# Molecular and functional analysis of *Caenorhabditis elegans* CHIP, a homologue of Mammalian CHIP

Liakot A. Khan[a,b], Nobuyuki Nukina[a,*]

[a]*Laboratory of Structural Neuropathology, RIKEN-Brain Science Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan*
[b]*Department of Biochemistry, University of Chittagong, Chittagong, Bangladesh*

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**Abstract A recently identified molecule C-terminus of Hsc70 interacting protein (CHIP) has been reported to be an E3 ubiquitin ligase collaborating with molecular chaperones for the degradation of misfolded or unfolded proteins. The physiological roles of CHIP in animal and plant development remain largely unknown. Here, we show that the knockdown of CeCHIP by RNAi and knockout by a deletion mutation arrests the development of the animal at the larval stage. CeCHIP expresses ubiquitously in all tissues but there are tissue specific variations of expression level. CeCHIP produces dose dependent phenotypes in vivo. Over expression of CHIP causes embryonic lethality, while a comparatively lower level of over expression causes locomotion and egg laying defects, and the CHIP over expressed animals form dauers at a higher temperature.**


*Keywords:* CHIP; Nematode; RNAi; Arrest; Egl; Unc; Dauer

## 1. Introduction

Chaperones and proteases appear to form a cellular surveillance system that monitors protein quality. Recent findings have provided insight into the molecular mechanisms that underlie the interplay of chaperones and proteases [1]. Chaperones participate in the folding of newly synthesized proteins. They also protect proteins during cellular stress and intracellular protein trafficking [2–4]. It has also been reported that Hsp70 and Hsp90 cooperate with degradation machinery [5–7]. However, the underlying molecular mechanisms of their dual roles remain largely elusive.

C-terminus of Hsp70 interacting protein (CHIP) was identified as a Tetratricopeptide repeats (TPRs) domain containing protein and was shown to modulate chaperone activity [8]. Three tandem TPR domains located at the N-terminus of CHIP, together with a highly charged adjacent α-helix, form a chaperone adaptor. CHIP utilizes this single adaptor to interact with either Hsp70 or Hsp90 [8,9].

Interestingly, the C-terminus of CHIP displays structural similarities to components of the ubiquitin/proteasome system, a major protein degradation pathway in eukaryotic cells [1,8,10]. CHIP possesses a U-box domain, which is shown to participate in ubiquitin conjugation. The modular structure of CHIP may thus enable the cofactor to directly link molecular chaperones to the degradation machinery [1]. In support of this hypothesis, CHIP was recently shown to promote the degradation of chaperone substrates by the ubiquitin/proteasome system [9,11]. It was reported that CHIP induces ubiquitylation and degradation of chaperone substrates glucocorticoid receptor (GR), cystic fibrosis transmembrane conductance regulator (CFTR), and Pael receptor in a dose dependent fashion [9,11–14]. CHIP apparently targets diverse chaperone substrates to the ubiquitin/proteasome system and in this way modulates the balance of protein folding and protein degradation during protein quality control.

Recently, CHIP was knocked out in mice and it was found that 5% of the knocked out mice quickly cannibalized in the immediate peripartum period and the remaining offspring suckled normally. 20% of these mice died in the peripartum period. These dead mice had marked thymic atrophy. The surviving 80% *CHIP* (−/−) mice had no obvious morphologic abnormalities but they had impaired responses to thermal challenge. 100% of the *CHIP* (−/−) mice died rapidly, either during thermal challenge or immediately thereafter [15]. In another report it was shown that over expression of *AtCHIP* in *Arabidopsis* leads to increased sensitivity to temperature stress conditions [16]. These findings suggest that CHIP might be playing important roles in cellular functions and imbalance of its expression may lead to serious consequences in stress tolerance.

Several neurodegenerative diseases are caused by protein misfolding or protein aggregation, for example Huntington's disease, Parkinson's disease, Spinocerebellar ataxia types 1, 2, 3, 6, 7, and 17 [17]. We have shown that chaperones suppress expanded polyglutamine aggregation and cellular toxicity [18,19]. It was reported that parkin cannot fully ubiquitylate Pael receptor on its own, and CHIP is required for efficient ubiquitylation with parkin [14]. We assume that CHIP, together with chaperones, will play an important role in neurodegenerative diseases.

Thus, it is worth to investigate the biological and physiological roles of CHIP using different model animals. We have used a genetically tractable animal, *Caenorhabditis elegans*, to investigate the physiological role of CHIP.

We demonstrate that knockdown of *C. elegans* CHIP by RNAi and knockout by deletion mutation cause larval arrest, and that over expression of CHIP causes dose-dependent effects. At a higher dose, CeCHIP is lethal, and at a comparatively

* Corresponding author. Fax: +81-48-462-4796.
*E-mail addresses:* khan@brain.riken.jp (L.A. Khan), nukina@brain.riken.jp (N. Nukina).

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lower dose, the transgenic animals develop with various behavioral abnormalities. At elevated temperature (25 °C), the transgenic animals form dauers. These in vivo data extend the earlier studies and suggest that the expression of CHIP is under tight regulation.

Our RNAi data demonstrate that CHIP is necessary for the postembryonic development of *C. elegans* and over expression data show for the first time that CHIP exerts dose-dependent effects in vivo.

## 2. Materials and methods

### 2.1. Maintenance of nematode strains

Worms were cultured using standard methods [20]. The standard N2 Bristol strain was used as wild type in this study. All strains were usually grown at 20 °C, on an *E. coli* OP50 lawn.

### 2.2. Backcrossing of ok459

First, strain CB61 *dpy-5 (e61)* was backcrossed once with wild-type N2. These backcrossed *dpy-5 (e61)* hermaphrodites were crossed with wild-type males. *dpy-5(e61)*/+ males were crossed with VC372 hermaphrodites. Non-Dpy, non-GFP F1 hermaphrodites were picked and cloned. The line that segregated Dpy and non-Dpy, non-GFP F2 progeny was maintained as the canonical strain of allele *ok459*. The deletion in this outcrossed strain was confirmed by PCR. This strain segregates Dpy, non-Dpy growing progeny and L1 arrested progeny.

Primers for detecting the deletion in the mutant allele *ok459*:
External left: 5′-GACTTGCGTGCCATAAAGGT-3′
External right: 5′-GTCTTGTTTGGTACCGGGAA-3′
Internal left: 5′-TGCTGCACAATTTCTTCTCG-3′
Internal right: 5′-CCTCAGCTGCACGTTTAACA-3′

### 2.3. RNAi procedure

A PCR fragment (−105 to +50 bp) of the *chn-1* gene was subcloned into the *Hin*dIII–*Bam*HI site of the vector pLITMUS28i (New England BioLabs, catalog#E2000S). Double-stranded (ds) RNA was synthesized in a single reaction (as described in the instruction manual

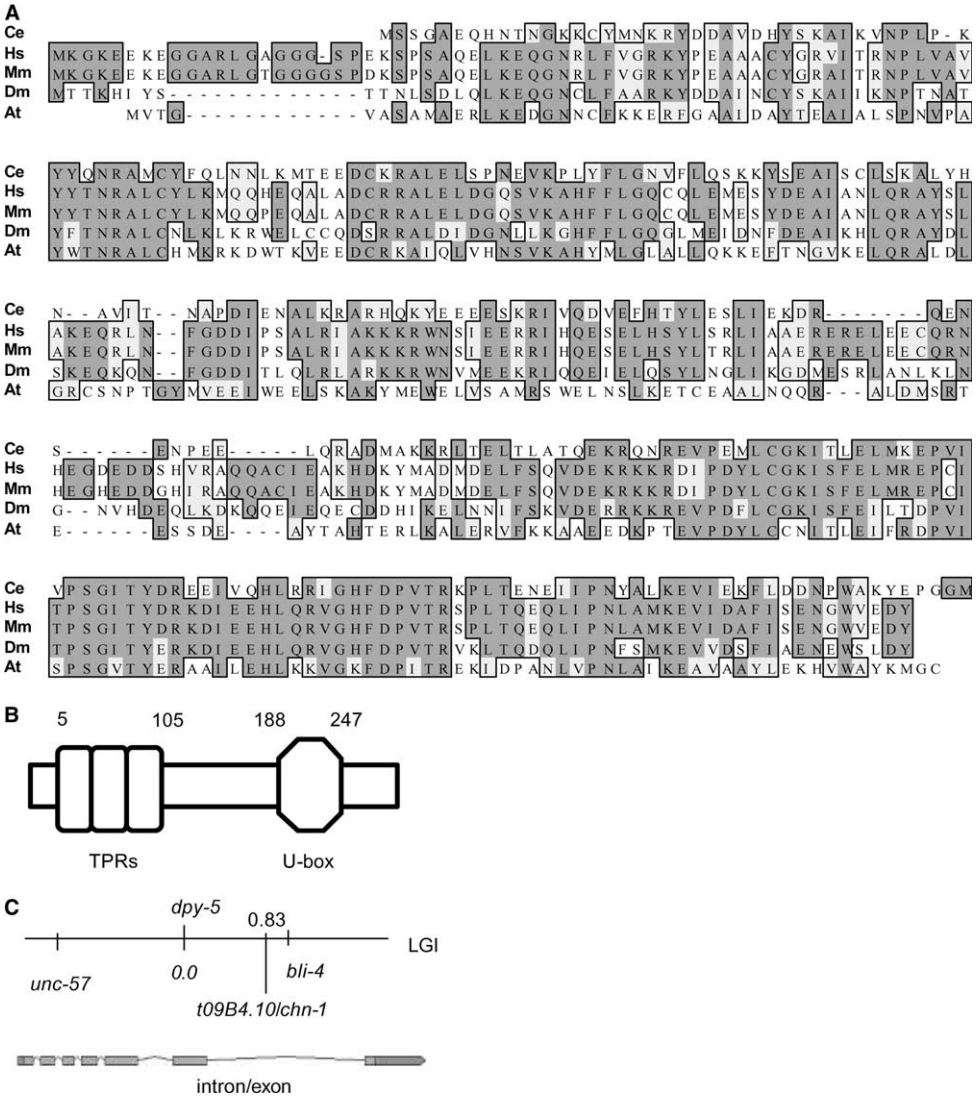


Fig. 1. Amino acid alignment, domains and genetic location of CeCHIP. (A) Amino acid sequence comparison of *C. elegans* CHIP with other animals and plant CHIPs. Ce, *C. elegans* (Accession: AAG50227): Hs, *Homo sapiens* (AAD33400): Mm, *Mus musculus* (AAD33401): Dm, *D. melanogaster* (AAD33399): At, *A. thaliana* (AAK68747). (B) Schematic diagram showing the domains of *C. elegans* CHIP, produced using the SMART program [29]. (C) Genetic location and structure of the gene *chn-1*. *C. elegans* CHIP *chn-1* is located on the right side of the linkage group I, about 0.83 map unit away from the center (http://www.wormbase.rog/), genetic markers are shown on both sides to compare the relative position of *chn-1*. The gene contains six introns and seven exons, the 3′ UTR is 320 bp.

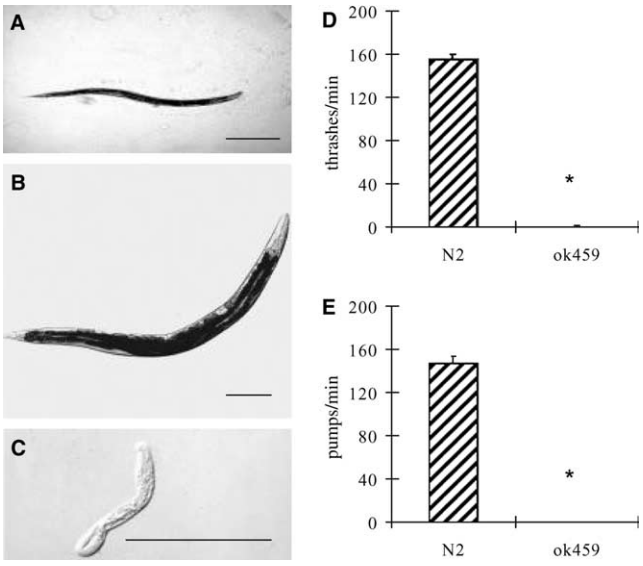


Fig. 2. CHIP RNAi and knockout arrested development. (A) Animals developmentally arrested by RNAi of CeCHIP, (B) wild-type animals, (C) *ok459* allele, developmentally arrested at the L1 stage, (D) body thrashing rate of wild type (N2) and *ok459* animals, data are presented as means ± S.E.M. (*$P < 0.0001$), (E) pharyngeal pumping rate of wild type (N2) and *ok459* animals, data are presented as means ± S.E.M. (*$P < 0.0001$). *P*-values were calculated using student's *t*-test. Scale bar: 100 μm.

of NEB). This RNA was purified by a RNA purification kit (QIAGEN) and then dissolved in DEPC-treated water. dsRNA was injected into the intestine of wild-type nematodes at a concentration of 4.0 μg/μl [21]. The injected animals were incubated at 20 °C for recovery and then the viable animals were cloned on new NGM plates and incubated at 25 °C. The phenotypes, including dead eggs, Unc, Egl, dauer, slow growth, and arrest, of the F1 progeny were carefully observed. For control RNAi, *Bam*HI–*Eco*RI fragment (this fragment contains the *gfp* gene) of the plasmid pPD95.77 was subcloned into the vector pLITMUS28i, and dsRNA was made as described above. *gfp* dsRNA was injected into the wild-type animals at a concentration of 4.0 μg/μl.

### 2.4. Recombinant DNA techniques

All recombinant plasmids were constructed by standard methods [22] and all constructs were sequenced to verify their integrity. A PCR product (−896 to +50 bp) was subcloned in frame into the *Sph*I–*Bam*HI site of the GFP vector pPD95.75 to investigate the spatial and temporal expression of the *chn-1* gene. A PCR product (−896 to +2580 bp) was subcloned into the vector pPD95.75, and this genomic fragment contains the whole *chn-1* gene including the 896 bp promoter and the 320 bp 3′ UTR. Transgenic animals were generated by standard transformation techniques [23].

Primers for promoter fusion and subcloning the full-length *chn-1* gene:

1. 5′-CGGGATCCCGCATATAACACTTTTTGCCAT-3′
2. 5′-ATGAGGTTCTGAAATCACTC-3′
3. 5′-CGGGATCCCGGTTATAGACCTTCCTATTGG-3′

### 2.5. RT-PCR

RT-PCR was carried out to confirm that CHIP was over expressed in the transgenic animals. Total RNA was isolated from, *chn-1* full-length + *Pchn-1::gfp* and *Pchn-1::gfp*, transgenic animals by TRIzol reagent (Invitrogen) and the isolated RNA was treated with RQ1 RNase-free Dnase I (Promega). Single stranded cDNA was made from 2.0 μg of total RNA using Superscript II RT (Invitrogen). PCR experiments were performed on 5× and 10× dilutions of the cDNAs. 22 to 36 rounds of PCR were carried out for each primer set at the conditions that had been optimized to ensure that reactions were carried in the linear range. PCR products were visualized on an agarose gel stained with ethidium bromide. *act-1* (actin) was used as a control gene. The RT-PCR experiment was repeated three times from two independent transgenic lines.

Primers for RT-PCR:

*act-1* f: 5′-GTGTGACGACGAGGTTGCCGCTCTTGTTGTAGAC-3′
*act-1* r: 5′-GGTAAGGATCTTCATGAGGTAATCAGTAAGATCAC-3′
*chn-1* f: 5′-AGAGGAGTTGCAAAGAGCAG-3′
*chn-1* r: 5′-ATGGGTTGTCGTCAAGAAAC-3′

### 2.6. Embryonic lethality analysis

The full-length *chn-1* gene, including promoter and 3′ UTR, was injected into the wild-type animals at different concentrations. *Pchn-1::gfp* was injected together as a transformation marker. *chn-1* full-length gene was injected (50.0 ng/μl) with *gfp* plasmid (50.0 ng/μl). More than 50 animals were injected and about 20 animals laid transgenic embryo (GFP positive). Total number of GFP-positive eggs was counted. The *chn-1* full-length gene was injected at a concentration of 25.0 ng/μl with *Pchn-1::gfp* (75.0 ng/μl), and the number of unhatched GFP-positive eggs and hatched GFP-positive larva were counted. In control animals, *Pchn-1::gfp* was injected at a concentration of 100.0 ng/μl.

### 2.7. Locomotion, body thrashing and pharyngeal pumping assay

Transgenic L4-stage animals were picked and transferred to a new NGM plate and allowed to crawl for 1 h at room temperature (25 °C). Body bends/min were counted under microscope [24]. Every time 10 animals were taken and the experiment was repeated three times. Body thrashing and pharyngeal pumping were counted as described in [25]. To count the body thrashing, an animal of L1 stage was placed in microtiter plate with 50 μl M9 buffer and allowed to recover for 2 min. Subsequently, thrashes were counted for 1 min. Pharyngeal pumping rates were determined by counting the number of pumps per minute. Wild type and *ok459* animals of the same stage were placed on NMG agar plates seeded with thin layer of OP50. Total numbers of pumps in 1 min were counted. Every time 10 animals were taken and the experiment was repeated at least three times.

### 2.8. Egl behavior assay

Egg-laying defective behavior was analyzed by counting the number of eggs in the uterus [26]. Single adult animals were picked and placed in M9 buffer on a glass slide. The animal was then dissected with a razor. In this way, most of the embryos come out of the uterus and few remain inside the uterus. All the embryos, outside and inside the uterus, were counted. In each experiment, 10 animals were dissected and this experiment was repeated three times.

### 2.9. Dauer assay

*chn-1* transgenic animals were seeded on NGM plates and allowed to lay about 100 eggs at 25 °C, then the parents were removed from the plate. The eggs were then incubated at 25 °C for 2 days. Total transgenic animals were counted using GFP as a marker. The animals that were grown to the L4 stage were separated from the younger larvae. The transgenic animals that stayed at the L1/L2 stages were kept in the same plate. These arrested animals were incubated for 7 days at 25 °C to confirm their arrested state. Their dauer phenotype was confirmed by their resistance to 1% SDS [27].

## 3. Results and discussion

### 3.1. Molecular characterization of C. elegans CHIP

Genome search shows that *C. elegans* contain one homologue of mammalian CHIP, which is named *chn-1* (cosmid product T09B4.10) (http://www.wormbase.org/). A comparison of the amino acid sequences of *C. elegans* CHIP with human, mouse, *Drosophila melanogaster* and *Arabidopsis thaliana* is shown in Fig. 1A. A BLAST search [28] showed that *C. elegans* CHIP CHN-1 is the only homologue of mammalian CHIP. SMART analysis [29] showed that *C. elegans* CHIP contains three TPRs and a U-box domain (Fig. 1B). Alignment between human and *C. elegans* CHIPs showed that 40%

of the amino acids were identical and 75% were similar. Based on the homology, we assumed that *C. elegans* CHIP may function in a similar manner as mammalian CHIPs. To understand its physiological role in the animals, we first attempted to knockdown the gene by RNAi.

### 3.2. *CHIP knockdown by RNAi arrests the development of* C. elegans

RNAi is an established method for gene knockdown in *C. elegans* [30] and we used this approach to knockdown the *chn-1* gene. We injected dsRNA of *chn-1* into the intestine of the wild type *C. elegans*. The injected viable animals were cloned and the F1 progenies were observed for phenotype. We found some slow-growing animals among the F1 progeny. In *C. elegans* RNAi effects were observed in F2 and the successive generations with lesser extent [21], so we decided to observe the effects of RNAi in the next generation. The slow-growing F1 animals were again cloned and F2 progenies were observed. We found that a fraction of the F2 progenies were arrested at L1/L2 stages (Fig. 2A). These larvae were picked ($n = 50$) and seeded on fresh NGM plates, 90% of these larvae remained in L1/L2 stages for about two weeks and then died. Some of these arrested larvae were able to grow slowly and produce F3 progeny. Again, arrested larvae were present among the F3 progeny. We did not see any arrested larvae in the F4 generation. As a control, we injected GFP dsRNA into the wild-type animals and we did not see any arrested larvae in the suc-

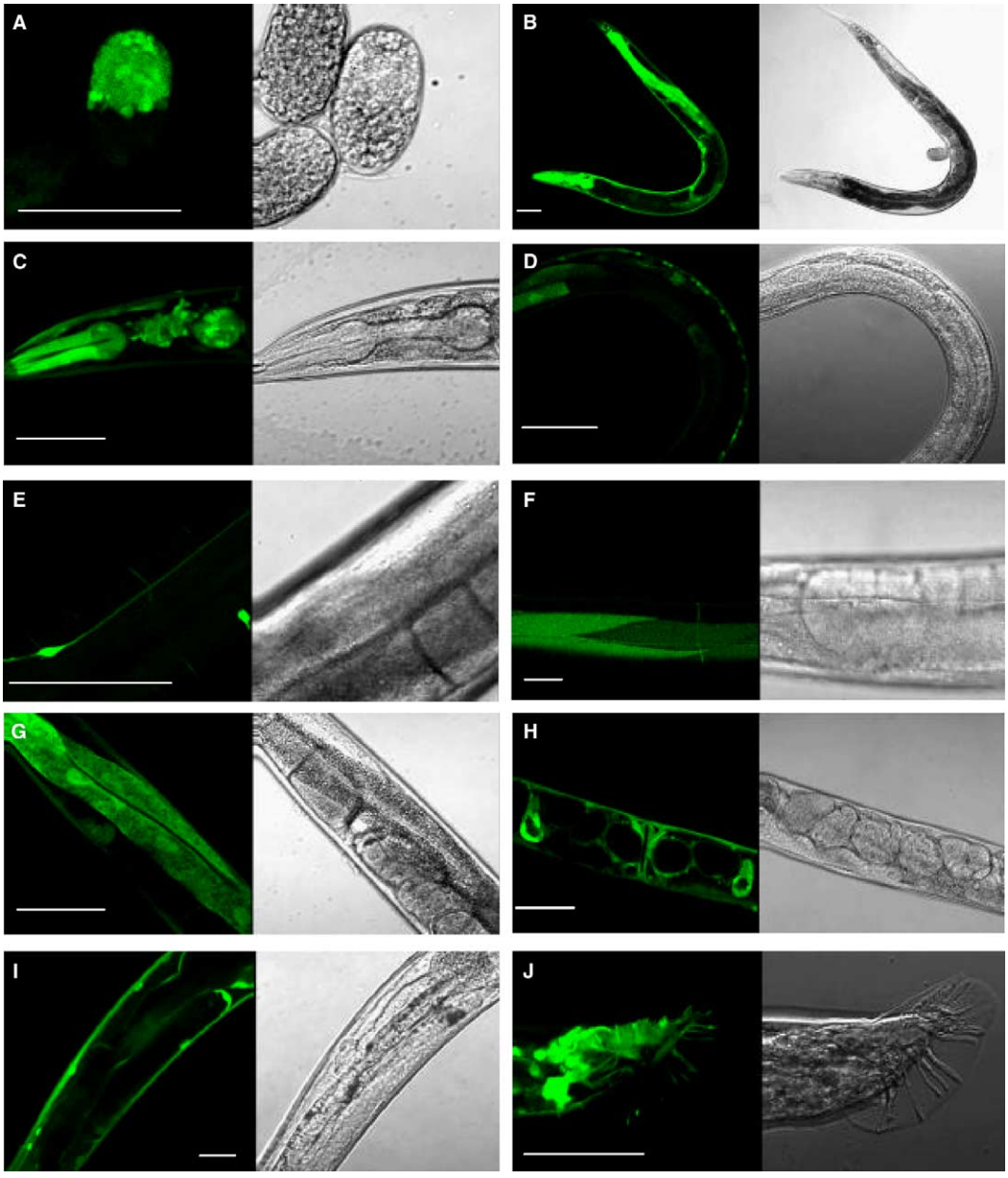


Fig. 3. Expression pattern of CeCHIP. Expression is shown by GFP fluorescence: (A) embryo, (B) whole animal, (C) pharynx and head neurons, (D) ventral cord neurons, (E) neuronal cell body and its process, (F) muscles, (G) intestine, (H) spermatheca, (I) tip of the gonad arms, (J) male tail rays. In each picture, left side is fluorescence and right side is phase contrast. Scale bar: 50.0 μm.

ceeding generations (data not shown). A few months after our RNAi experiment, we obtained a deletion allele of *chn-1* from the gene knockout consortium. The deletion mutant allele *ok459* is maintained as a heterozygote (*ok459*/*ok59*), we characterized the allele and found that the homozygous *ok459* larvae arrested at L1 stage (Fig. 2C). (The allele *ok459* contains deletion in *chn-1* and a part of its neighboring gene *t09b4.2*. Since *chn-1* RNAi arrests the growth at early stage, we assumed that the phenotype of *ok459* is due to deletion in *chn-1* gene.) *ok459* animals bend their body occasionally but they do not have the exploratory behavior. They were either lethargic or unable to move. We examined their body thrashing rate and found that their body thrashing rate is very low compared to wild type animals (Fig. 2D). We also examined the contraction rate of the pharynx of *ok459* animals. It was found that the *ok459* animals were completely unable to contract pharynx (Fig. 2E). The gross morphology of the pharynx of *ok459* animals was seen like wild type animal (data not shown). It is possible that in the absence of CHIP the nematode cannot contract their pharyngeal muscles and hence cannot pump food into the intestine, as a result the animals are unable to grow. Since CeCHIP expresses very highly in the pharynx, probably dsRNA caused dysfunction of the pharynx. Due to pharyngeal dysfunction animals could not eat and remained arrested at the larval stage. It is possible that in the escaping animals dsRNA was not transported into the pharynx but was transported in to other tissues, as a result the animals could eat and grow.

It was reported that CHIP (−/−) mice had impaired response to thermal challenge [15], we also examined whether the CHIP (−/−) nematode shows any sensitivity to heat shock. CHIP (−/−) nematode arrested at the L1 larval stage, they thrash their body very rarely (1–5 times/min, Fig. 2D) but can bend their head (forage) very frequently. We found that 25% ($n = 120$) of the heat shocked CHIP (−/−) nematodes larvae were dead (no movement at all). On the other hand, 100% ($n = 530$) of the heat shocked control animals (wild type) were alive (contract pharynx and thrash body). 4.9% ($n = 121$) of the CHIP (−/−) larvae grown at 20 °C (optimum growth temperature) were also dead (no movement at all). Our observations suggest that CeCHIP is necessary for the postembryonic development of the nematode. Our data, together with the previous observations, also suggest that CHIP may have important physiological role in stress tolerance.

### 3.3. chn-1 (CeCHIP) is expressed ubiquitously in all tissues

The promoter of the *chn-1* gene was fused with GFP (see Section 2) and the recombinant DNA was injected into the wild-type animals. GFP was observed from the embryo stage through to the adult stages. In the adult animal it was expressed in the pharynx, intestine, body wall muscle, gonad, distal tip cells and/or germ cells, nervous system, male tail rays, and spicule (Fig. 3). *chn-1* is probably expressed in all tissues. Human CHIP is also expressed in all tissues with higher expression in skeletal muscles and the heart [8]. CeCHIP is also very highly expressed in the pharynx, which is functionally similar to the heart (pharynx continuously pumps food into

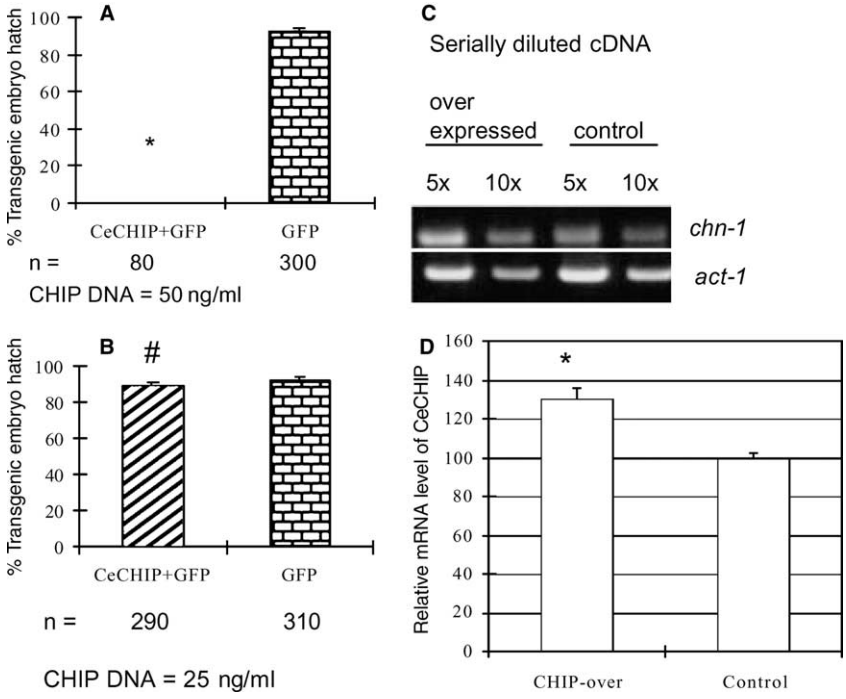


Fig. 4. Effect of CHIP over expression is dose-dependent. (A) Left bar, injection of CHIP DNA at a concentration of 50.0 ng/μl with 50.0 ng/μl *gfp* plasmid is lethal; right bar, injection of *gfp* plasmid at a concentration of 100.0 ng/μl is not lethal. Data are presented as means ± S.E.M. (*$P < 0.0001$). (B) Left bar, injection of CHIP DNA at a concentration of 25.0 ng/μl with 75.0 ng/μl of *gfp* plasmid is not lethal; right bar, injection of *gfp* plasmid at a concentration of 100.0 ng/μl. Data are presented as means ± S.E.M. ($^{\#}P = 0.35$). (C) RT-PCR was performed to confirm the over expression of CeCHIP in the transgenic animals. Lane 1, 2: RNA was isolated from the animals that carried *chn-1* full-length gene + *Pchn-1::gfp*; lanes 3, 4: RNA was isolated from the control animals that carried only *Pchn-1::gfp*. Lanes 1 and 3, 5× dilution of cDNA; lanes 2 and 4, 10× dilution of cDNA. (D) Relative mRNA level in CeCHIP over expressed animals (left bar) and control animals (right bar), calculated from the average band intensities. Data are presented as means ± S.E.M. (*$P < 0.002$).

the intestine) [31]. Expression data indicate that hCHIP and CeCHIP may perform similar function in the heart and in the pharynx, respectively.

### 3.4. Over expression of CeCHIP causes dose-dependent effects

In the cellular system, it was demonstrated that CHIP degrades GR in a dose-dependent fashion and over expression of CHIP enhances the degradation of CFTR [9,11]. To see if CeCHIP exerts similar effects in vivo, we over expressed CeCHIP in live animals. We injected wild-type *chn-1* DNA at different doses into the wild-type animals. First, the *chn-1* full-length gene was injected at a concentration of 50.0 ng/μl with 50.0 ng/μl of GFP marker plasmid (see Section 2). We found that 100% of the transgenic (GFP-positive) embryos remained unhatched (Fig. 4A). Since the transgenic embryos remained unhatched, we injected only GFP plasmid DNA (100.0 ng/μl) as a control. We found that 95% of the control transgenic (GFP-positive) embryos hatched. Then, we reduced *chn-1* DNA concentration to 25.0 ng/μl and injected with GFP marker plasmid (75.0 ng/μl). We found that 95% of the CHIP transgenic (GFP-positive) embryos hatched (Fig. 4B). Over expression of CeCHIP in the survived transgenic lines was confirmed by RT-PCR analysis (Fig. 4C and D). These data suggest that CHIP causes dose dependent effects in vivo.

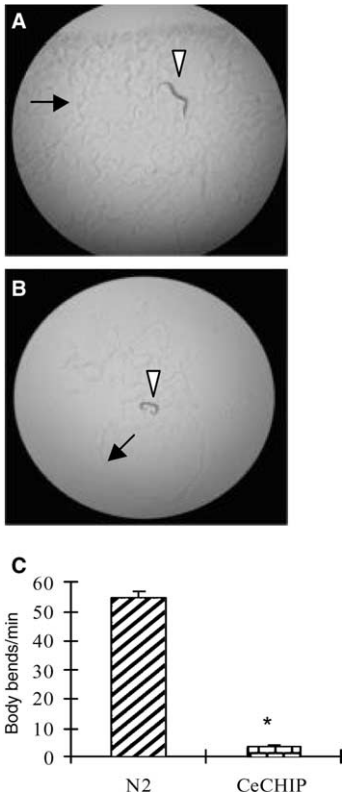


Fig. 5. Over expression of CHIP causes locomotion defect. (A) Tracks produced by a wild type animal on the *E. coli* lawn, *Pchn-1::gfp* transgenic animal (arrowhead) made huge tracks (arrow) all over the lawn during overnight incubation. (B) Tracks produced by a CeCHIP transgenic animal on the *E. coli* lawn, the CeCHIP transgenic animal (arrowhead) produced only few tracks (arrow) during overnight incubation. (C) Left bar, body bends/min of wild type animals: right bar, body bends/min of CHIP transgenic animals. Data are presented as means ± S.E.M. (*$P < 0.0001$). Animals were incubated overnight on *E. coli* lawns to make tracks for observation.

### 3.5. Over expression of CeCHIP causes locomotion defect

When DNA was injected at a concentration of 25.0 ng/μl, 95% of the transgenic embryos were hatched (Fig. 4B), but all the transgenic animals (GFP-positive) were uncoordinated in locomotion (Unc) from the larval stage. The Unc phenotype was determined by watching the tracks produced by the *Pchn-1::gfp* and CHN-1 full transgenic animals on *E. coli* lawns (Fig. 5A and B) and by calculating the body bends/minute of the L4-stage animals (Fig. 5C). These data suggest that over expression of CeCHIP causes cellular dysfunction involved in the process of locomotion.

### 3.6. Over expression of CeCHIP causes egg-laying defect

The transgenic animals (GFP-positive) with over expressed CeCHIP were egg-laying defective (Egl) at adult stage at 20 °C. These animals could lay few eggs but huge eggs remained inside the uterus and ultimately many eggs hatched in the uterus (Fig. 6A–D). We counted the number of eggs inside the uteruses of wild-type and CHIP transgenic animals. Usually, the wild-type animals have 10–12 eggs inside the uterus and the eggs are in the early stage of their development [26]. We found that the CHIP transgenic animals contained 40–45 eggs inside the uterus (Fig. 6E). Moreover, many eggs were in the pretty late stages, such as two- or three-folded stages. These data suggest that over expression of CHIP causes cellular dysfunction involved in the process of egg laying. CHIP is expressed in all tissues, so egg laying defective behavior may arise

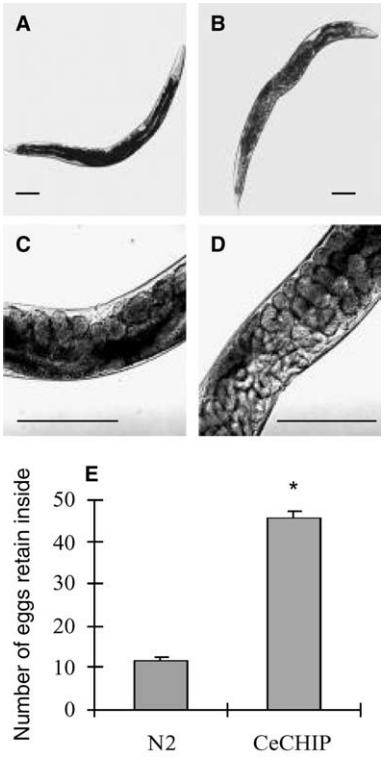


Fig. 6. Over expression of CHIP causes egg-laying defect. Confocal micrograph of (A) wild-type, (B) CHIP transgenic animals. Confocal micrograph at higher magnification of (C) wild type, very few eggs inside the animals, (D) CHIP transgenic animals, huge eggs inside the animal and the eggs are at late stage. (E) Number of eggs retained inside the uterus: left bar, wild type animals: right bar, CHIP transgenic animals. Data are presented as means ± S.E.M. (*$P < 0.0001$). Scale bar: 100 μm.

from dysfunction of neurons, muscles, the uterus, or all of these.

We also expressed the human CHIP under the control of the CeCHIP promoter. The HsCHIP transgenic animals were partially Egl (data not shown). This phenomenon also supports the idea that both HsCHIP and CeCHIP may play similar physiological roles. Since transgenic animals reared at 20° were Unc and Egl, those reared at elevated temperatures may behave differently. We examined the behavior at 25 °C.

### 3.7. CHIP over expressed animals form dauers at elevated temperatures

We incubated the CHIP transgenic animals at 25 °C to assess the effect of temperature. It was found that most of the transgenic F1 progeny grew with Unc and Egl phenotypes, but about 30% of the F1 remained in the L1/L2 stage (Fig. 7 and Table 1). We treated the arrested animals with 1% SDS and found that they are resistant to SDS. These data indicate that these arrested larvae are dauers [27]. When shifted from 25 to 15 °C, these dauers did not return to a normal life cycle. Therefore, CHIP transgenic animals form irreversible dauers at 25 °C. The other 70% of the transgenic animals grew into adults. Since the transgenic lines had the *chn-1* gene as an extra chromosome, the expression level may not be equal in all the progeny. Therefore, some animals grow into adults and others form dauers, depending on the expression level.

Many *C. elegans* mutants behave like wild-type at 20 °C but form dauers at 25 °C. Wild-type *C. elegans* also form dauers when the culture plate is overcrowded or food supply is insufficient. Dauers are formed when environmental conditions are inadequate for successful reproduction [32]. CHIP transgenic animals can continue reproductive life at 20 °C with developmental abnormalities (Unc and Egl at 20 °C), but higher temperatures might produce additional stress to the CHIP over expressed animals, and as a result, the transgenic animals switch to form dauers instead of continuing the reproductive life cycle. A similar phenomenon was also observed in the case of *A. thaliana*. Over expression of AtCHIP leads to increased sensitivity to both low- and high-temperature conditions [16]. Together with our data, this suggests that both higher and lower levels of CHIP affect cell functions both in animals and plants.

Based on experimental evidence, it was assumed that some membrane channel proteins might be substrate for AtCHIP [16]. CeCHIP expressed in all tissues with very high expression in the pharynx. We found that CHIP knocked out animals were unable to contract pharynx. Since different channel proteins ($Ca^{++}$, $K^{+}$ and $Cl^{-}$ channels) are involved in pharyngeal contraction [33], we assume that those channel proteins could be substrates for CeCHIP. Further studies will address these possibilities.

Our results are consistent with the previous models for the function of CHIP [1,34]. In those models, it was proposed that CHIP helps in degradation of unfolded or misfolded proteins by ubiquitin–proteasome system. It was also proposed that CHIP inhibits the folding of nascent proteins into functional proteins [34]. We observed that CHIP knocked down and knocked out animals could not grow beyond larval (L1) stage, this arrest could be due to accumulation of misfolded or unfolded proteins in the cells. We also observed that over expression of CHIP in *C. elegans* leads to serious consequences (death or defective behavior depending on dose of over expression). It is possible that when over expressed, CHIP might compete with Hop [1] and nascent proteins were degraded instead of folding. Further genetic and biochemical investigations will be necessary to understand the role of CHIP.

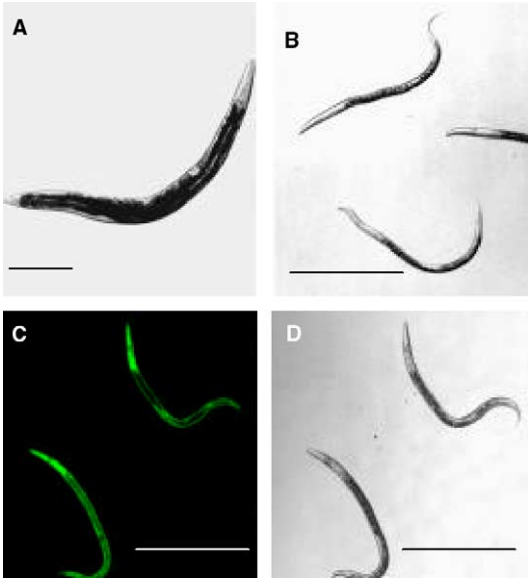


Fig. 7. CHIP transgenic animals form dauers at 25 °C. (A) Wild-type animals developed at 25 °C, (B) wild type dauers from crowded starved plate, (C, D) CHIP transgenic animals form dauers at 25 °C, (C) fluorescence and (D) phase contrast micrograph of the same animals. Scale bar: 200.0 μm.

Table 1
CHIP transgenic animals forming dauers at 25 °C

| Transgenic lines | % forming dauers | *P*-value |
|---|---|---|
| *Pchn-1::gfp* | $1 \pm 0.57$ ($n = 600$) | |
| *chn-1* full-length *Pchn-1::gfp* | $34.33 \pm 2.33$ ($n = 400$) | $P < 0.003$ |

Data are presented as means ± S.E.M. of three independent observations. *P*-value was calculated by student's *t*-test.

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## References

[1] Hohfeld, J., Cyr, D.M. and Patterson, C. (2001) EMBO Reports 2, 885–890.
[2] Hartl, F.U. (1996) Nature 381, 571–580.
[3] Frydman, J. and Hohfeld, J. (1997) Trends Biochem. Sci. 22, 87–92.
[4] Bakau, B. and Horwich, A.L. (1998) Cell 92, 351–366.
[5] Schneider, C., Sepp-Lorenzino, L., Nimmesgern, E., Ouerfelli, O., Danishefsky, S., Rosen, N. and Hartl, F.U. (1996) Proc. Natl. Acad. Sci. USA 93, 14536–14541.
[6] Bercovich, B., Stancovski, I., Mayer, A., Blumenfeld, N., Mayer, A., Blumenfeld, N., Laszlo, A., Schwartz, A.L. and Ciechanover, A. (1997) J. Biol. Chem. 272, 9002–9010.

[7] Dul, J.L., Davis, D.P., Williamson, E.K., Stevens, F.J. and Argon, Y. (2001) J. Cell Biol. 152, 705–716.
[8] Ballinger, C.A., Connell, P., Wu, Y., Hu, Z., Thompson, L.J., Yin, L. and Patterson, C. (1999) Mol. cell Biol. 19, 4535–4545.
[9] Connell, P., Ballinger, C.A., Jiang, J., Wu, Y., Thompson, L.J., Hohfeld, J. and Patterson, C. (2001) Nature Cell Biol. 3, 93–96.
[10] Murata, S., Minami, Y., Minami, M., Chiba, T. and Tanaka, K. (2001) EMBO Report 2, 1133–1138.
[11] Meacham, G.C., Patterson, C., Zhang, W., Younger, J.M. and Cyr, D.M. (2001) Nature Cell Biol. 3, 100–105.
[12] Loo, M.A., Jensen, T.J., Cui, L., Hou, Y., Chang, X.B. and Riordan, J.R. (1998) EMBO J. 17, 6879–6887.
[13] Meacham, G.C., Lu, Z., King, S., Sorscher, E., Tousson, A. and Cyr, D.M. (1999) EMBO J. 18, 1492–1505.
[14] Imai, Y., Soda, M., Hatakeyama, S., Akagi, T., Hashikawa, T., Nakayama, K.I. and Takahashi, R. (2002) Molecular Cell 10, 55–67.
[15] Dai, Q., Zhang, C., Wu, Y., McDonough, H., Whaley, R.A. and Godfrey, V., et al. (2003) EMBO J. 22, 5446–5458.
[16] Yan, J., Wang, J., Li, Q., Hwang, J.R., Patterson, C. and Zhang, H. (2003) Plant Physiol. 132, 861–869.
[17] Ross, C.A. (1995) Neuron 15, 493–496.
[18] Jana, N.R., Tanaka, M., Wang, G. and Nukina, N. (2000) Hum. Mol. Genet. 9, 2009–2018.
[19] Jana, N.R., Zemskov, E.A., Wang, G. and Nukina, N. (2001) Hum. Mol. Genet. 10, 1049–1059.
[20] Brenner, S. (1974) Genetics 77, 71–94.
[21] Grishok, A., Tabara, H. and Mello, C.C. (2000) Science 287, 2494–2497.
[22] Sambrook, J., Fritsch, E.F. and Maniatis, T. (2001) in: Molecular Cloning: A Laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
[23] Mello, C.C., Kramer, J.M., Stinchcomb, D. and Ambros, V. (1991) EMBO J. 10, 3959–3970.
[24] Robatzek, M. and Thomas, J.H. (2000) Genetics 156, 1069–1082.
[25] Moorman, C. and Plasterk, R.H.A. (2002) Genetics 161, 133–142.
[26] Trent, C., Tsung, N. and Horvitz, R. (1983) Genetics 104, 619–647.
[27] Cassada, R.C. and Russell, R.L. (1975) Dev. Biol. 46, 326–342.
[28] Altschul, S.F., Warren, G., Webb, M., Eugen, W.M. and David, J.L. (1990) J. Mol. Biol. 215, 403–410.
[29] Schultz, J., Milpetz, F., Bork, P. and Ponting, C.P. (1998) PNAS 95, 5857–5868.
[30] Fire, A., Xu, S., Montogomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998) Nature 391, 806–811.
[31] Wood, W.B. (1988) The nematode *Caenorhabditis elegans*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[32] Riddle, D.L. and Albert, P.S. (1997) in: *C. Elegans* II, Genetic and environmental regulation of dauer larva development (Riddle, D.L., Blumenthal, T., Meyer, B.J. and Priess, J.R., Eds.), pp. 739–790, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[33] Avery, L. and Thomas, J.H. (1997) in: *C. Elegans*, Feeding and Defecation (Riddle, D.L., Blumenthal, T., Meyer, B.J. and Priess, J.R., Eds.), pp. 679–716, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[34] Patterson, C. (2002). A new gun in town: The U box is a ubiquityn ligase domain. Science's STKE 116, PE4.