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A Novel Calcineurin-interacting Protein, CNP-3, Modulates Calcineurin Deficient Phenotypes in *Caenorhabditis elegans*

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Calcineurin (Cn) is a calcium/calmodulin-dependent serine/threonine protein phosphatase that has diverse functions in different cell types and organisms. We screened proteins interacting with the *C. elegans* CnA homolog, TAX-6, by the yeast two-hybrid system. CNP-3 (Calcineurin interacting protein-3) is a novel protein that physically interacts with the catalytic domain of TAX-6. It is strongly expressed in the nuclei of intestine, hypodermis, dorsal uterine regions and spermatheca. Expression begins around the 60-cell stage and proceeds during all larval stages and the adult. To elucidate the biological function of *cnp-3* we isolated a *cnp-3* deletion mutant. Since *CNP-3* binds CnA, we looked at factors associated with calcineurin loss-of-function mutants, such as brood size, body size, serotonin- and levamisole-mediated egg-laying behavior. The *cnp-3(jh145)* single mutant had no gross defects compared to wild-type animal. However, the phenotypes of the double mutants, *tax-6(p675);cnp-3(jh145)* and *cnb-1(jh103);cnp-3(jh145)*, were more severe in terms of brood size, body size and serotonin-mediated egg-laying defects than *tax-6(p675)* and *cnb-1(jh103),* respectively. These results suggest that dysfunction of *cnp-3* enhances certain calcineurin loss-of-function phenotypes in *C. elegans*.

Keywords: Calcineurin (Cn); Calcineurin Binding Protein; *C. elegans*; CNP-3; TAX-6.

Introduction

Calcineurin (Cn) is a serine/threonine protein phosphatase which plays diverse roles in different cell types and diverse organisms (Crabtree, 1999; Klee et al., 1979; 1998; Stewart et al., 1982). It is highly conserved from yeast to humans and is composed of the catalytic calcineurin A (CnA) subunit and the regulatory calcineurin B (CnB) subunit (Kincaid, 1993). The enzyme is tightly regulated by Ca^{2+}/Calmodulin (CaM), and plays critical roles in several calcium-mediated signal transduction pathways (Lee et al., 2004). The catalytic subunit, CnA, contains a catalytic and a regulatory domain. The latter contains the CnB-binding, CaM-binding and auto-inhibitory (AI) domains (Hashimoto et al., 1990; Hubbard and Klee, 1989). In the absence of Ca^{2+}/CaM, the AI domain occludes the catalytic site thereby inhibiting enzyme activity. Binding of Ca^{2+}/CaM with Cn causes conformational changes displacing the AI domain away from the active site, and thereby activating the enzyme. When the auto-inhibitory domain is deleted, the enzyme is transformed into a Ca^{2+}/CaM-independent, constitutively active form (Hashimoto et al., 1990; O'Keefe et al., 1992).

The *C. elegans* orthologs of CnA and CnB are designated *tax-6* and *cnb-1*, respectively. We and others have previously reported on the *in vivo* functions of *tax-6* and *cnb-1* (Bandyopadhyay et al., 2002; Kuhara et al., 2002; Lee et al., 2004). Calcineurin plays many roles in *C. elegans,* in reproduction, body size regulation, movement, egg laying in response to serotonin, *etc*. For example, we have previously reported that RCN-1 (regulator of calcineurin) can physically bind to and inhibit Cn in *C. elegans* (Lee et al., 2003a). In order to understand the additional proteins that might either regulate or be regulated by Cn, we screened for proteins interacting with TAX-6 by the yeast two-hybrid system. We have named the pro-

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teins interacting with TAX-6 as CNP-1, CNP-2, CNP-3 *etc.*, where CNP denotes calcineurin interacting protein and the numbers refer to the order of their isolation. The protein encoding T23C6.3 is named CNP-3 and here we report the characterization of the interaction between *tax-6* and *cnp-3*.

cnp-3 is a novel gene with very limited homology to other animal genes. We have isolated and characterized a *cnp-3* deletion mutant and describe the genetic interaction between *tax-6* and *cnp-3*. Our results indicate that TAX-6 physically interacts with CNP-3, and that *cnp-3* specifically enhances a subset of phenotypic abnormalities associated with Cn loss-of-function mutants.

Materials and Methods

***C. elegans* strains and maintenance** The Bristol N2 and *tax-6(p675)* strains were obtained from the *Caenorhabditis elegans* Genetic Center (CGC) at the University of Minnesota, USA. *cnb-1(jh103)*, *tax-6(jh107)* and *cnp-3(jh145)* were isolated by reverse genetic methods (Hope, 1999). Worm breeding and handling were conducted as described (Brenner, 1974).

Isolation of the *cnp-3* deletion mutants and generation of double mutants Deletion mutants were isolated by the TMP (Trimethylpsoralen)/UV method as described previously (Hope, 1999; Lee et al., 2003b). Primers were designed based on predicted sequences spanning the full genomic DNA of *cnp-3*: outer upstream primer (5′-CTG TGT CCT TAA GAA CAC TCC GTT GTT-3′) and outer downstream primer (5′-GAA ATG ACA TTT GTC AGA CCG TCA CG-3′), inner upstream primer (5′-CAG TTG ACA GTC CTA CAG TAC CCT GAA C-3′) and inner downstream primer (5′-CAT TGC TGA CCT TAG ATT TCT TGA CG-3′). Homozygous mutants for an 879 bp deletion relative to the wild type were isolated and outcrossed six times with wild type animals. The double mutants *tax-6(jh107);cnp-3(jh145)*, *tax-6(p675);cnp-3(jh145)* and *cnb-1(jh103);cnp-3(jh145)* were generated by standard genetic methods. PCR was used to detect the *tax-6(jh107)* and *cnb-1(jh103)* alleles. The point mutation in the *tax-6(p675)* mutant was confirmed by sequencing the *tax-6* PCR product.

Yeast two-hybrid assays Yeast two hybrid screening was conducted using full length *tax-6* cloned into pAS2-1 as bait, and a *C. elegans* cDNA library cloned into pACT2 as prey vector (Clontech). Screening was performed as per the manufacturer's protocol.

GFP fusion construction and microinjection Cosmid, T23C6 was obtained from Dr. A. Coulson (Sanger Center, UK). For studying the expression pattern, the promoterless *gfp* vector, pPD95.79 (provided by Dr. A. Fire) was used. The 2.2 kb 5′ upstream region and the full coding region of *cnp-3* except for the stop codon were cloned into pPD95.79 using the *Bal*I and *Pst*I restriction sites. The resulting plasmid was mixed with a transformation marker, pRF4 (dominant *rol-6*) and injected into the gonads of young adults as described previously (Mello et al., 1991).

Phenotypic analysis N2, c*np-3(jh145), tax-6(jh107), tax-6(jh107); cnp-3(jh145)*, *tax-6(p675), tax-6(p675);cnp-3(jh145)* and *cnb-1 (jh103), cnb-1(jh103);cnp-3(jh145)* were characterized microscopically. The brood sizes of wild-type and mutants were determined by placing individuals on seeded plates and allowing them to self-fertilize at 20°C. The P_0 mother was then transferred to a fresh plate at 24 h intervals each of the next six days. Total F1 progeny on the plates were counted. Body volume was calculated from body length and width. To measure the length and width of adults, L4 larvae were picked to fresh plates and incubated at 20°C for 48 h. Worms were then visualized under a Zeiss dissecting microscope. All measurements were done using the free Java image processing program Image J (http://rsb.info.nih. gov/ij/) (Morck and Pilon, 2006). To calculate body volume, worms were treated as approximate cylinders ($v = \pi r^2 l$) (McCulloch and Gems, 2003).

Serotonin- and levamisole-mediated egg-laying phenotypes were examined as described (Trent et al., 1983). Briefly, one-day-old adult worms were immersed in control M9 solution (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml of 1M $MgSO_4$ per liter) or in a 12.5 mM serotonin (5-HT) or 12.5 mM levamisole solution (Sigma) for 90 min, after which the number of eggs laid by each worm was counted.

Results and Discussion

TAX-6 physically interacts with CNP-3 A full length cDNA of *tax-6* was cloned into a bait vector and screened for proteins interacting with TAX-6 by the yeast two-hybrid method. Sequencing and subsequent database search revealed that seven independent clones that we isolated were T23C6.3 (GenBank accession No. NM_ 078375). We named the corresponding gene *cnp-3*. In order to identify the region of TAX-6 that interact with CNP-3, we used truncated forms of TAX-6 lacking the auto inhibitory [TAX-6 ΔAI] or catalytic [TAX-6 (Cat)] domain of calcineurin as bait and tested their interaction with CNP-3 (Fig. 1A). The results of this experiment with the appropriate controls indicate that the catalytic domain of TAX-6 is sufficient for the interaction with CNP-3. Currently, we do not know the significance of this interaction. It is possible that CNP-3 is a substrate of TAX-6. In accord with this view we have identified three putative Ser/Thr phosphorylation motifs in CNP-3 (Fig. 2A). Furthermore, CNP-3 also contains a canonical Cn docking site-like motif (PxIxIT). Further experiments are needed to examine the significance of these motifs.

cnp-3* encodes a novel nematode-specific protein in *C.

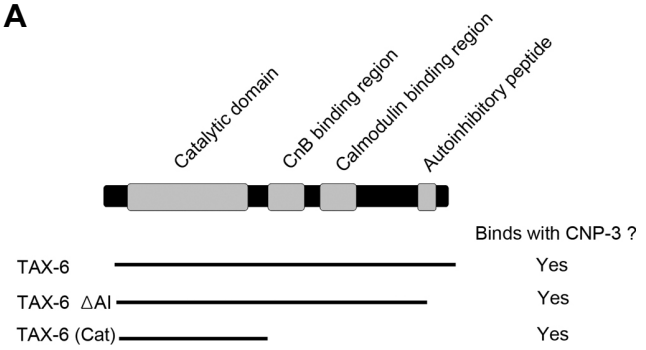

Fig. 1. Physical interaction between TAX-6 and CNP-3. **A.** Schematic diagram showing the major features of TAX-6. The regions of TAX-6 included in the bait vectors are indicated as solid lines. **B.** Yeast two-hybrid analysis of the physical interaction between TAX-6 and CNP-3. The bait vector and prey vector co-transformed into yeast is indicated as 'bait/prey' above each lane. The lower lanes indicate the ability of the co-transformants to grow on synthetic dropout (SD) medium lacking Trp (W) and Leu (L). The upper lanes indicate the ability or inability of the transformants to activate two independent reporter genes as assayed by the presence or absence of growth on the SD medium lacking Trp (W), Leu (L), His (H), and Ade (A). The interaction between p53 and T-antigen was used as positive control, and the interaction between p53 and Lamin C was used as negative control.

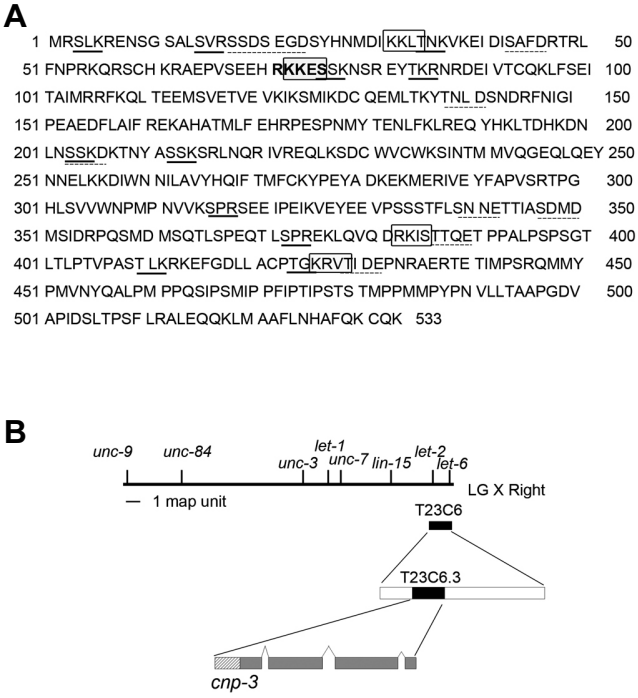

Fig. 2. A. Deduced amino acid sequence of CNP-3. The nuclear localization signal (NLS) is indicated in bold fonts. Putative consensus sites for protein kinase C (PKC) phosphorylation are underlined by solid lines. Dotted lines depict putative consensus sites recognized by casein kinase II. And putative consensus sites recognized by cAMP- and cGMP-dependent protein kinases are indicated in boxes. **B.** Genetic map of the *cnp-3* locus showing the major markers. The cosmid T23C6 includes the indicated region of linkage group X. The organization of *cnp-3* is shown with boxes for exons and thin lines connecting the boxes for introns.

elegans *cnp-3* maps to the X-chromosome and is present in cosmid T23C6. It is composed of four exons and three introns (Fig. 2B). BLASTp search using CNP-3 as query against the non redundant database of proteins retrieved only one ortholog with a significant E-value; it was from the closely related species, *Caenorhabditis briggsae*. Thus, CNP-3 appears to be specific to either the genus *Caenorhabditis* or nematodes in general. Alternatively, CNP-3 may have undergone substantial divergence such that BLASTp failed to detect more distant homologs of CNP-3. We therefore performed a PSI-BLAST search to identify possible distant homologs of CNP-3, and after the third to fourth iteration of PSI-BLAST, many nuclear proteins such as polymerases and transcription factors were retrieved. Interestingly, CNP-3 has a putative nuclear localization signal (Fig. 2A, bold fonts). Hence, it is a poorly conserved protein that remotely resembles members of the transcription factor family.

Spatial and temporal expression pattern of CNP-3 in *C. elegans* In order to understand when and where CNP-3 is expressed, we subcloned a region of *cnp-3* containing about 2 kb upstream plus the full length gene in frame with the GFP coding region in pPD95.79. We injected the construct into the gonads of wild-type worms and examined the spatial and temporal expression of GFP in the transgenic animals. As shown in Figs. 3A and 3B, the earliest detectable expression begins around gastrulation (60 to 100 cell stages); the GFP appears to be present in most of the cells at this stage. From the comma stage onwards, expression is predominantly confined to the nuclei of the intestine (Figs. 3C–3F). In early larval stages, it is also found in hypodermal and intestinal nuclei (Figs. 3G and 3H). Interestingly, it is transiently expressed in the spermatheca and dorsal uterus of the L3 larva (Figs. 3I–3L) and disappears at the onset of the developmental progression from the L4 to adult stage (Fig. 3M), suggesting a possible role in the spermatheca and dorsal uterus in L3.

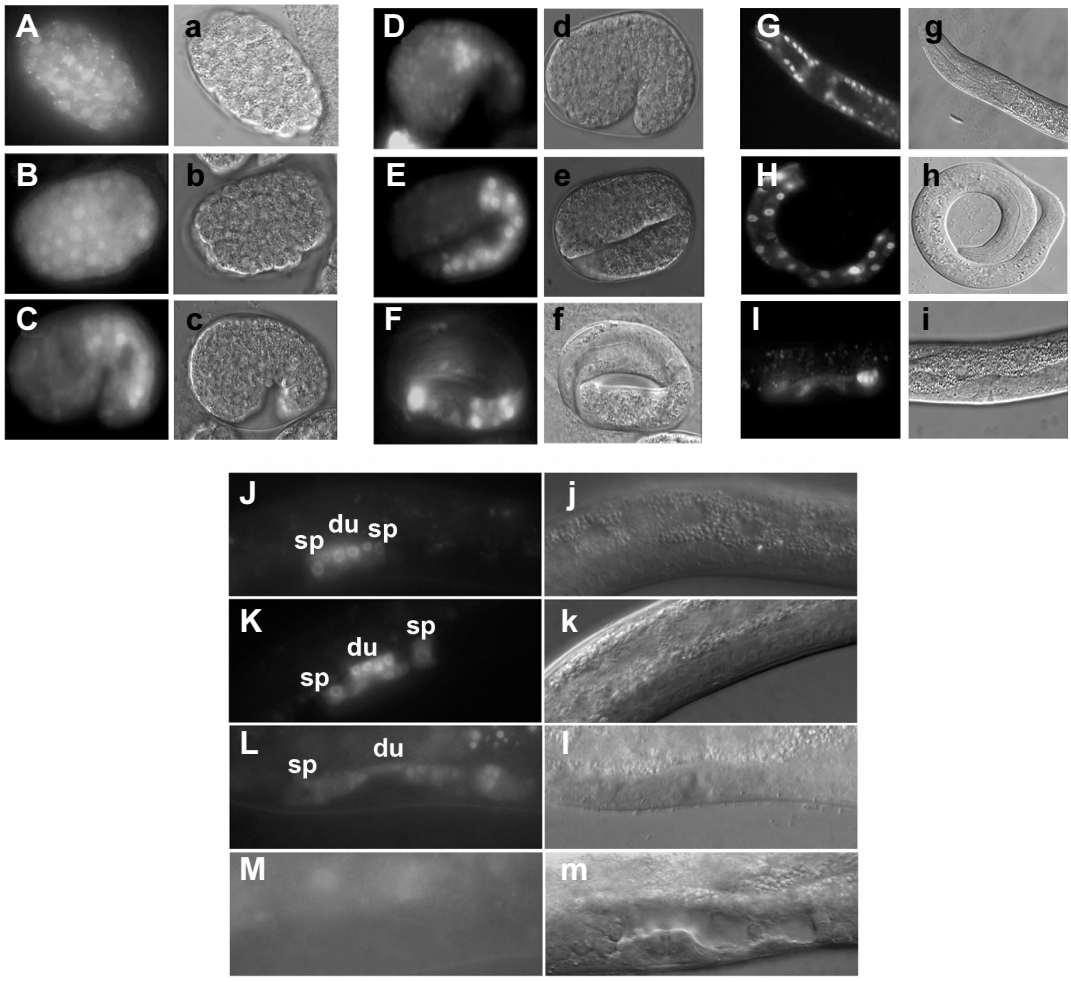

Fig. 3. Spatial and temporal expression of *cnp-3*. Fluorescence microscopic images (A to M) and corresponding Nomarski images (a to m) of various developmental stages of transgenic *C. elegans* expressing a *gfp* reporter under the control of the *cnp-3* promoter. **A.** 60-cell stage, **B.** 100-cell stage, **C.** Comma stage, **D.** 1.5-fold stage, **E.** 2-fold stage, **F.** 3-fold stage, **G, H.** L1 to L2 stage, **I.** L3 to L4 stage, **J.** Early L3 stage, **K.** Mid L3 stage, **L.** Late L3 stage, **M.** L4 stage to young adult stage. Expression of *cnp-3::gfp* begins at the 60-cell stage (A). Until around the 100-cell stage, expression is found in most cells of the embryo. From the comma stage onwards, strong expression of *cnp-3* is seen in the nuclei of the intestine (C–F). The *cnp-3* is also expressed in the nuclei of hypodermis (G), intestine (H), dorsal uterus and spermatheca (I). Expression of *cnp-3* in dorsal uterus (du) and spermatheca (sp) is confined to the L3 to early L4 stages (J–L). Expression of *cnp-3* in dorsal uterus and spermatheca is absent from the L4 to young adult stage (M).

We and others have previously reported that calcineurin is expressed in hypodermis, intestine and spermatheca, in addition to other tissues (Bandyopadhyay et al., 2002; Kuhara et al., 2002; Lee et al., 2004). Since calcineurin and CNP-3 have at least partially similar spatial expression patterns, we believe that the physical interaction between calcineurin and CNP-3 is biologically relevant.

Isolation and characterization of a *cnp-3(jh145)* deletion mutant In order to define the *in vivo* roles of *cnp-3*, we isolated a deletion mutant by PCR-based TMP/UV mutagenesis. The *cnp-3(jh145)* allele bears an 879 bp internal deletion that removes a part of the second exon, all the second intron and a part of the third exon (See Fig. 4A). The *cnp-3(jh145)* allele is expected to generate a mutant protein lacking the amino acids corresponding to the deleted region plus additional downstream amino acids due to frame shifting and premature termination. Hence we believe that *cnp-3(jh145)* is a strong loss-of-function mutant. *cnp-3(jh145)* homozygotes are viable, and do not have any obvious phenotype. This could be because *cnp-3* affects fitness in soil - its native environment - but is not important in the pampered laboratory environment. Alternatively, it may be genetically redundant with some other gene(s). Indeed, the *C. elegans* genome contains a gene K09E3.7 encoding a protein that is 52% identical and 66% similar to CNP-3, raising the possibility that K09E3.7 and CNP-3 perform a common func-

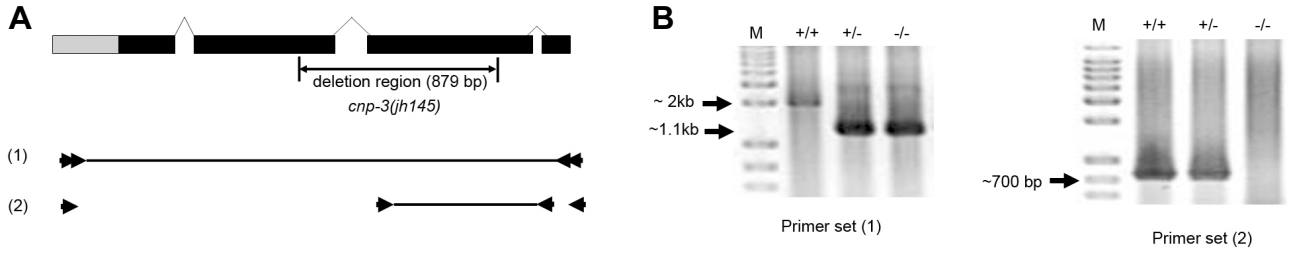

Fig. 4. Isolation of a *cnp-3(jh145)* deletion mutant. **A.** Genomic organization of *cnp-3* and location of the target regions of the primers designed to isolate and characterize the *cnp-3(jh145)* allele are shown. *cnp-3* is composed of four exons (boxes) and three introns (thin lines). A region comprising a part of the second exon, all the second intron and a part of the third exon, is deleted in *cnp-1(jh145)* (indicated by double headed arrow between vertical bars). The two primer sets used for analyzing the *cnp-3* locus are indicated as (1) and (2). **B.** Representative gel image of the DNA amplified from single worms of the indicated genotypes. Using primer set (1), the wild-type (+/+) yielded a ~2kb band while the *cnp-3(jh145)* heterozygote (+/–) and homozygote (–/–) yielded a ~1.1 kb band, confirming the internal deletion. For checking, primer set (2) was used to check homozygosity; the wild-type and *cnp-3(jh145)* heterozygote yield a ~700 bp band while the *cnp-3(jh145)* homozygote yields no band.

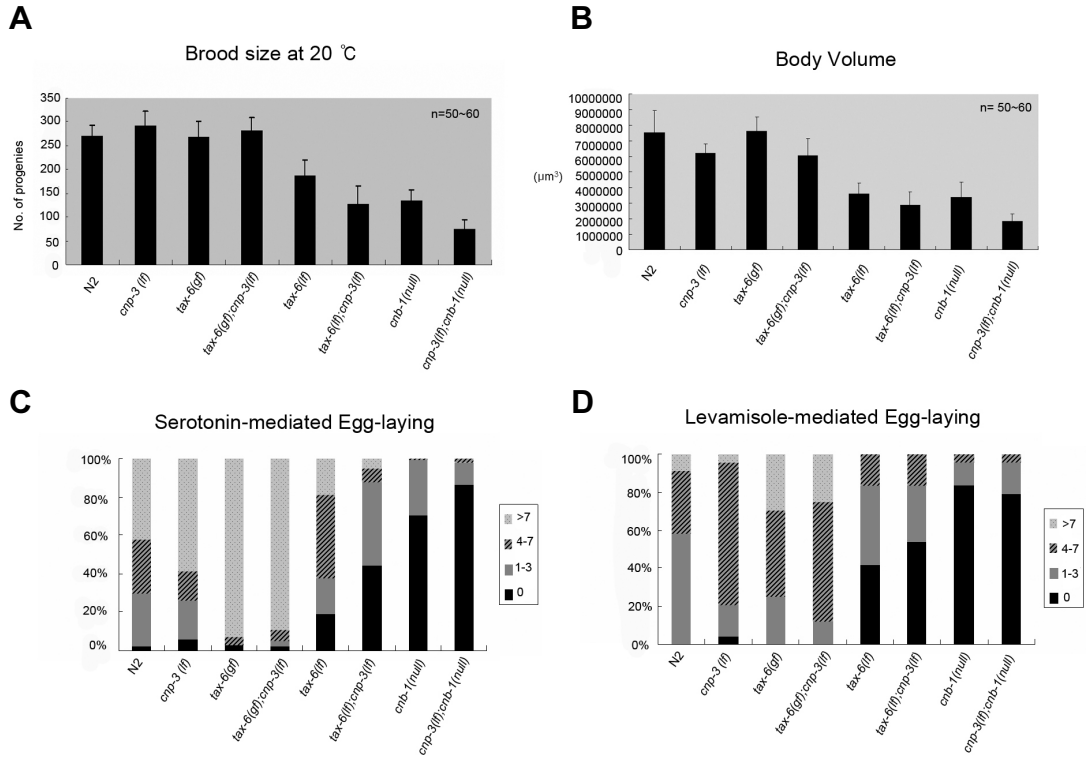

Fig. 5. *cnp-3(jh145)* enhances calcineurin loss-of-function phenotypes. **A.** Brood size of wild type worms (N2) and the indicated mutants (n = 50 to 60). **B.** Body volume of wild type and mutants (n = 50 to 60). The body length and width of one-day adult worms was measured and body volume was calculated from the formula, $v = \pi r^2 l$, where r = body width and l = body length. **C.** Serotonin-mediated egg-laying behavior of wild type and mutants (n = 50 to 60). The percentage of worms laying the indicated ranges of egg numbers in response to 12.5 mM serotonin are shown in the form of a bar diagram. **D.** Levamisole-mediated egg-laying behavior of wild type and mutants (n = 50 to 60). The percentage of worms laying the indicated ranges of egg numbers in response to 12.5 mM levamisole are shown in the form of a bar diagram.

tion. We are currently trying to isolate a double mutant to test this interesting possibility.

***cnp-3(jh145)* enhances calcineurin loss-of-function phenotypes** Since our intention was to understand the relationship between calcineurin and CNP-3, we examined the *cnp-3(jh145)* homozygotes to see if they were altered in any of the processes affected in calcineurin mutants, such

as brood size, body volume, serotonin-mediated egg-laying and levamisole-mediated egg-laying. As shown in Fig. 5A, the brood size of *cnp-3(jh145)* mutants is indistinguishable from that of the wild type. However, the brood sizes of the double mutants *tax-6(lf);cnp-3(jh145)* and *cnb-1(null);cnp-3(jh145)* mutants are significantly lower than those of the *tax-6(lf)* and *cnb-1(null)* single mutants, respectively. This implies that *cnp-3(jh145)* enhances calcineurin loss-of-function phenotypes. The data in Fig. 5B indicate that *cnp-3(jh145)* also enhances the effect of calcineurin loss-of-function on body volume. We also characterized the serotonin-mediated egg-laying phenotype of calcineurin and *cnp-3* single and double mutants. As we have reported previously (Lee et al., 2004) and show here (Fig. 5C), calcineurin positively regulates the expulsion of eggs from the uterus in response to extraneous serotonin. The *tax-6(lf)* and *cnb-1(null)* mutants lay smaller number of eggs in response to serotonin than wild type worms and thus exhibit a serotonin-resistant phenotype. Interestingly, the *tax-6(lf);cnp-3(jh145)* and *cnb-1(null);cnp-3(jh145)* double mutants are hyper-resistant to serotonin, i.e. they enhance the phenotype of *cnp-3(jh145)* with respect to serotonin-mediated egg-laying.

Finally, we examined levamisole-mediated egg-laying phenotype of the calcineurin and *cnp-3* mutants. Interestingly, *cnp-3(jh145)* has little or no enhancing effect on the phenotype of the calcineurin mutants with respect to this behavior (Fig. 5D). We conclude that *cnp-3(jh145)* modifiers a specific subset of calcineurin loss-of-function phenotypes.

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