

Published in final edited form as:

J Mol Biol. 2011 March 25; 407(2): 222–231. doi:10.1016/j.jmb.2011.01.039.

PKN-1, a homologue of mammalian PKN, is involved in the regulation of muscle contraction and force transmission in *C. elegans*

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Abstract

To examine the *in vivo* functions of protein kinase N (PKN), one of the effectors of Rho small GTPases, we used the nematode *Caenorhabditis elegans* as a genetic model system. We identified a *C. elegans* homologue (*pkn-1*) of mammalian PKN and confirmed direct binding to *C. elegans* Rho small GTPases. Using a GFP reporter, we showed that *pkn-1* is mainly expressed in various muscles and is localized at dense bodies and M-lines. Over-expression of the PKN-1 kinase domain and loss-of-function mutations by genomic deletion of *pkn-1* resulted in a loopy Unc phenotype, which has been reported in many mutants of neuronal genes. The results of mosaic analysis and body wall muscle specific expression of PKN-1 kinase domain suggests that this loopy phenotype is due to the expression of PKN-1 in body wall muscle. The genomic deletion of *pkn-1* also showed a defect in force transmission. These results suggest that PKN-1 functions as a regulator of muscle contraction-relaxation and as a component of the force transmission mechanism.

Keywords

C. elegans; muscle; Rho GTPase; PKN; contraction

Introduction

The small GTPases have two convertible forms; GDP bound inactivated and GTP bound activated¹. The two states are regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). The GEFs have been shown to accelerate release of

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GDP from GTPases, resulting in binding to GTP in the cell, thus activating the GTPase². Rho family small GTPases include Rho, Cdc42 and Rac. Each of these GTPases, in their GTP bound forms, preferentially interact with specific effector proteins. So far, many effectors of Rho GTPases have been identified³. Among them, protein kinase N (PKN) was the first identified effector of Rho GTPase⁴. PKN was identified as novel protein kinase containing a protein kinase domain similar to protein kinase C (PKC)⁵. It has been reported that PKN is involved in many physiological functions, such as cytoskeletal regulation, cell adhesion, vesicle transport, apoptosis, and cell cycle regulation⁶. In *Drosophila*, a PKN homologue has been reported to be involved in a signaling cascade that regulates dorsal closure and development^{7, 8}. Nevertheless, the mechanisms by which PKN participates in these various cellular functions are not well understood. We used *C. elegans* as a genetic model for investigation of in vivo functions of PKN.

Biochemical characterization of a *C. elegans* PKN ortholog, PKN-1

We identified F46F6.2 as the single *C. elegans* ortholog of mammalian Protein Kinase N (PKN) by an homology search using the WormBase database (<http://www.wormbase.org/>). WormBase predicts two isoforms, F46F6.2a and b, by alternative splicing. We confirmed the splicing pattern of F46F6.2a by sequencing of EST cDNA clones (Figure 1A). We named the gene *pkn-1* (*C. elegans* protein kinase N). The *C. elegans* PKN-1 protein shares structural features with mammalian PKN; three Hr1 repeats at the N-terminus, a C2-like domain in the middle, and a serine/threonine kinase domain at the C-terminus (Figure 1B). The human and nematode PKN proteins are 38% identical in overall protein sequence and 71% identical within the protein kinase domains. Since mammalian PKN is one of the effectors of Rho GTPase, we examined whether PKN-1 could bind to *C. elegans* Rho GTPases in vitro. We prepared GST fusions of human RhoA and *C. elegans* Rho type GTPases (RHO-1 as RhoA, CED-10 and MIG-2 as Rac, and CDC-42 as cdc42) with GTP γ S (a non-hydrolyzable form of GTP) and GDP, and an MBP fusion of PKN-1 N-terminus (residues 1-291) containing the three Hr1 repeats, a region which has been reported to be essential for the interaction of mammalian PKN with RhoA⁴. By using those GST fusion proteins (Figure 1D), MBP-PKN-1 N-terminus showed binding to GST-RHO-1 and GST-RhoA, and weaker binding to GST-CED-10 and GST-MIG-2, but no binding to GST-CDC-42 (Figure 1C). Binding was much higher with GTP γ S than with GDP. These results suggest that *C. elegans* PKN-1 is also one of the effectors of RhoA type GTPases. It has been reported that PKN can bind to Rac type GTPases in mammalian cells⁹ and *Drosophila*⁸. However, in *C. elegans*, *pkn-1* is expressed mainly in muscle (see below) and *ced-10* and *mig-2* are not expressed in muscle based on anatomical expression data. Thus, we suggest that PKN-1 is only regulated by RHO-1 GTPase in muscle cells.

Expression pattern of the *pkn-1* gene and localization of the PKN-1 protein

To examine the expression pattern of *pkn-1*, we used GFP fused to the *pkn-1* promoter region (Figure 1A). GFP fusion containing 5 Kb upstream and first and second exons of *pkn-1* showed expression in various muscle cells; pharyngeal muscle, body wall muscle, vulval muscle (Figure 2A), and some other tissues including neurons (data not shown). To characterize the localization of PKN-1 in body wall muscle, we expressed a GFP fusion of full-length *pkn-1* cDNA under the control of a heat shock promoter and immunostained with anti-GFP and antibodies to proteins of known sarcomeric location. GFP::PKN-1 co-localized with α -actinin¹⁰ and UNC-89¹¹, indicating localization at dense bodies (arrowheads in Figure 2B) and M-lines (arrows in Figure 2B). In the major striated muscle of *C. elegans*, residing in the body wall, thick filaments are organized around M-lines, and thin filaments are attached at dense bodies, equivalent to Z-disks (lines) in vertebrate striated muscle. Many proteins are localized to both M-lines and dense bodies (e.g. PAT-3 (β -

integrin), UNC-112 (Kindlin) etc.), whereas others are localized only to M-lines (e.g. UNC-89 (obscurin), UNC-98, UNC-96), and others are localized only to dense bodies (e.g. DEB-1 (vinculin), ATN-1 (α -actinin), ALP-1 (ALP/Enigma) etc.)^{12, 13}. Since GFP fusion of the first and second exons of *pkn-1* also localized to dense bodies and M-lines in body wall muscle (Figure 2A, bottom left), the N-terminal 138 residues of PKN-1a might be sufficient for its localization to dense bodies and M-lines. Muscle expression of *pkn-1* is supported by SAGE data available on WormBase (<http://www.wormbase.org/>). SAGE data showed that the *pkn-1* also expresses in neurons, hypodermis, pharynx, gonad, and gut cells. It has been reported that *rho-1* and *unc-89* (encoding a giant protein that includes a RhoA GEF domain, and is localized to M-lines), function in muscle in the regulation of sarcomere organization¹⁴. Therefore, it is possible that at sarcomeric M-lines, the RHO-1 / PKN-1 interaction is a part of this UNC-89 / RHO-1 pathway in *C. elegans* striated muscle. A major component of nematode muscle dense bodies is α -actinin^{15, 16}. Therefore, the observed dense body localization of PKN-1 is consistent with the known interaction of vertebrate PKN with α -actinin¹⁷. Whether nematode PKN-1 interacts with α -actinin will require additional experiments.

Activation and inactivation of *pkn-1* in *C. elegans*

We examined sarcomeric organization in an intragenic deletion of *pkn-1* (*pkn-1* (*ok1673*)) using a battery of antibodies to sarcomeric proteins. The *ok1673* is a 1064 bp deletion and 18 bp insertion beginning in the 4th exon and ending in the 7th exon (Figure 1A), resulting in a premature stop inside of C2-like domain. Thus, if the truncated protein from *ok1673* were stable, it would lack the kinase domain. Compared to wild type, we observed no defects in the organization of thin filaments, thick filaments, and in both the deep and surface regions of muscle attachment structures (dense bodies and M-lines) (Figure 3).

Next, we examined the phenotype of overexpressing of PKN-1 kinase domain as a type of gain of function mutation for *pkn-1*. We prepared transgenic animals harboring PKN-1 kinase domain expressed by the control of a heat shock promoter (hsp). After heat shock, animals with hsp-PKN-1 kinase showed locomotion with more exaggerated bending (“loopy”)(Figure 4A). Heat shock itself had no effect on animal movement (Figure 4A). To characterize this loopy Unc phenotype quantitatively, we counted swimming or thrashing (beats per minute) and omega turn frequency (omega turn per minute) in M9 liquid. An omega turn is a deep ventral bend in the animal that is shaped like the Greek letter omega. It usually is at the end of a large reversal (defined as more than two heads wings backwards) and the animal changes direction of locomotion when it completes an omega turn¹⁸. With heat shock, both wild type and PKN-1 kinase overexpressed worms showed a slight reduction in motility (Figure 4B). Most significantly, we observed that omega turn frequency increased after heat shock of worms harboring hsp-PKN-1 kinase domain (Figure 4B). Query of WormBase database indicates that there are many mutants that have a “loopy” phenotype: *baf-1* (encoding novel protein), *eat-16* (encoding RGS protein, regulator of heterotrimeric G protein), *egl-8* (encoding phospholipase C beta), *flp-1* (encoding FMRFamide-related peptides), *gpb-2* (encoding beta subunit of heterotrimeric G protein), *gsa-1* (encoding alpha subunit of heterotrimeric G protein), *kin-2* (encoding cAMP-dependent protein kinase), *rab-3* (encoding rab GTPase), *sync-3* (not yet identified), *unc-43* (encoding Ca²⁺-Calmodulin dependent protein kinase), *unc-77* (not yet identified), and *unc-117* (not yet identified). Since almost all of these mutants are reported to have defects in neuronal functions (for example, described previously¹⁹) and some *pkn-1* is expressed in neurons, we conducted two types of experiments to determine whether expression of *pkn-1* in muscle or neurons is required for display of the loopy Unc phenotype. The first

experiment was mosaic analysis using a transgenic animal harboring an extrachromosomal array containing hsp-PKN-1 kinase and *sur-5::nls::gfp*²⁰. In *C. elegans*, as development proceeds, at a low frequency, extrachromosomal arrays are randomly lost when cells divide, resulting in mosaic expression of each gene on the array²¹. In our experiment, the maintenance of the extrachromosomal array was scored by the existence of *sur-5::nls::gfp* in each tissue's nuclei. After heat shock of transgenic animals, we picked 42 “loopy” worms and 6 non-“loopy” worms and determined whether *sur-5::nls::gfp* was present in neurons or muscle cells. In all 42 “loopy” animals, *sur-5::nls::gfp* was maintained in muscle cells, but was missing in some neurons (such as those in the neural ring). In all 6 non-“loopy” worms, *sur-5::nls::gfp* was missing in muscle, but present in neuronal cells. Therefore, the loopy phenotype is exactly correlated with overexpression PKN-1 in muscle. Second, we prepared transgenic worms harboring the PKN-1 kinase domain driven by a muscle specific promoter, from the gene *myo-3*, and examined worm locomotion. When the PKN-1 kinase domain was expressed by the *myo-3* promoter (strain NR150), transgenic worms showed a loopy phenotype with reduced motility and increased frequency of omega turn (Figure 4B). When PKN-1 kinase domain was overexpressed by *myo-3*, muscle structure was normal, as assessed by polarized light microscopy (data not shown). Taken together, we conclude that overexpression of the PKN-1 kinase domain in the muscle cells causes the loopy Unc phenotype. Although intragenic deletion of *pkn-1*, *ok1673*, showed normal muscle structure (Figure 3), *ok1673* (strain VC1414) also displayed a weak loopy Unc phenotype, including reduced motility and increased frequency of omega turns (Figure 4B). This weak loopy phenotype was also observed in worms subjected to RNAi mediated knockdown of *pkn-1* (data not shown).

In addition to characterizing worm behavior in liquid, we characterized worm crawling on NGM agar plates using a recently established video recording and analysis system. We characterized precise movement of wild type (N2), *pkn-1* genomic deletion mutant (VC1414), and constitutively over-expressed PKN-1 kinase domain in body wall muscle worm (NR150). We found that the maximum bending amplitude was reduced in the *pkn-1* deletion mutant, as compared to wild type or worms overexpressing PKN-1 kinase in muscle (Figure 4C), similar to loss of function of *atn-1* (α -actinin)²². This reduction in maximal bending suggests a role for PKN-1 in the transmission of force, and is consistent with the fact that PKN-1 co-localizes with ATN-1 (α -actinin) at dense bodies (Figure 2B). We also found that the average bending angle of worms overexpressing PKN-1 kinase in muscle was reduced as compared to wild type and genomic deletion (Figure 4C). This defect might explain the slow motility of this strain (Figure 4B). Theoretically the “loopy” phenotype of *pkn-1* mutants might result from worms making more deeper turning angles or changing direction more frequently. The later possibility is more likely since: (1) *pkn-1* mutants display normal or reduced maximum bending and average bending angles, and (2) *pkn-1* mutants display more frequent omega turns.

We found that both gain of function and loss of function of *pkn-1* in muscle cells results in a loopy phenotype. The loopy phenotype that has been reported for loss of function for many neuronal genes has been explained as too much contraction caused by de-regulated neurotransmitter release¹⁹. In the case of *pkn-1*, we propose two hypotheses to explain this loopy phenotype. First, the PKN-1 kinase could phosphorylate and modify the function of regulators of muscle contraction-relaxation, resulting in too much bending of the worm and thus a loopy Unc phenotype. Such regulators, phosphorylated by PKN-1, are likely to be located at sarcomeric dense bodies and M-lines, the sites of PKN-1 localization. In mammalian cells, it has been reported that PKN can phosphorylate a regulatory subunit of myosin phosphatase and that this phosphorylation results in a decrease in the activity of myosin phosphatase²³. Reduced myosin phosphatase activity could result in increased

phosphorylation of regulatory myosin light chains, and thus increased myosin activity and contraction. This is plausible for nematode body wall muscle which has both thin filament and myosin based calcium regulation²⁴. Second, PKN-1 could be involved in retrograde signaling from muscle to neurons to enhance the release of neurotransmitter. It has been reported that muscle expression *aex-1*, a gene involved in neuronal function, is essential for its neuronal function²⁵.

That both loss of function and gain of function mutations in *pkn-1* result in motility defects suggests that a tightly controlled level of PKN-1 activity is required for proper regulation of muscle activity. One explanation for sharing the same phenotype for both gain-of-function and loss-of-function mutations, is some defect in the cycling of two interconvertible states. For example, mutations in small GTPase activators (GEFs) and inactivators (GAPs) show the same phenotype^{26, 27}. In the muscle cells, it is possible that the defect in contraction-relaxation cycle might be caused by unbalanced contraction or relaxation, resulting in a loopy phenotype. Thus, we prefer the first hypothesis. Further examination of the mechanism of loopy phenotype caused by mutation of *pkn-1* through the identification of genetic interactors and PKN-1 substrates may provide insight into the role of PKN-1 in regulating muscle contraction-relaxation.

Acknowledgments

We thank Dr. Alan Coulson (Wellcome Trust Genome Campus) for cosmids clones; Dr. Yuji Kohara (National Institute of Genetics) for EST cDNA clones; Andy Fire (Stanford University School of Medicine) for GFP and ectopic expression vectors; Dr. Min Han (University of Colorado) for pTG96 plasmid; Dr. Robert Barstead (Oklahoma Medical Research Foundation) for *C. elegans* cDNA library RB2; Dr. Kristy J. Wilson (Emory University) for making the worm tracings for Fig. 4A; and Dr. Shinya Kuroda (University of Tokyo) for helpful discussion. We also thank Dr. Ken Norman (Albany Medical College) for help with initial characterization of confocal microscopic images, and Dr. Carlos Moreno (Emory University) for advice on statistical analysis. Some of the strains used in this work were provided by the *Caenorhabditis elegans* Genetics Center, which is funded by the National Institutes of Health (NIH) Center for Research Resources. HQ, MA, and KK were supported by grants-in-aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan, by the Japan Society of the Promotion of Science Research for the Future, by the Human Frontier Science Program. GMB was supported by NIAMS / NIH grant AR051466. HL is supported by NIH (NIBIB-R21EB012803, NIA-R01AG035317), NSF (CBET/CAREER-0954578), and the Alfred P. Sloan Foundation.

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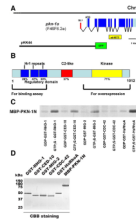


Figure 1. Direct binding between PKN-1 and Rho type small GTPases

A: Genomic structure of *pkn-1*. The exon-intron structure of *pkn-1a* (F46F6.2a) is depicted, below which is shown the location of the intragenic deletion *ok1673*, and the location of the region used for the promoter-GFP fusion construct (pKK44) expressed in transgenic worms (Figure 2A). The red box denotes the trans-spliced leader RNA SL1, the grey boxes denote the 5' and 3' UTR sequences, and the blue boxes denote exons.

B: Schematic showing organization of domains within PKN-1 (F46F6.2a). PKN-1 has three Hr1 repeats, a C2-like domain, and a serine/threonine kinase domain, similar to the human PKN protein in the types, numbers and organization of domains. Percentages under each domain denote the identity between the *C. elegans* and human PKN proteins. Lower brackets indicate regions used for binding assay (Figure 1C) and for overexpression (Figure 4).

C: Regulatory region of PKN-1 can bind to *C. elegans* Rho-type GTPases. Purified MBP-PKN-1 N-terminus (residues 1-295) was mixed with glutathione beads coated with GST fusions of Rho-type GTPases (GST-RHO-1, GST-CED-10, GST-MIG-2, GST-CDC-42, and GST-RhoA (human)) loaded with GTP γ S or GDP. After extensive washes, binding proteins were eluted with glutathione, subjected to SDS-PAGE separation, and visualized by Coomassie Blue staining⁴. The MBP-PKN-1 N-terminus binds to GTP forms of GST-RHO-1 and GST-RhoA, and GTP forms of GST-CED-10 and GST-MIG-2 more weakly. All proteins were expressed in *E. coli* and purified using glutathione agarose (for GST fusions) or amylose resin (for MBP fusion). Expression plasmids were constructed by cloning of corresponding cDNA fragments amplified by PCR from a cDNA library (RB2) into pGEX-4T-1 (GE Healthcare) or pMAL-c2 (New England Biolab) bacterial expression vectors²⁸.

D: Coomassie Blue staining of an SDS-PAGE containing the GST-RHO-1, GST-CED-10, GST-MIG-2, GST-CDC-42, GST-RhoA (Hs), and MBP-PKN-1 N-terminus used in this assay. 2 μ g proteins were loaded on 10% SDS-PAGE gel, and stained with Coomassie Brilliant Blue.

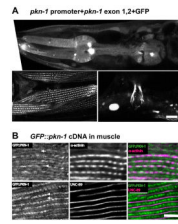


Figure 2. The expression and localization of PKN-1 in muscle

A: GFP expressed by the *pkn-1* promoter was observed in many types of muscle. We injected a mixture of pKK44 plasmid (5 Kb of upstream putative promoter, the first and second exons, and the first intron of *pkn-1* was cloned into pPD95.75, a gift from Dr. Fire) and a *lin-15* rescuing plasmid into *lin-15* (*n765*) (temperature sensitive multinulva (Muv)) worms. To obtain transgenic animals, we picked non-Muv worms at 25°C. In transgenic animals, GFP driven by *pkn-1* promoter was observed in pharynx including pharyngeal muscle (upper panel), body wall muscle (lower left panel), and vulva muscle (lower right panel). Scale bar, 10 μ m.

B: GFP tagged PKN-1 is localized at dense bodies (arrowheads) and M-lines (arrows). We prepared transgenic worms harboring an extrachromosomal array containing both a plasmid expressing GFP tagged full-length *pkn-1* cDNA under the control of heat shock promoter, and a plasmid for the *rol-6* (roller) dominant marker. Worms were fixed by the Nonet method²⁹. Antibody staining with anti-GFP (Invitrogen A11122, 1/200 dilution), and MH35 (1/200 dilution; α -actinin) or MH42 (1/200 dilution; UNC-89) was performed as described previously²⁸. All images were captured at room temperature with a Zeiss confocal system (LSM510) equipped with an Axiovert 100M microscope using an Apochromat 63x/1.4 oil objective, in 2.5x zoom mode. The color balances of the images were adjusted by using Adobe Photoshop. Scale bar, 10 μ m.

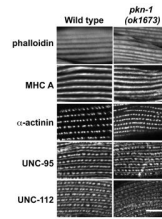


Figure 3. Muscle structure in a *pkn-1* loss of function mutant

pkn-1 intragenic deletion mutant (*pkn-1 (ok1673)*) and wild type worms were stained with phalloidin (actin thin filaments), anti-MHC A (5–6, myosin thick filaments and deep part of M-lines³⁰), anti- α -actinin (MH35, deep part of dense bodies¹⁰), anti-UNC-95 (a LIM domain protein³¹ located at the membrane proximal portion of dense bodies and M-lines²⁸), and anti-UNC-112 (mammalian Kindlin ortholog³² located at the membrane proximal portion of dense bodies and M-lines³³). As shown, the organization of all these sarcomeric components is normal. Worms were fixed by the Nonet method²⁹ and immunostaining was performed as described previously²⁸. All images were captured at room temperature with a Zeiss confocal system (LSM510) equipped with an Axiovert 100M microscope using an Apochromat 63x/1.4 oil objective, in 2.5x zoom mode. The color balances of the images were adjusted by using Adobe Photoshop. Scale bar, 10 μ m.

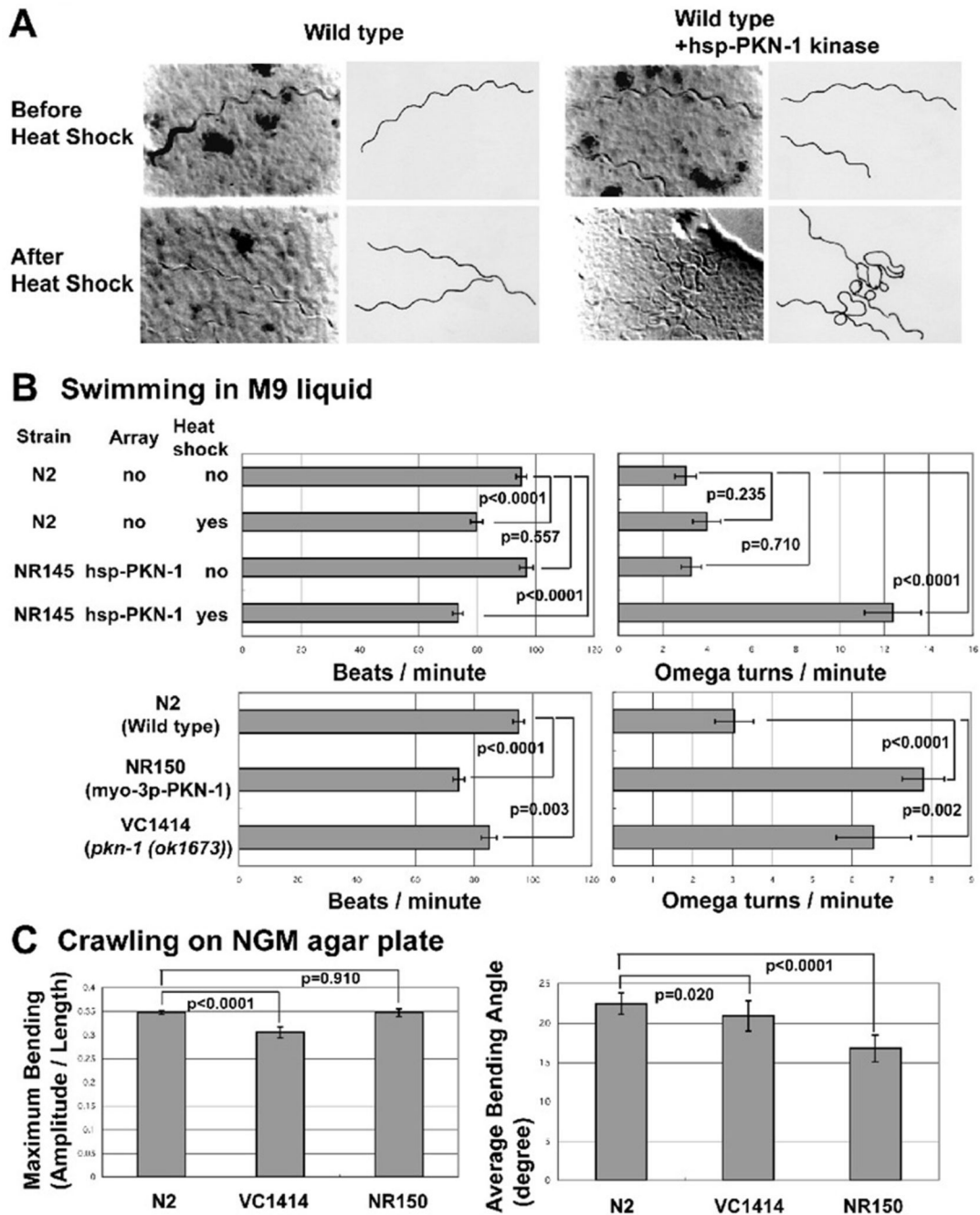


Figure 4. Either gain of function or loss of function of *pkn-1* results in a “loopy Unc phenotype”

A: Images (left) together with tracings (right) of worm tracks made along a bacterial lawn, from wild type, or wild type with hsp (heat shock promoter)-PKN-1 kinase before or after heat shock (30°C for 2 hrs). Without heat shock, wild type and wild type with hsp-PKN-1 kinase domain worms showed normal movement. After heat shock, only wild type with hsp-PKN-1 kinase domain worms showed more loopy tracks on the bacterial lawn surface.

Transgenic worms harboring an extrachromosomal array consisting of hsp-PKN-1 kinase domain and *sur-5::nls::gfp* was constructed by injection³⁴ of DNA mixture of pPD49.78-PKN-1 kinase, pPD49.83-PKN-1 kinase, and pTG96 (*sur-5::nls::gfp* marker plasmid) into N2 wild type, and selecting progeny showing GFP expression. The extrachromosomal array

was integrated by UV irradiation³⁵. To construct pPD49.78-PKN-1 kinase and pPD49.83-PKN-1 kinase, PCR amplified *pkn-1* cDNA containing the kinase domain (see Figure 1A) was cloned into pPD49.78 or pPD49.83.

B: Bar graphs showing the results of two assays of adult worm swimming in M9 liquid: beats per minute (left) and omega turns per minute (right), each with means and standard errors (SEs) (n=20). Pair-wise comparisons for statistical significance are indicated with brackets and p values. These comparisons were made with one-way ANOVA using the post hoc Tukey HSD test, on StatPlus (AnalystSoft, Inc.). NR145 (harboring hsp-PKN-1 kinase) with heat shock, NR150 (harboring myo-3p-PKN-1 kinase), VC1414 (*pkn-1* genomic deletion) show increased omega turn frequency. NR150 and VC1414 show reduced beats per minute. NR150 is an integrated line of the myo-3p-PKN-1 kinase array. The *myo-3* promoter is reported to be expressed only in muscle³⁶. To construct the plasmid expressing the PKN-1 kinase domain under the control of the *myo-3* promoter (pPD95.86-PKN-1 kinase), a PCR amplified cDNA fragment for *pkn-1* kinase domain was cloned into pPD95.86. Transgenic worms harboring extrachromosomal arrays were obtained by injection³⁴ of myo-3p-PKN-1 kinase plasmid with pTG96 (*sur-5::nls::gfp*) into wild type worms. The extrachromosomal array was integrated by UV irradiation³⁵. We obtained VC1414 from Caenorhabditis Genetics Center.

C: Bar graphs showing the maximum bending amplitude during an induced reversal (left) and the average bending angle during normal forward locomotion (right) of N2 (wild type), VC1414 (*pkn-1* genomic deletion), and NR150 (integrated myo-3p-PKN-1 kinase), each with means and SEs. For maximum bending amplitude, n=20 except for N2 (n=65). For average bending angle, n=15 for each strain. Pair-wise comparisons for statistical significance are indicated with brackets and p values. These comparisons were made with one-way ANOVA using the post hoc Bonferroni test for the graph on the left (Maximum Bending), the post hoc Tukey HSD test for the graph on the right (Average Bending Angle), on StatPlus (AnalystSoft, Inc.). Each assay was performed on a blank NGM agar plate without bacteria. The average bending angle is the average of the absolute values of the angles of the worm when it is divided into 12 equal segments (13 points). The angle is between consecutive segments and relative to 180 degrees. The deeper the bend, the higher the angle. The numbers shown are averages over a 6–15 second interval when the animals were moving forward. VC1414 shows a reduction of maximum bending ability. NR150 shows a reduction of average bending angle. The methods for these measurements were described previously^{22, 37, 38}.