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Pleiotropic roles of calumenin (*calu-1*), a calcium-binding ER luminal protein, in *Caenorhabditis elegans*

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ABSTRACT

Calumenin is a Ca^{2+} binding protein localizing at the lumen of the endoplasmic reticulum (ER). Although it has been implicated in various diseases, the in vivo functions of calumenin are largely unknown. Here, we report that calumenin has pleiotropic roles in muscle and cuticle function in *Caenorhabditis elegans*. Mutant analysis revealed that the *calu-1* is required for regulating fertility, locomotion and body size. In addition, *calu-1* is important for two behaviors, defecation and pharyngeal pumping, consistent with its ability to bind Ca^{2+}. The genetic analysis further suggested the possibility that *calu-1* regulates the pharyngeal pumping together with the inositol 1,4,5-triphosphate (IP_3) receptor encoded by *itr-1*. Taken together, our data suggest that calumenin is important for calcium signaling pathways in *C. elegans*.

1. Introduction

The CREC (Cab45, Reticulocalbin, ERC-45, Calumenin) family consists of several proteins that contain multiple Ca^{2+} binding EF hand motifs and localize at the endoplasmic reticulum (ER) secretory pathway. They have been implicated in a variety of diseases such as cancer, neuromuscular and cardiovascular diseases [1]. Therefore, CREC family members have been recently suggested as promising disease biomarkers, even though many aspects of pathological functions of the proteins remain unknown [1]. Calumenin, a member of the CREC family, contains EF hand motifs and a C-terminal ER retention signal [2–4]. It is ubiquitously expressed in all tissues but highly expressed in heart, lung, muscle and placenta [3–5]. Similar to other CREC proteins, calumenin has been implicated in various diseases. It has been shown that calumenin is differentially expressed in cardiomyopathy [6], cells induced apoptosis [7], squamous tumor cells [8] and astrocytes expressing human immunodeficiency virus (HIV) Tat protein [9], indicating that calumenin is related to these various malignant diseases. However, the mechanisms for calumenin regulation in vivo are still unknown. Thus, it is important to know the specific in vivo functions of calumenin by using appropriate model organism.

Calumenin is well conserved from worm to human [10]. Previously, Honore and Vorum [10] have identified a *Caenorhabditis elegans* homolog of calumenin, termed CCB-39 (Ca^{2+} binding protein of 39 kDa). In this report, we renamed it as *calu-1* in order to avoid possible confusion. *C. elegans* has a single homolog of calumenin annotated as M03F4.7, which shows 45% identity and 62% similarity to human calumenin in amino acid sequence. High throughput analysis of *C. elegans* gene expression revealed that the *calu-1* is broadly expressed in pharynx, intestine, reproductive system, developing vulva, hypodermis and body wall muscle [11].

In this study, we have characterized a calumenin deletion mutant, *calu-1*(*tm1783*) and further examined its expression profile in individual developmental stages. We show here that *calu-1* is required for normal cuticle formation and/or maintenance. We also

Abbreviations: ER, endoplasmic reticulum; IPTG, isopropyl β-D-1-thiogalactopyranoside; SERCA, sarcoplasmic reticulum Ca^{2+}-transporting ATPase; HIV, human immunodeficiency virus; pBoc, posterior body contraction; SEM, scanning electron microscopy; RNAi, RNA interference; IP_3, inositol 1,4,5-triphosphate; PDI, protein disulfide isomerase; PPIase, peptidyl prolyl cis–trans isomerase

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show that *calu-1* regulates Ca^{2+} mediated behaviors such as pharyngeal pumping and defecation.

2. Materials and methods

2.1. Cultivation of C. elegans

The following strains were obtained from the *Caenorhabditis* Genetics Center (CGC): Bristol N2, PS2366 *itr-1*(*sy328*) *unc-24*(*e138*) IV and JT73 *itr-1*(*sa73*) IV. The *eat-3*(*tm1077*) II and *calu-1*(*tm1783*) X mutants were obtained from the National BioResource Project, Japan, and out-crossed more than six times. The strain BC12836 *dpy-5*(*e907*)/*dpy-5*(*e907*);*sIs11268*[*rCes M03F4.7*::*GFP* + *pCeh361*] was obtained from the Baille lab [11].

2.2. Phenotype analysis

One-day adults were used for all phenotypic analyses. The brood sizes were measured by counting progeny (F_1) after placing and daily transferring individual worms (P_0) on seeded plates at 20 °C. Egg laying rate was determined by counting the number of eggs laid by a single animal for 1 h. Thrashing assay was conducted for 20 s twice in an individual well containing 150 μl of M9 buffer, and averaged [12]. Body length was measured as described previously [13]. Briefly, animals were immobilized on a 2% agarose pad with 10 mM levamisole and then length was measured under a Normaski microscope (Carl Zeiss Axio Imager. A1). Pharyngeal pumping was measured by counting terminal bulb contractions for 20 s twice at 20 °C (Modified from [14]). Defecation cycles were observed at room temperature (25 °C) and the interval time was measured from one posterior body contraction (pBoc) to the next pBoc as described [15]. The percentage of enteric muscle contractions per cycle was scored as described previously [15].

2.3. RNA interference (RNAi)

The *calu-1* RNAi clone was purchased from Geneservice. For the bacteria-mediated RNAi, L4 or young adult worms (P_0) were transferred onto plates seeded with *Escherichia coli* HT115 (DE3) strain harboring L4440 control plasmids or *calu-1* RNAi plasmids. Phenotypes were analyzed by observing F_2 to F_4 progenies.

2.4. Expression and purification of GST, GST::CALU-1 and GST::CNB-1

The full length of *calu-1* cDNA was cloned into pGEX4T-1 (Amersham) to construct a GST-fused CALU-1 plasmid. The recombinant protein was over-expressed in *E. coli* BL21 (DE3) at 37 °C in the presence of 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) (2 h induction after OD_{600} of the culture reached 0.5). The fusion protein was then purified using a glutathione-Sepharose column and eluted with the elution buffer. The expression and purification of GST and GST::CNB-1 (Calcineurin B subunit) was done as described previously [16].

2.5. Ca^{2+} binding assay and quantification of signal

The Ca^{2+} binding assay was performed as described previously [17]. Briefly, GST, GST::CALU-1 and GST::CNB-1 (Calcineurin B subunit) was expressed, purified from bacteria, separated by SDS–PAGE, transferred to nitrocellulose membrane and probed with $^{45}Ca^{2+}$. Densitometry for quantification was carried out by Image J. The absolute intensity of each sample band was divided by the absolute intensity of standard to determine a relative intensity for each sample band.

2.6. Preparation of polyclonal antibody and Western blot

The affinity purified GST::CALU-1 fusion protein was used to immunize rabbits to generate anti-CALU-1 antibody. Worm protein extracts were prepared by lysis and sonicating of wild type and *calu-1*(*tm1783*) mutant worms. Protein samples were then used for Western blotting as described previously [16,18].

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from N2 and *calu-1*(*tm1783*) was isolated by using TRI reagent (MRC). Oligo(dT) primer was utilized to synthesize the first strand of cDNA by reverse transcription. The *calu-1* cDNA was amplified by using SL1 primer (5′-GGTTTAATTACCCAAGTTTGAG-3′) and *calu-1* specific primer (5′-TTAGAGCTCGGCTGGGTCGT-3′). The resultant cDNA was cloned into pGEM-T Easy vector (Promega) and sequenced.

3. Results

3.1. C. elegans calumenin (CeCALU-1) is a Ca^{2+} binding protein

A *C. elegans* homolog of calumenin (M03F4.7) has been identified previously [10], and we have renamed this gene as *calu-1* according to *C. elegans* nomenclature. CALU-1 shares 45% identity and 62% similarity in amino acid sequences with its human counterpart (Fig. 1A). CALU-1 shows five putative EF hands motifs for Ca^{2+} binding motifs, whereas the human homolog contains six of them. It also has the C-terminal ER retention signal sequence, PAEL, as described previously [10], and the critical C-terminal two residues (EL) are conserved as in other homologs [2].

In order to test the ability to bind Ca^{2+}, we performed a Ca^{2+} overlay assay. As shown in Fig. 1B, GST::CALU-1 binds with Ca^{2+}, even though the binding appeared to be weaker than that of CNB-1 (Calcineurin B subunit). We further quantified the binding signal of CALU-1. Although a little bit more amount of GST::CALU-1 proteins were used, GST::CALU-1 showed much weaker signal (0.37 ± 0.09) compared to GST::CNB-1 (2.30 ± 0.01) as shown in Fig. 1C. The ability of CALU-1 to bind Ca^{2+} was further confirmed by Stains-all (data not shown). Thus, our results confirm that indeed CALU-1 is a Ca^{2+} binding protein which shows relatively low affinity.

3.2. The calu-1 gene is expressed in pharynx, intestine, muscle and hypodermis during development

A transcriptional GFP fusion was used to observe the expression pattern of *calu-1* during development. As shown in Fig. 2, *calu-1* is mainly expressed in pharynx, intestine, body wall muscle, and hypodermis. Interestingly, we found that the *calu-1* is predominantly expressed in muscle cell nuclei of L1 larvae (Fig. 2A; indicated with arrows). The same expression of *calu-1* was observed in males (Fig. 2F).

3.3. The calu-1(tm1783) is a loss-of-function mutant

In order to understand the in vivo functions of *calu-1*, we characterized a deletion mutant, *calu-1*(*tm1783*). The deletion removes an approximately 500 bp region containing the 5′-UTR, the first two exons, and the splice donor site of the second intron (Fig. 3A). A homozygous *calu-1*(*tm1783*) mutant was confirmed by nested and internal PCR using the specific primer sets (Fig. 3A) as shown in Fig. 3B and C.

Next, we tested whether *calu-1* mRNA is transcribed in *calu-1*(*tm1783*) mutant or not. RT-PCR results revealed that *calu-*

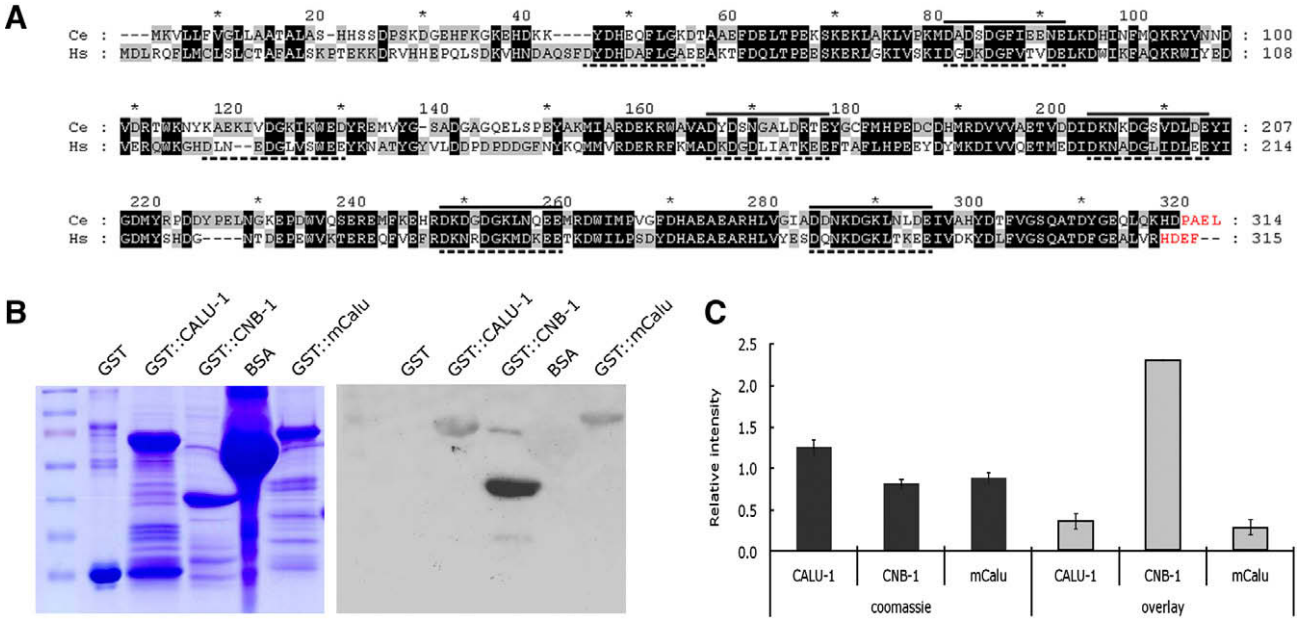

Fig. 1. *Caenorhabditis elegans* calumenin is a Ca^{2+} binding protein. (A) Pairwise, global alignment of human calumenin (Hs) and *C. elegans* calumenin (Ce). Solid lines and dotted lines indicate the Ca^{2+} binding EF hand motifs of the *C. elegans* and human calumenin, respectively. C-terminal tetra-peptides for endoplasmic reticulum (ER) retention are shown in red. (B) Ca^{2+} overlay assay. Left panel shows the coomassie blue staining of the polyacrylamide gel. Right panel shows the autoradiography of the membrane incubated with $^{45}Ca^{2+}$. BSA, bovine serum albumin, was used for negative control. mCalu, mouse calumenin. (C) Quantification of binding signal of coomassie gel and autoradiography.

1(*tm1783*) mutant produced a shorter transcript (~0.7 kb) compared to wild type (~0.9 kb) (Fig. 3D). We then asked whether the mutant transcripts are successfully translated into protein. As shown in Fig. 3E, CALU-1 antibodies detected a smaller product (~28 kDa) from protein extracts of *calu-1*(*tm1783*) mutants. This indicates that *calu-1*(*tm1783*) mutant can make a truncated CALU-1 protein lacking N-terminal 71 amino acids. This opens the possibility that truncated CALU-1 could act as a neomorph. This possibility is further discussed in Section 4.

3.4. The calu-1(tm1783) mutant exhibits pleiotropic defects

The *calu-1*(*tm1783*) mutants are slightly dumpy and show uncoordinated movement compared to wild type (Fig. 4A and B), indicating possible roles in muscle function and cuticle formation and/or maintenance. In order to observe the cuticle defects in detail, we used scanning electron microscopy (SEM). As shown in Fig. 4C–F, wild type animals showed well organized alumni and furrow of cuticle (Fig. 4C) whereas *calu-1*(*tm1783*) mutant animals displayed disorganized cuticle structures such as aberrant alumni and furrow (Fig. 4D). Furthermore, *calu-1* (*tm1783*) mutants exhibited deformed alae (Fig. 4F) compared to wild type (Fig. 4E). Thus, these results suggest that *calu-1* is important for normal cuticle structure formation. Consistently, we found that *calu-1*(*tm1783*) mutant animals showed smaller body length, which could be caused by cuticle malformation [19–21] (Table 1).

We also monitored the locomotion defects of *calu-1*(*tm1783*) mutants, since they displayed uncoordinated movement (Table 1). The body bends of *calu-1*(*tm1783*) mutants were reduced by 50% compared to that of wild type. This quantitatively confirmed the defective locomotion of *calu-1*(*tm1783*) mutants, suggesting that the *calu-1* is required for normal muscle function. In addition, *calu-1*(*tm1783*) mutants showed dramatically reduced brood size and egg laying rate (Table 1). This indicates that *calu-1* is also important for fertility. Taken together, our results suggest that *calu-1* participates in multiple aspects of *C. elegans* physiology.

3.5. RNAi knock-down of calu-1 phenocopies the calu-1(tm1783) mutant

calu-1(*tm1783*) mutant animals showed a severe defect in cuticle so that a rescue experiment by DNA microinjection was not successful. Therefore, we tested by knocking down *calu-1* whether the pleiotropic defects of *calu-1*(*tm1783*) mutant was indeed due to the mutation. As shown in Table 1, wild type animals fed with *calu-1* RNAi showed reproducible phenotypes of reduced brood size, egg laying rate, reduced body bends and smaller body length compared to wild type animals fed with control vector (L4440). This phenotypic similarity between the knock-down and knock-out of *calu-1* indicates that specific loss of *calu-1* is indeed responsible for the observed defects.

3.6. calu-1 regulates pharyngeal pumping and defecation cycle

CALU-1 is a Ca^{2+} binding protein in ER, and we demonstrated that *C. elegans* CALU-1 indeed binds to Ca^{2+} in vitro. Therefore, we examined two representative behaviors which are regulated by intracellular Ca^{2+} signaling in *C. elegans*: pharyngeal pumping [22] and defecation [22,23]. Interestingly, *calu-1*(*tm1783*) mutants showed significantly decreased pharyngeal pumping and prolonged defecation cycle compared to wild type (Table 2), suggesting that the *calu-1* may function in regulating these Ca^{2+} mediated behavioral rhythms. Previously it has been reported that mutation in the *C. elegans* inositol 1,4,5-triphosphate (IP_3) receptor gene *itr-1* leads to a decreased pharyngeal pumping and a prolonged defecation cycle [22,23]. In addition, *itr-1* has been shown to be expressed in pharynx and intestine [24] similar to the patterns of *calu-1* expression. Therefore, we tested the genetic interaction between *itr-1* and *calu-1* in these particular behaviors.

In order to test the genetic epistasis between *itr-1* and *calu-1*, a double mutant *itr-1*(*sy328*)(*gf*);*calu-1*(*tm1783*) was constructed. At the same time, *itr-1*(*sa73*) loss-of-function mutants were fed with bacteria expressing *calu-1* RNAi plasmid. As shown in Table 2,

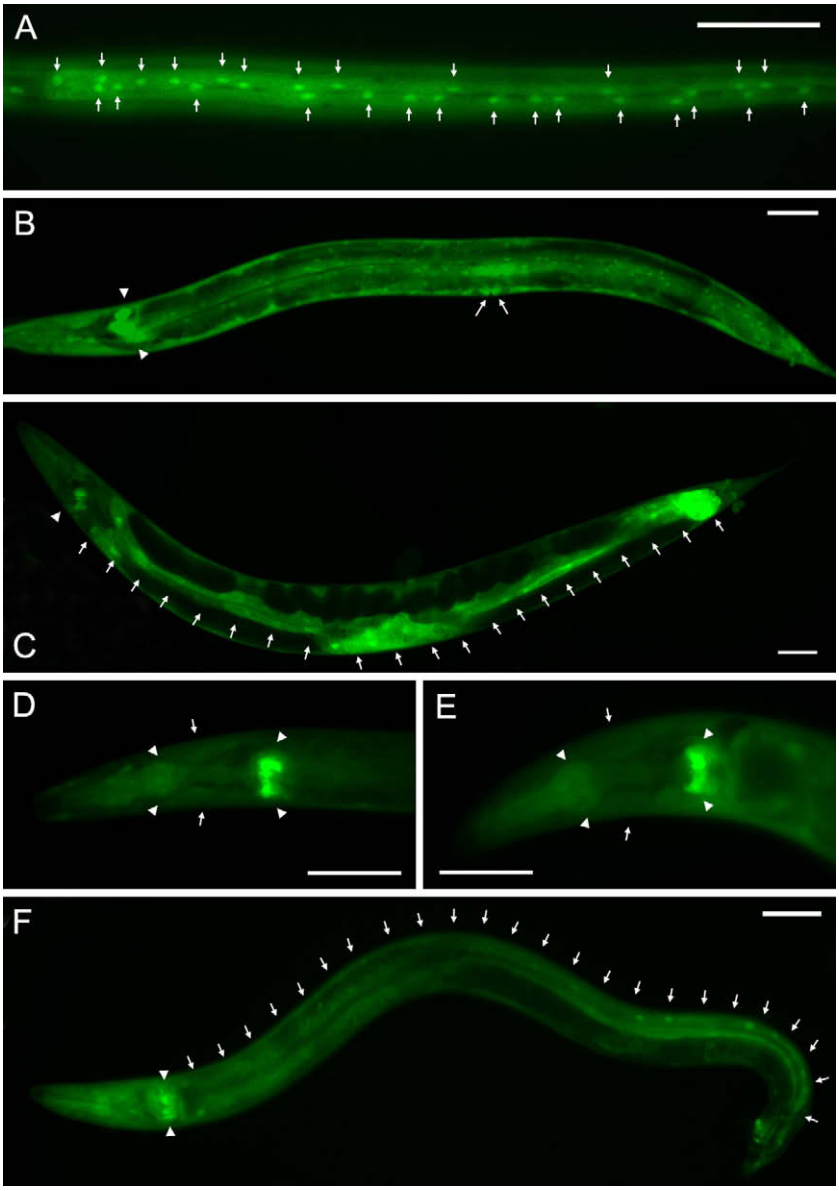

Fig. 2. *calu-1* is expressed in pharynx, intestine, muscle and hypodermis. (A) L1 larval stage. Muscle cell nuclei are indicated by arrows. (B) L4 larval stage. Expression was observed in pharynx (arrowheads), developing vulva (arrows) and hypodermis. (C) Adult stage. Intestinal expression was predominant (arrows). Arrowhead indicates pharynx. (D and E) *calu-1* expression in pharyngeal muscles (arrowheads) and hypodermis (arrows) was clearly shown in L4 larva (D) and adult worm (E). (F) Strong expression was observed in pharynx (arrowhead) and intestine (arrows) in male. Scale bars, 50 μm.

itr-1(*sy328*) gain of function mutants displayed normal pharyngeal pumping as previously reported [22]. *itr-1*(*sy328*)(*gf*);*calu-1*(*tm1783*) double mutants showed reduced pharyngeal pumping similar to *calu-1*(*tm1783*) mutant. On the other hand, *itr-1*(*sa73*)(*lf*) mutants fed with *calu-1* RNAi plasmid did show a slightly additive reduction of pharyngeal pumping when compared to *itr-1*(*sa73*)(*lf*) fed with control vector. Judging from the variation of feeding RNAi efficiency in pharyngeal muscle it would be difficult to suggest any additive or synergistic effect of these two genes. Possible interpretations of these results are discussed in Section 4.

We next observed the defecation cycle of mutants. *itr-1*(*sy328*)(*gf*);*calu-1*(*tm1783*) double mutants exhibited intermediate defecation cycles compared to each single mutant (Table 2). In addition, *itr-1*(*sa73*)(*lf*) mutant fed with *calu-1* RNAi plasmid showed a synergistically prolonged defecation cycle, even though an extreme variation was observed among individual worms (Table 2). These results indicate that *itr-1* and *calu-1* may function in more than one pathway to regulate defecation cycles. This possibility was further supported by the additive EMC (enteric muscle contraction) defects observed in double mutants (Table 2).

4. Discussion

Here we report for the first time the in vivo functions of *calu-1* gene encoding calumenin in *C. elegans*. CeCALU-1 shows high similarity to its human counterpart and indeed binds to Ca^{2+}. The Ca^{2+} binding affinity of CALU-1 appeared to be lower than that of CNB-1. In fact, calcineurin B, the regulatory subunit of calcineurin binds four molecules of Ca^{2+} with high affinity ($K_d \leqslant 10^{-6}$ M) [25] whereas CREC family members including human calumenin [4] have rather low Ca^{2+} affinity ($K_d = 10^{-4}$–10^{-3} M) [10]. Consistent with previous reports, our results showed that the binding affinity of CALU-1 is much lower than that of CNB-1, demonstrating that

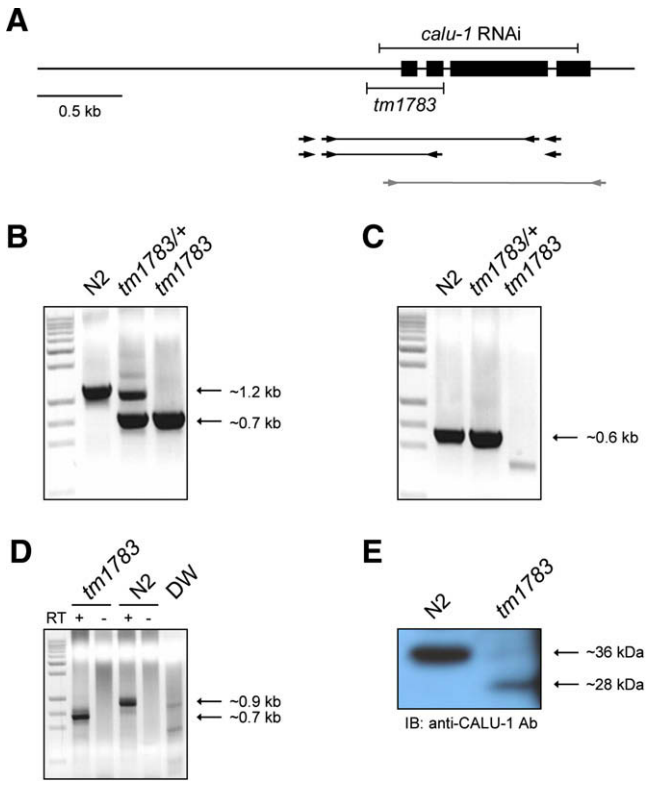

Fig. 3. *calu-1*(*tm1783*) is a loss-of-function mutant. (A) Schematic diagram of the genomic organization of *calu-1*. Black boxes indicate exons. The region of deletion in *calu-1*(*tm1783*) is indicated as a black line flanked by vertical lines. Primer sets used to identify the genotype of *calu-1*(*tm1783*) are indicated as black arrows. Primer pairs utilized for RT-PCR are indicated as gray arrows. Scale indicates 0.5 kb. Genomic DNA fragment amplified by using nested PCR primer sets (B) and internal PCR primer sets (C) were shown. Wild type N2 (+/+), heterozygous *calu-1*(*tm1783*/+) and homozygous *calu-1*(*tm1783*) showed indicated PCR products. No band was observed in homozygous *calu-1*(*tm1783*) by internal PCR. (D) RT-PCR product of wild type N2 or *calu-1*(*tm1783*) mutant. (+) and (−) indicate the addition or omission of reverse transcriptase in reaction, respectively. DW indicates the addition of distilled water instead of RT product as a template in PCR. (E) Western blot analysis of total proteins isolated from wild type N2 or *calu-1*(*tm1783*) worms using anti-CALU-1 polyclonal antibody.

this biochemical property of calumenin is well conserved among organisms. This is further supported by similar Ca^{2+} binding capacity of mouse calumenin (GST::mCalu) which we have shown in Fig. 1B (0.29 ± 0.09).

We have shown here that the *calu-1*(*tm1783*) mutant exhibits pleiotropic defects, implying multiple roles of calumenin in various signaling pathways. The defective phenotypes of mutants were phenocopied by *calu-1* RNAi treatment, indicating that specific loss of *calu-1* function is indeed responsible for such abnormalities. This also rules out the possibility that truncated CALU-1 may act as a neomorph. However, reduced brood size and smaller body length were reproduced after feeding *calu-1* RNAi only at the F_4 generation. This might be due to the incomplete knock-down of *calu-1* in corresponding tissues in the F_2 or F_3 generations.

The decreased body bends of *calu-1*(*tm1783*) mutants suggests that *calu-1* may function in muscle. Consistently, *calu-1* is expressed in muscle during development. Furthermore, recent studies showed that calumenin interacts with and inhibits (RyR) ryanodine receptor and sarcoplasmic reticulum Ca^{2+}-transporting ATPase (SERCA), which are responsible for muscle contraction and relaxation, respectively [26,27]. Therefore, we suggest that *C. elegans calu-1* may also play important roles in muscle function similar to other homologs.

More interestingly, we found that *calu-1* is expressed in muscle cell nuclei in early larval stage (L1). This is very intriguing, because there have been no reports describing the localization of mammalian CREC proteins in the cell nucleus. However, this observation is based on the transcriptional fusion of *calu-1* gene. Therefore, further biochemical characterizations of calumenin localization are needed.

Consistent with the expression in reproductive system and developing vulva, *calu-1*(*tm1783*) mutants displayed reduced brood size and abnormal egg laying process suggesting a possible role in fertility and/or ovulation. It has been also shown that mammalian calumenin is highly expressed in placenta, and affinity purification of placenta proteins uncovered calumenin binding proteins such as amyloid P component [28] and TSP-1 (thrombospondin-1) [29], relating the function of calumenin to immunologic defense system and pathological process. However, the specific function of calumenin in fertility or ovulation has not yet been reported related to these binding partners or other new partners. Thus, it might be interesting to further reveal the function of calumenin in the reproductive system.

The nematode cuticle is a multi-functional exocytoskeleton composed of collagens, and is required for maintenance of body morphology and integrity [30]. Here we have provided evidence indicating that probably *calu-1* functions in the formation and/or maintenance of cuticle in *C. elegans*. First of all, *calu-1* gene is expressed in hypodermal seam cells which secrete cuticle. Secondly, the *calu-1*(*tm1783*) mutant showed slightly dumpy morphology, which according to previous reports can be due to mutations in collagen genes and components in their biosynthetic pathway [30,31]. Finally, *calu-1*(*tm1783*) mutants indeed have severe cuticle defects including aberrant alumni, furrow and deformed alae. Thus, these results suggest that the *calu-1* is important for cuticle formation and/or maintenance. These cuticle defects may also explain the smaller body length of the *calu-1*(*tm783*) mutant. In addition, cuticle defects may be somehow responsible for locomotion defects, because cuticle is critical for locomotion via attachments to body wall muscle [30].

Collagen biosynthesis is a complex process involving chaperones and modifying, folding, and processing enzymes. Simply, procollagen is modified in the ER and further processed after secretion from the ER [30]. ER resident enzymes such as protein disulfide isomerase (PDI) and peptidyl prolyl cis–trans isomerase (PPIase) are important for procollagen modification. Interestingly, the activity of these enzymes has been reported to be regulated by Ca^{2+} [32–35]. Thus, we assume that the *calu-1* may be involved in maintaining Ca^{2+} level in the ER, thereby affecting those enzymes whose function is needed to properly modify procollagen. Alternatively, it could be possible that CALU-1 itself binds to those enzymes to regulate their activities.

Lastly, we examined pharyngeal pumping and defecation to test the Ca^{2+} mediated functions of *calu-1*. These behaviors are controlled by Ca^{2+}, and IP_3 receptors are important to regulate behaviors. Our genetic analysis of interactions between *itr-1* and *calu-1* are consistent with several possibilities: First, *calu-1* may function downstream of *itr-1* to regulate pharyngeal pumping. However, we also cannot exclude the possibility that *calu-1* may act in a parallel pathway to regulate pharyngeal pumping. This should be further confirmed by *itr-1*(*lf*);*calu-1*(*lf*) double mutant. On the other hand, there could be more than one pathway regulating the defecation cycle, in which *calu-1* might be independently involved. However, we cannot rule out the possibility that *itr-1* and *calu-1* may share some components to regulate defecation cycle because *itr-1*(*sa73*)(*lf*) mutants treated with *calu-1* RNAi displayed a pattern of defecation somewhat similar to that of *itr-1*(*sa73*)(*lf*) mutants.

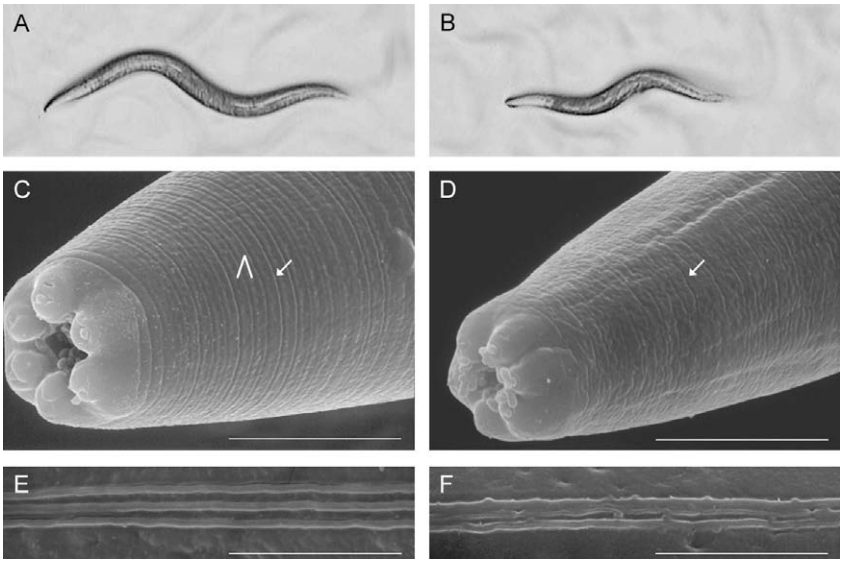

Fig. 4. The cuticle defects of *calu-1*(*tm1783*) mutant. (A) Morphology of wild type N2. (B) Morphology of *calu-1*(*tm1783*) mutant. Mutant showed semi-dumpy morphology. (C–F) Scanning electron microscopy (SEM) images of animals. (C) Wild type displayed regularly organized annuli (Λ) and furrow (arrow) of cuticle in the head region. (D) The *calu-1*(*tm1783*) mutant displayed abnormal cuticle structure in the head. Furrows (arrow) are disconnected. (E) Wild type showed three distinct lines of alae. (F) *calu-1*(*tm1783*) mutant showed deformed alae. Scale bars, 50 μm (A and B) and 10 μm (C–F).

Table 1
Summary of phenotypic defects of *calu-1*(*tm1783*) and *calu-1* RNAi.

	Brood size[a]	No. of eggs laid (h)	Body bends (s)	Body length (μm)[a]
N2	298 ± 26 (38)	8.4 ± 3.5 (40)	2.0 ± 0.2 (30)	758 ± 57 (30)
calu-1(*tm1783*)	170 ± 52 (33)	3.5 ± 1.2 (60)	1.0 ± 0.2 (30)	552 ± 44 (20)
N2_L4440	228 ± 30 (12)	9.6 ± 2.5 (13)	2.1 ± 0.2 (24)	705 ± 35 (27)
N2_*calu-1* RNAi	187 ± 20 (18)[b]	5.5 ± 2.8 (30)	1.0 ± 0.4 (24)	547 ± 26 (13)[c]

Mean ± S.D.
n = number is shown in parenthesis.
N2_L4440 vs. N2_*calu-1* RNAi.
[a] F_4 generation was tested for phenotype after feeding RNAi.
[b] $P < 0.05$.
[c] $P < 0.0001$.

Table 2
Ca^{2+} related phenotypes of *calu-1*(*tm1783*) and *calu-1* RNAi.

	Pharyngeal pumping (pumps/min)	Defecation interval (s)	EMC/cycle (%)
N2	241 ± 15 (20)	46 ± 01 (25)	97 ± 08
eat-3(*tm1077*)	96 ± 25 (20)	N.D	N.D
calu-1(*tm1783*)	179 ± 22 (20)	82 ± 08 (51)	96 ± 08
itr-1(*sy328*)(*gf*)	247 ± 20 (20)	54 ± 07 (30)	94 ± 11
itr-1(*sy328*)(*gf*);*calu-1*(*tm1783*)	164 ± 24 (20)	66 ± 07 (35)[a]	63 ± 21
itr-1(*sa73*)(*lf*)	175 ± 32 (20)	112 ± 51 (13)	89 ± 23
N2_L4440[b]	237 ± 08 (20)	51 ± 04 (12)	90 ± 13
N2_*calu-1* RNAi[b]	205 ± 15 (20)[c]	66 ± 05 (12)[d]	100 ± 00
itr-1(*sa73*)(*lf*)_L4440[b]	185 ± 26 (20)	123 ± 32 (16)	94 ± 12
itr-1(*sa73*)(*lf*)_*calu-1* RNAi[b]	162 ± 25 (20)[e]	184 ± 79 (11) [f]	66 ± 34

Mean ± S.D.
n = number is shown in parenthesis.
[a] *calu-1* vs. *itr-1*(*gf*);*calu-1*, $P<0.0001$; *itr-1*(*gf*) vs. *itr-1*(*gf*) vs. itr-1(*gf*);*calu-1*, $P<0.0001$.
[b] F_4 generation was tested for phenotypes after feeding RNAi.
[c] N2_L4440 vs. N2_*calu-1* RNAi. $P<0.0001$.
[d] N2_L4440 vs. N2_*calu-1* RNAi. $P<0.0001$.
[e] *itr-1* (*lf*)_L4440 vs. *itr-1* (*lf*)_*calu-1* RNAi, $P<0.05$.
[f] N2_*calu-1* RNAi vs. *itr-1* (*lf*)_*calu-1* RNAi, $P<0.001$.

The CREC proteins are increasingly drawing research interests because of their involvement in various diseases. However, the physiological and pathological mechanisms are not fully understood. Our report showing the in vivo functions of calumenin therefore may provide very useful hints for future studies.

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