cuticle in these EM transverse sections. In interfered dauer larvae, where the alae are missing, the external layer of the lateral cuticle was smooth, followed the internal layer and was easily measured. In normal dauer larvae the external layer of the lateral cuticle was instead measured following the contour of the five pointed alae. Despite the different shape, the length of the external layer of the lateral portion of the cuticle did not change significantly between normal and interfered dauer larvae (Table 3). In summary, interference with *cut-1* causes a dorso-ventral extension (>3×) of the internal layer of the lateral cuticle resulting in an increased diameter of the worm. The external layer is instead not increased. This layer is not folded over the internal one to make the five ridges of the alae as in wild-type dauer larvae but is distended and parallel to the internal layer resulting in the absence of the alae.

Table 3
Length of different sections of the cuticle

	Wild type	*cut-1(RNAi)*
dorsal cuticle	27 ± 4 μm (n = 24)	28 ± 5 μm (n = 22)
ventral cuticle	29 ± 4 μm (n = 24)	28 ± 5 μm (n = 22)
lateral cuticle (each side)		
internal layer	3 ± 2 μm (n = 24)	10 ± 2 μm (n = 22)*
external layer	11 ± 5 μm (n = 15)	10.5 ± 3 μm (n = 22)

Ventral, dorsal and lateral cuticle as well as external and internal layer are described in Fig. 8.
The means ± the standard deviations are indicated.
* Indicates a statistically significant difference (t test) with the wild-type strain ($P < 0.001$).

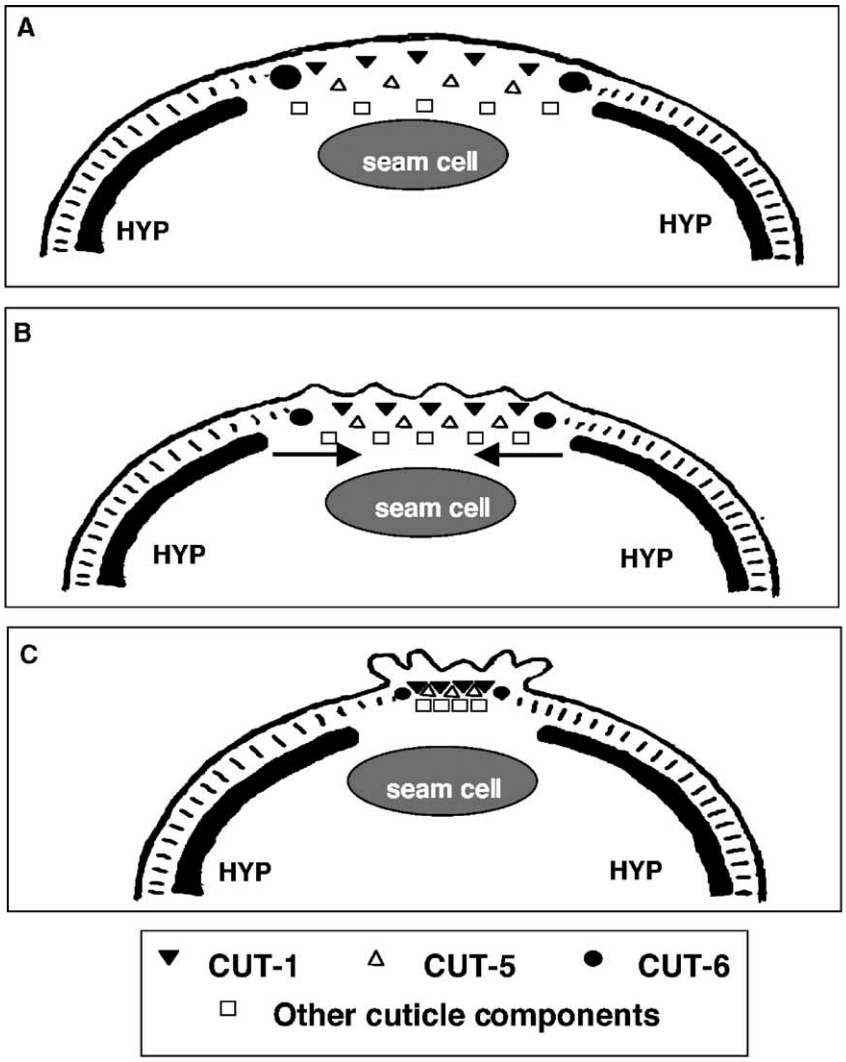
The cuticle measurements reported above suggest a model (Fig. 9) in which the reduction of the body diameter and the formation of the alae of dauer larvae are strictly correlated and appear to be dependent on the expression of CUT-1. When the dauer cuticle is first secreted, the internal and external layers of the lateral cuticle are, as in *cut-1* mutant worms, parallel to each other and extended. The body diameter is still large and the alae are not present. In a second step, one which fails in the absence of *cut-1* expression, the internal layers of the lateral sections of the cuticle shrink circumferentially resulting in a reduction of the body diameter. Differential interference contrast (DIC) images of the lateral cuticle during alae formation in dauer larvae were consistent with this hypothesis. Alae formation starts with the appearance of thin longitudinal ridges running orthogonally to the circumferential annuli which are interrupted at the borders of the lateral cuticle with the dorsal and ventral ones (Fig. 9D). In the beginning the ridges are widely spaced becoming progressively more pronounced and closer to each other until they attain the much more compact shape of the mature ala (Figs. 9E and F).

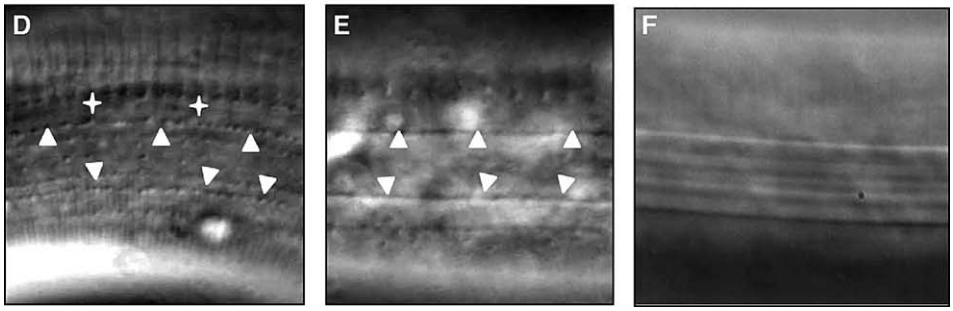
Discussion

CUT-1, CUT-3 and CUT-5 play stage-specific, essential roles in the development of larval alae

The organization and the shape of extra cellular matrices depend on the proteins and on the other macromolecules of which they are composed but also on events occurring in the cells which secrete the matrix. An example is given, in *C. elegans*, by the formation of the annuli, the major visible differentiation of the cuticle, beside the alae. Patterning of the annuli is achieved by the hypodermal cells through specific modifications of their cytoskeleton and apical membranes and through the coordinated regulation of transcription and secretion of the cuticle components (Costa et al., 1997; McMahon et al., 2003). Our study is focused on the alae, longitudinal, symmetric ridges of the cuticle, which run along the sides of L1 and dauer larvae and adults and have stage-specific shape.

In this paper we have not addressed the cellular activities underlying the development of the alae but rather the role in this process of some protein components of the lateral





cuticle after they have been secreted. We show that CUT-1 is required for the formation of the alae of dauer larvae, CUT-3 for those of L1 larvae while CUT-5 is required for both. CUT-6, which is necessary for the dauer larvae body shape (Muriel et al., 2003), is instead not strictly required for alae formation. Finally CUT-4 is required in adults for proper assembly of the cuticle but not for the formation of the alae. These CUT-1-like proteins, all have a signal peptide for secretion, a Zona Pellucida domain and, at the C-terminus, a transmembrane region followed by a short cytoplasmic tail (Figs. 2A, M. R. Sapio and P. Bazzicalupo unpublished and http://www.wormbase.org/). The domain organization of CUT-6 is more complex as it also contains a VWFA-domain at its N-terminus immediately following the signal peptide. The ZP domain (Bork and Sander, 1992) has been conserved in evolution and is present in many extra-cellular proteins with widely different functions ranging from receptorial to structural and mechanical (Chung et al., 2001; Jazwinska et al., 2003; Wassarman et al., 2001; Wilkin et al., 2000). The ZP domain itself appears to be necessary for polymerization and assembly of the proteins containing it (Jovine et al., 2002). CUT-1 and CUT-6 have been previously shown to be cuticle components. Because of the similarity in structure, expression and function, it is reasonable to think that also the newly identified proteins are components of the cuticle.

The temporal pattern of expression of the corresponding genes suggests that stage-specific transcription underlie, at least in part, their stage-specific functions. In addition we have shown that, at the appropriate stages, *cut-1* and *cut-5* are expressed by the seam cells which are the epithelial cells that secrete the alae. The results of RNA interference and the expression during late embryogenesis suggest that *cut-3* exerts in L1 larvae the same function that *cut-1* exerts in dauer larvae. This conclusion is strengthened by the observation that CUT-3, driven by the *cut-1* regulatory sequences, could rescue the phenotype of dauer larvae depleted of CUT-1 by RNA interference (Fig. 4). Despite the very similar structure, the function of CUT-5 must instead be different and not redundant with that of CUT-3 or CUT-1 since it is required for alae formation even when CUT-3 in L1s or CUT-1 in dauer larvae are present.

Development of larval alae and reduction of body diameter are strictly connected

By analyzing animals with reduced or no expression of the CUT-1-like proteins we could show that alae formation and the reduction of the body diameter, which normally occur in L1 and dauer larvae, are two consequences of a single process, the circumferential shrinking of the internal layer of the lateral cuticle, such that the distance between the ends of the dorsal and ventral internal striated layers is drastically reduced. This shrinking cannot occur in the absence of CUT-1 and CUT-5 in dauer larvae and of CUT-3 and CUT-5 in L1 larvae. The mechanism by which the larval alae form is thus the folding/wrinkling of the external layer of the lateral cuticle over the internal one (Fig. 9).

Laser ablation and genetic studies on the alae of adults have shown that the alae form in separate sections along the antero-posterior axis, with each section corresponding to a single seam cell. Killing or genetically changing the fate of one seam cell causes the interruption of the alae in the region only above that cell (Koh and Rothman, 2001; Singh and Sulston, 1978). Our studies show that the same is true for the alae of dauer larvae and of L1 larvae and that the reduction of the body diameter also occurs in segments corresponding to single seam cells (e.g., *cut-5(RNAi)* larvae; Fig. 5), further supporting the hypothesis that alae development and reduction of body diameter are strictly correlated.

There are several reasons to believe that the mechanism of alae formation that we hypothesize for L1 and dauer larvae does not apply to the formation of adult alae. First, the shape itself of the adult alae is different, with the three ridges protruding straight out from the main cuticle and forming a right angle with it. Second, there is no evident shrinking of the adult body diameter after the formation of the adult cuticle, which also lacks an internal striated layer. Finally we have so far failed to identify a *cut-1*-like gene that has, on the alae of adults, the effect that *cut-1*, *cut-3* and *cut-5* have on larval alae. *cut-4*, which is specifically

Fig. 9. A model for larval alae formation. The drawings (A, B and C) depict successive steps in the formation of the dauer alae and focus on what happens in the lateral cuticle. In the formation of the alae of L1 larvae the function of CUT-1 would be exerted by CUT-3. In the model, after the most external layers of the cuticle have been secreted, CUT-1-like proteins are deposited in the internal layer of the lateral cuticle (A). CUT-1 and CUT-5 are secreted by the seam cells underneath where the alae will eventually form, while CUT-6 is deposited by the body hypodermis along the dorsal and ventral margins of the lateral cuticle (Muriel et al., 2003). Initially, these ZP domain containing proteins assemble with themselves and with other interacting proteins (collagens, other cuticlins or other unknown proteins) in ordered, non-covalent, supra-molecular aggregates. In a second step (B), one or more biochemical modifications (e.g., a cross-linking reaction) begin to compact the non-covalently assembled network of components of the lateral cuticle and generate the force, arrows, to pull together the internal striated layers of the dorsal and ventral cuticle, reducing the width of the lateral cuticle. At the end of the process (C), the reduced width of the lateral cuticle is responsible for the reduced diameter of the larva and for the formation of the alae which results from a sort of folding or wrinkling of the external layer of the lateral cuticle over the internal one. CUT-1, CUT-3 and possibly CUT-5 participate in this mechanism by acting as substrates for the cross-linking activity. In the absence of CUT-1 or CUT-3 or CUT-5 (e.g., RNAi worms) the cross-linking does not occur because a necessary substrate is missing. The dorsal and ventral halves of the cuticle are not pulled together, the diameter of the worm remains larger, no wrinkling occurs and no ala forms. The role and the localization of CUT-6 appear to be different from those of CUT-1, CUT-3 and CUT-5 (see Fig. 7 and Discussion section). (D–F) DIC images of three successive moments during alae maturation in a wild-type dauer larva. (D) The annuli are already interrupted (stars); the longitudinal ridges (arrowheads) are just beginning to appear as a series of aligned thicker dots, barely rising above the surface of the cuticle. (E) The dots have became a continuos line (arrowheads) rising more clearly above the surrounding cuticle. (F) The formation of the ala is complete.

expressed in adults and contributes to the assembly of the adult cuticle, is not required for the formation of the alae.

A model for the roles of CUT-1-like proteins in alae formation

The results of Muriel et al. (2003) with CUT-6 and our analysis with the anticuticlin antibodies and at the EM of *cut-6* interfered dauer larvae (Fig. 7) indicate that CUT-1 and CUT-6 have different functions in the organization of the lateral cuticle of dauer larvae. The main body hypodermis secretes CUT-6 in two strips which flank the lateral cuticle along its borders with the dorsal and ventral cuticles. The presence of CUT-6 at this location appears to mark the point where the circumferential annuli are interrupted such that in the absence of CUT-6, but not in the absence of either CUT-1 or CUT-5, the annuli appear to extend over the sides of the larva (Fig. 7D). CUT-1 is instead secreted by the seam cells in the internal layer of the cuticle and forms a ribbon, possibly between the two strips of CUT-6. In the model depicted in Fig. 9, after these and other components, including CUT-5, are assembled in the cuticle, some biochemical mechanism(s) compacts the lateral internal layer. As a consequence the striated layers of the dorsal and ventral cuticle are pulled toward each other resulting in a dorso-ventral reduction of the lateral cuticle (dumpyish body shape) and in the folding of the external layer over the compacted, internal one (alae formation). This step does not occur without CUT-1 or CUT-5. It is possible that the presence of CUT-6 is necessary for coupling the internal layers of the dorsal and of the ventral cuticle to the internal layer of the lateral one. Without this coupling the compacting of the CUT-1 network fails to pull together the dorsal and ventral halves of the cuticle and the body diameter is not reduced, although the alae do form.

The proposal of Muriel et al. (2003), that CUT-6 might interact/bind to collagens of the dorsal and ventral cuticle with its VWFA-domain and to CUT-1-like proteins of the lateral cuticle with its ZP domain, is compatible with this scenario.

The model in Fig. 9 predicts that one or more biochemical reactions compact the internal layer of the lateral cuticle once its components have been deposited and have assembled in ordered supramolecular aggregates. Covalently cross-linking the protein components of the cuticle (CUT-1, CUT-3, CUT-5 and possibly other proteins) may be responsible for this step. CUT-1 is indeed found in a highly cross-linked, non-reducible form in cuticlin, the insoluble residue of the cuticle (Sebastiano et al., 1991). Non-reducible, covalent, inter-protein links, compatible with the properties of cuticlin, could result from the formation of glutamyl-lysine and/or of dityrosine bridges. While both are potential candidates, dityrosine has been shown to be abundant in cuticlin (Fetterer and Rhoads, 1990; Fetterer et al., 1993) and was also shown to have a role in the cross-linking, in vitro, of CUT-2, a *C. elegans* cuticlin component with a domain organization different from the one of the proteins discussed in this paper (Lassandro et al., 1994; Parise and Bazzicalupo, 1997). Thus, enzymes capable of promoting the formation of intermolecular dityrosine bridges, such as peroxidases or phenol oxidases, may be responsible for this step of the process. Other possible candidates are enzymes, such as transglutaminases, which could catalyze the formation of intermolecular glutamyl-lysine bridges. In this model CUT-1-like proteins would function as necessary substrates for the cross-linking activities of these enzymes. These hypotheses can clearly be tested experimentally.

The model we describe is aimed at explaining the general mechanics for the formation of the alae but does not account for the difference in the shape between the alae of L1 and dauer larvae. In the experiment in which CUT-3 substitutes for CUT-1 during dauer larva formation (Fig. 4), the shape of the rescued alae is not that typical of L1s but that of dauer larvae. This suggests that, although these proteins are necessary for the formation of the alae, the difference in shape between the alae of dauer and of L1 larvae is independent of whether CUT-1 or CUT-3 is present. From this point of view, these CUT-1-like proteins act as permissive substrates and not as instructive extracellular proteins. In analogy to what has been found for the patterning of the annuli (Costa et al., 1997; McMahon et al., 2003), we think that the mechanisms underlying the patterning of the five pointed alae of dauer larvae and of the three-foil, mushroom-shaped alae of L1 larvae are likely to be based on cellular events, still to be explored, which occur in the seam cells.

Acknowledgments

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