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An NAD⁺ biosynthetic pathway enzyme functions cell non-autonomously in *C. elegans* development

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Abstract

Background—Disruption of cellular metabolite levels can adversely impact development. Specifically, loss-of-function of the *C. elegans* NAD⁺ salvage biosynthesis gene PNC-1 results in an array of developmental phenotypes. Intriguingly, PNC-1 and its functional equivalent in vertebrates are secreted, but the contributions of the extracellular enzymes are poorly understood. We sought to study the tissue-specific requirements for PNC-1 expression and to examine the role of the secreted isoform.

Results—A thorough analysis of PNC-1 expression did not detect expression in tissues that require PNC-1 function. Limited expression of both the secreted and intracellular PNC-1 isoforms provided function at a distance from the tissues with phenotypes. We also find that the secreted isoform contributes to *in vivo* PNC-1 activity. Furthermore, *uv1* cell survival has the most stringent requirements in terms of PNC-1 expression pattern or level.

Conclusion—Using careful promoter analysis and a restricted expression approach we have shown that both the secreted and the intracellular PNC-1 isoforms function cell non-autonomously, and that the PNC-1a isoform is functionally relevant *in vivo*. Our work suggests a model where PNC-1 function is provided cell non-autonomously by a mix of intra and extracellular activity, most likely requiring NAD⁺ salvage metabolite transport between tissues.

Keywords

NAD⁺ salvage biosynthesis; Nampt; Pnc1; nicotinic acid; vitamin B3; nicotinamide

Introduction

Nicotinamide adenine dinucleotide (NAD⁺) is central to metabolism in all cells, both as a co-factor in redox reactions, including oxidative phosphorylation and glycolysis, and as a substrate of NAD⁺ consumer enzymes. Differentiation of muscle and immune cells in mammals is disrupted when NAD⁺ biosynthesis is perturbed (Fulco et al., 2008; Skokowa et al., 2009; Yin et al., 2012), highlighting the importance of NAD⁺ and its biosynthesis in development. Similarly, we have demonstrated that perturbing NAD⁺ biosynthesis results in very specific effects during development of the invertebrate *C. elegans*, establishing *C.*

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C. elegans as an important model for studying NAD⁺ biosynthesis and metabolism during development (Vrablik et al., 2009; Vrablik et al., 2011).

In its role as a cofactor, NAD⁺ is interconverted between its oxidized and reduced forms but these reactions do not affect the total concentration of NAD⁺ available to the cell. However, NAD⁺ consumers hydrolyze NAD⁺, creating a need to replenish the key molecule via biosynthesis (Landry et al., 2000; Tanny and Moazed, 2001; Kim et al., 2004). NAD⁺ may be resynthesized from nicotinamide (NAM), a byproduct of NAD⁺ consumers, in a process called salvage biosynthesis (Rongvaux et al., 2003). It can also be synthesized from dietary nicotinic acid (NA) via the Preiss-Handler pathway (Preiss and Handler, 1958a; Preiss and Handler, 1958b), from dietary nicotinamide riboside (NR) (Bieganski and Brenner, 2004), or synthesized *de novo* from tryptophan (Gazzaniga et al., 2009). Two types of pathways carry out salvage biosynthesis. Vertebrates use nicotinamide phosphoribosyltransferase (Nampt) to convert nicotinamide into nicotinamide mononucleotide (NMN), which is then converted into NAD⁺ by NMN adenylyltransferase (Nmnat). Invertebrates convert nicotinamide (NAM) into nicotinic acid (NA) using a nicotinamidase, which is then fed into the Preiss-Handler pathway (Magni et al., 1999; Rongvaux et al., 2003). Although Nampt and nicotinamidases are enzymatically distinct, both catalyze the conversion of NAM to the next metabolite in their respective NAD⁺ salvage biosynthesis pathways and both would be expected to modulate NAM levels, making them biologically functionally analogous.

C. elegans uses a nicotinamidase to catalyze the first step of salvage NAD⁺ biosynthesis (van der Horst et al., 2007; Vrablik et al., 2009; French et al., 2010). The genomic locus of the *C. elegans* nicotinamidase gene *pnc-1* is complex, encoding two isoforms that encode proteins which differ only in regards to the presence of a cleavable signal peptide, thus creating a secreted PNC-1a isoform and an identical intracellular PNC-1b isoform. The PNC-1a isoform is expressed from a distinct promoter (Vrablik et al., 2009), and we show in this study that two promoters appear to direct expression of PNC-1b (Fig. 1). We sought to understand the tissue specific functions of PNC-1 and the contributions of the secreted and intracellular isoforms to development. Given the role of salvage biosynthesis in the maintenance of NAD⁺ levels and its importance in general metabolism, it came as a surprise that neither expression of Nampt in mice nor nicotinamidases in *Drosophila* and *C. elegans* is ubiquitous (Chintapalli et al., 2007; Revollo et al., 2007b; Vrablik et al., 2009). Like *C. elegans*, which encodes a secreted PNC-1 isoform, vertebrates express an intracellular (iNampt) and extracellular (eNampt) (Yonezawa et al., 2006; Revollo et al., 2007a; Ocón-Grove et al., 2010). NAD⁺ biosynthetic capacity might be provided to tissues that lack Nampt or nicotinamidase expression by cell-autonomous expression of enzymes in other NAD⁺ biosynthetic pathways. Alternatively, exchange of metabolites such as NAM, NMN and NA between tissues could contribute to NAM clearance and NAD⁺ biosynthesis in tissues that don't express the key salvage enzyme. Alternatively, there is evidence that extracellular conversion of substrate to product and transport of product to tissues in need could play a role (Revollo et al., 2007b; Vrablik et al., 2009; Vrablik et al., 2011). Although Nampt is critical for NAD⁺ salvage (Rongvaux et al., 2002; van der Veer et al., 2005), the specific role of eNampt is contentious and the need for extracellular activity of eNampt has been controversial (Revollo et al., 2007b; Hara et al., 2011).

PNC-1 has multiple functions in promoting proper development of specific tissues in *C. elegans*. For example, adequate PNC-1-mediated NAD⁺ biosynthesis is required to prevent a delay in development of the gonad with respect to the soma (Vrablik et al., 2009). The body wall muscles also require PNC-1 activity during development for optimal function during adulthood. In this tissue, PNC-1 activity is required both to promote biosynthesis of NAD⁺ and to prevent accumulation of NAM (Vrablik et al., 2011). In addition, PNC-1 promotes egg-laying and survival of uv1 (uterine-vulval) cells located within the gonad by preventing accumulation of NAM that is toxic to the egg-laying muscles and the uv1 cells (Vrablik et al., 2009; Vrablik et al., 2011). In our preliminary expression analysis we were surprised to find no PNC-1 expression in the tissues that manifest *pnc-1* phenotypes (Vrablik et al., 2009). Given this disconnect between sites of PNC-1 expression and functional requirement, the presence of a secreted PNC-1a isoform in *C. elegans* and also the controversy over the role of the secreted eNampt in vertebrate metabolism, we addressed the following questions: Can specific PNC-1 developmental functions be attributed to specific expression sites? Does PNC-1 function cell non-autonomously? And is the secreted PNC-1a isoform necessary and / or sufficient for any specific PNC-1 function?

Results

Is PNC-1 expressed in tissues that exhibit phenotypes?

We previously examined expression of the *pnc-1* isoform-specific promoters 1a and 1b.1 and were surprised to find no expression in the body-wall muscle and limited expression in the gonad, two tissues where the global absence of PNC-1 function has developmental consequences (Vrablik et al., 2009). This suggests that *pnc-1* may have cell non-autonomous functions. We first aimed to ensure that we are not missing potential regulatory elements that direct *pnc-1* expression in the tissues that display phenotypes in the null mutant by constructing several additional GFP reporter genes. We included the native 3' regulatory region in new translational reporters using the 1a and 1b promoters. We also made transcriptional and translational reporters to test for activity of a predicted promoter upstream of exon 2, in the region between exon 1b and exon 2 (Fig. 1), a promoter region we have dubbed 1b.2. Finally, we made a translational fusion reporter gene in the context of the entire genomic region. This *pnc-1 genomic* construct includes 1.4 kbp upstream of the first exon and all introns, with the exception of a portion of intron 2 that could not be cloned (Fig. 1).

We examined two to four individual transgenes for each construct and identified each GFP-positive cell (Table 1). We consistently observed expression predominantly in the head, specifically in a large number of neurons, pharyngeal muscle cells and hypodermal cells (Fig. 2A,B,C,D). The promoter for the secreted isoform has the most restricted expression pattern, with expression largely confined to the head, consistent with previous analysis (Vrablik et al., 2009). Transgenes encoding the secreted PNC-1a isoform occasionally had faint diffuse expression in the gonad extracellular space (Fig. 2E, F and Table 1), suggesting provision of the secreted isoform to this tissue by other cells. We find that the intracellular PNC-1b isoform is expressed from two different promoters with overlapping but not identical expression patterns (Table 1). The genomic construct, which has the most

regulatory elements presumably in their intact orientation, has the broadest expression pattern, which is broader than three individual promoters together (Table 1). In the animals with the *pnc-1 genomic* and *pnc-1b.2* promoter transgenes, we detected expression in the anterior cells of the intestine, a tissue not previously reported to express PNC-1 (Fig. 2B). Nonetheless, expression of PNC-1 is not ubiquitous. In particular, we failed to detect expression in several tissues that manifest *pnc-1* mutant phenotypes, including the gonad and the gonadal uv1 cells, the body wall muscles, and the egg-laying muscles. Therefore we considered whether the PNC-1 activity that promotes development of the gonad, survival of the uv1 cells or function of the muscle could be provided by non-autonomous expression of *pnc-1* in other tissues.

Can PNC-1 function cell non-autonomously?

We speculated that the secreted PNC-1a isoform may be critical for providing function to tissues that don't express PNC-1. To address this hypothesis, we first asked if the secreted PNC-1a isoform was sufficient to provide function to the tissues that have detectable phenotypes in the absence of *pnc-1* function. We tested the ability of transgenes that express the secreted PNC-1a isoform from the native *pnc-1a* promoter to rescue the egg-laying defect, the gonad developmental delay and the uv1 necrosis of the *pnc-1* null mutants. These *pnc-1a* transgenes, which express *pnc-1* message at levels comparable to wild type (Fig. 3B), partially rescued all three phenotypes (Fig. 3A). We also engineered animals to express a higher amount of PNC-1a by injecting the vector at a 60-fold higher concentration. This "high copy" array directed higher levels of mRNA transcription (Fig. 3B) and rescued each phenotype almost completely (Fig. 3A). Thus, the expression of the secreted PNC-1a isoform from endogenous expression sites (Fig 2A, E, F and Table 1) is sufficient to provide function to the egg-laying muscles, the gonad and the uv1 cells (Fig. 3A).

As a more rigorous test of whether the PNC-1 can function cell non-autonomously we expressed the secreted and intracellular isoforms from the highly restricted *daf-7* and *gcy-8* promoters which are expressed solely in the ASI and AFD neurons, respectively (Ren et al., 1996; Yu et al., 1997; Crook et al., 2010). Both the AFD and ASI neurons are sites of *pnc-1* expression from the *pnc-1 genomic* transgene (Table 1). These transgenes will result in PNC-1 expression as far from the egg-laying muscles, gonad and uv1 cells as possible.

We first confirmed that our constructs were being expressed in the expected neurons and no other cells. Expression was restricted to AFD in all *gcy-8* promoter lines; we observed GFP in no other cells. *gcy-8P::pnc-1b* was expressed diffusely within the cytoplasm of AFD in all transgenic animals. *gcy-8P::pnc-1a* expression was visible in AFD in only a minority of transgenic animals. In animals where GFP was visible, it was restricted to distinct cytoplasmic puncta. This pattern is consistent with localization of the protein to the secretory apparatus and secretion of the protein into the pseudocoelomic space. The *daf-7* promoter-driven constructs directed a lower level of GFP expression but once again expression was completely restricted to only the intended cell, ASI. *daf-7P::pnc-1b* was detected solely in the ASI neurons in a majority of transgenic animals in all transgenic lines. However, GFP from the *daf-7P::pnc-1a* transgenes was not detected in any of the four

transgenic lines examined, although it clearly functions, again consistent with secretion of this protein into the pseudocoelom.

We then tested these constructs for the ability to rescue the egg-laying (Egl), gonad developmental delay and uv1 necrosis phenotypes. We predicted that the secreted isoform of PNC-1 would show the most robust cell non-autonomous rescue. However, we found that the secreted and intracellular isoforms were both equally able to rescue the Egl and gonad developmental delay phenotypes when expressed in AFD or ASI neurons (Fig. 3C). Thus, both secreted PNC-1a and the intracellular PNC-1b isoform are sufficient for egg-laying muscle function and gonad development when expressed from limited sites. For the uv1 cells, only PNC-1b expressed in the ASI was able to rescue uv1 cell necrosis, and this rescue was not robust relative to the other phenotypes (Fig. 3C). These data show that expression of the secreted PNC-1a from its endogenous promoter or expression of either PNC-1 isoform from two restricted neuronal promoters is sufficient to provide function cell non-autonomously to the egg-laying muscles and the gonad.

Is the secreted PNC-1a isoform necessary *in vivo*?

We have shown that expression of the secreted PNC-1a or intracellular PNC-1b isoforms at a distance is sufficient to provide function to other tissues. This still leaves open the question about the importance of the secreted isoform. Is the secretion of PNC-1a necessary for development? To address this question we mutated three codons in the first exon of the *pnc-1 genomic* transgene to inactivate the *pnc-1a* signal sequence (see Materials and Methods) and compared the activity of the *pnc-1 genomic* transgene and the new *pnc-1 SS* transgene (Fig. 1). We predicted that the *pnc-1 genomic* arrays would rescue each phenotype and that the *pnc-1 SS* arrays would fail to fully rescue one or more of the phenotypes tested, due to the lack of secretion of the PNC-1a isoform.

We found that our *pnc-1 genomic* arrays, which express *pnc-1* message at levels comparable to wild type (Fig. 4C), rescued each of the tested phenotypes as expected (Fig. 4A,B). The *pnc-1 SS* arrays rescued the gonad developmental delay and the body wall muscle thrashing rate as well as the arrays that encoded the secreted version (Fig. 4A,B). Thus, the secreted isoform is not strictly necessary *in vivo* for optimal PNC-1 activity as it applies to the gonad or the body wall muscle. In the case of the Egl and uv1 necrosis phenotypes, the *pnc-1 SS* arrays provided robust rescue activity. However, this activity was significantly lower than the arrays with an intact signal sequence (note significant difference between values in Fig. 4A). Moreover, when we compared the standard deviations for the *pnc-1 genomic* and *pnc-1 SS* arrays using the F-test we found that they were significantly different for the Egl and uv1 necrosis phenotypes, with values of $p = 0.009$ and 0.039 , respectively, indicating more variability in rescue in the absence of the secreted version. *pnc-1* mRNA levels expressed from the genomic and *SS* arrays were not significantly different (Fig. 4C), suggesting that variation in expression levels between transgene types does not account for the differences in function. The less robust and more variable rescue with the *SS* arrays suggests that while the secreted PNC-1a isoform is not formally necessary for function it does contribute to optimal function *in vivo*. In particular, the

secreted isoform appears important in phenotypes that are caused by accumulation of NAM indicating the secreted isoform may play an important role in clearance of NAM.

Can functions be mapped to specific expression sites?

Given that PNC-1b is expressed from two promoters, we sought to test if expression from one or the other was more relevant to PNC-1 activity *in vivo*. We tested the *pnc-1b.1* and *pnc-1b.2* promoter translational fusion transgenes (Fig. 1) for their ability to rescue the Egl, gonad developmental delay and uv1 necrosis phenotypes. We observed significantly higher rescue of uv1 necrosis when PNC-1b was expressed from the *pnc-1b.2* promoter, compared with the partial rescue from its expression from the *pnc-1b.1* promoter (Fig. 5). This differential rescue suggests that PNC-1b expression from sites or at a level specific to the *pnc-1b.2* promoter is important for the survival of the uv1 cells. Expression from both promoters was predominantly neuronal with the most obvious difference unique to *pnc-1b.2* being expression in the intestine and some pharyngeal cells (Fig. 2B, C and Table 1). We did not see any preference for expression in a particular cell or tissue in terms of the egg-laying muscles or the gonad.

Discussion

PNC-1-mediated NAD⁺ salvage biosynthesis and clearance of nicotinamide are critical for optimal development and physiology of multiple tissues in *C. elegans*, including the gonad, the uv1 cells within the gonad, the body wall muscle and the egg-laying muscle (Vrablik et al., 2009; Vrablik et al., 2011). Previous work suggested that PNC-1 was not expressed by body wall muscle cells, and that while the development of the entire gonad was affected in the mutant, the gene was expressed in only a few cells in the gonad (Vrablik et al., 2009). That observation led to the suggestion that PNC-1 might function cell non-autonomously.

Because the expression pattern of PNC-1 was central to our hypothesis that it functions cell non-autonomously, we sought to perform a more rigorous expression analysis. We returned to transgenic reporter gene analysis to search for elements that might direct muscle or gonad expression. We successfully identified another promoter in the genomic locus and fully characterized the expression patterns of all three promoters in isolation as well as expression from a genomic transgene with all three promoters intact, which displayed the broadest expression pattern. Our results are consistent with previous observations that PNC-1 is predominantly expressed in the nervous system (Vrablik et al., 2009), and we revealed expression in the intestine for the first time. It is still formally possible that we have missed a relevant enhancer sequence that drives gonad expression. However, despite extensive effort with multiple transgenes, transgenic lines and different combinations of *pnc-1* regulatory sequences, we were unable to detect *pnc-1* expression broadly within the gonad or body wall muscle cells. We did detect the secreted PNC-1a::GFP in the uterus, further supporting a potential cell non-autonomous function. In seeking a second method to confirm PNC-1 expression patterns, we have raised antibodies to PNC-1, but these reagents have not been useful in immunohistochemistry experiments (unpublished data).

The presence of multiple promoters suggests the possibility for regulation involving the specific isoforms, *i.e.* providing PNC-1a versus PNC-1b activity to different tissues in

response to demand. While the issue of biologically relevant regulation has yet to be addressed, our analysis does suggest promoter-specific function. Specifically, function to the uv1 cells is provided more efficiently by expression of PNC-1b from the *Ib.2* promoter compared with the *Ib.1* promoter.

We also sought to experimentally test the hypothesis that PNC-1 can function cell non-autonomously. Our approach was to express the secreted PNC-1a and intracellular PNC-1b isoforms from promoters with a restricted expression pattern. We note that despite having the most restricted expression pattern of the three *pnc-1* promoters, *Ia*-driven expression of secreted PNC-1a was able to robustly rescue all the phenotypes we examined. We suggest that even though this promoter is most restricted in terms of number of tissues we could detect expression from, the pattern of protein localization is likely broader given that the secreted protein would have access to the entire animal via the pseudocoelomic space (see Fig. 2E,F). More surprisingly, expression of the intracellular PNC-1b from either of its endogenous promoters was able to provide function to the egg-laying muscles and gonad, although they differed in their ability to provide function to the uv1 cells. As a more rigorous test of our cell non-autonomous hypothesis we used two promoters to drive expression solely in a single pair of neurons. Expression of either secreted PNC-1a or intracellular PNC-1b in the ASI or AFD neurons was able to robustly rescue the egg-laying and gonad developmental delay phenotypes, although they were either ineffective or much less effective at rescuing the uv1 necrosis phenotype. These data provide strong support for our hypothesis that PNC-1 can function cell non-autonomously and revealed an unexpected role for the intracellular form in providing non-autonomous function. Cell non-autonomous function by the intracellular isoform suggests that the substrate and product of PNC-1 are moving between cells (see below).

Even though intracellular PNC-1 can function at a distance, we were still interested in understanding if secretion of PNC-1 is important to function in the animal, especially given the controversy over the role of secreted eNampt in vertebrates. Although salvage synthesis itself is accomplished *via* distinct proteins in vertebrates and invertebrates, secretion of the protein which metabolizes NAM is conserved between species. The *C. elegans* system thus provides a great experimental system to test if secretion is necessary for the organism. To determine if the secretion of PNC-1a is necessary we compared the rescue of two *pnc-1* genomic transgenes that were identical except for the presence or absence of a functional *pnc-1a* signal sequence. We found that *pnc-1* genomic transgenes lacking the PNC-1a signal sequence were still able to robustly rescue, which is consistent with our surprising result regarding cell non-autonomous function of intracellular PNC-1b. However, for two NAM-induced phenotypes our evidence hints that the secretion of PNC-1a was biologically and functionally relevant. The transgenic lines that lacked the signal sequence were mildly but statistically significantly reduced in activity and more variable in activity. The simple model we had originally envisioned, where the secreted PNC-1a provides the cell non-autonomous function and the intracellular PNC-1b provides the cell autonomous function is not supported. Instead, we have shown that intracellular PNC-1b is sufficient while the PNC-1a isoform makes a minor contribution, specifically in preventing accumulation of NAM.

Conditions that restore NAD⁺ biosynthetic capacity to the animal rescue gonad development (Vrablik et al., 2011) whereas NAM conversion to NA is likely responsible for rescue of uv1 cells (Vrablik et al., 2009). PNC-1 provides both of these activities, but how does PNC-1 provide function to a range of tissues when expressed at a distance from these tissues and in a limited number of cells? And why does uv1 cell survival seem to be the phenotype with the most stringent requirement for PNC-1 function, in terms of both sensitivity to the loss of the secreted isoform and requirement for a specific expression site (or level)? We have considered several possibilities for how PNC-1 functions at a distance. First, it may be that distant cells actually direct the development of the gonad or the activity of the muscle. Under this scenario those cells would have to be AFD and ASI acting redundantly. We consider it unlikely that these two pairs of sensory neurons have such distinct functions and previous experiments looking at function of these cells revealed no such activities (Ren et al., 1996; Schackwitz et al., 1996; Kimura et al., 2004; Clark et al., 2006; Beverly et al., 2011). Second, it may be that metabolites are moved between cells and metabolic activity by the intracellular isoform in only two neurons, including their processes which extend to ¼ the length of the animal is adequate to provide NA to the whole animal or at least to key control cells in the vicinity of these neurons. Both the PNC-1 substrate NAM and product NA move between cells both in yeast and in metazoans (Llorente and Dujon, 2000; Houtkooper et al., 2010; Belenky et al., 2011; Nikiforov et al., 2011). A nicotinic acid permease for NA import has been identified in yeast (Llorente and Dujon, 2000), but no known exporter and no NAM transporters have been identified. It is not surprising that widespread expression of intracellular PNC-1 could help prevent NAM, produced from NAD⁺ hydrolyzing enzymes in many cell types, from accumulating globally. However it was a surprise that uptake and processing of NAM by only two cells would be sufficient to keep NAM levels from accumulating to levels that are toxic to uv1 cells at a distance. Similarly, production of NA or a downstream metabolite in a pair of neurons appears to be sufficient to supply NAD⁺ biosynthetic precursors more broadly to the animal. In the case of the secreted isoform, it is likely that PNC-1a converts NAM to NA in the pseudocoelomic space, either to supply NA to nearby cells that can further process it to NAD⁺ or to reduce extracellular NAM to sub-toxic levels.

Why do uv1 cells have the most stringent requirement for PNC-1 activity? It is likely that the uv1 cells are peculiarly sensitive to NAM or that NAM levels in their vicinity are higher. However, the need to metabolize NAM also likely relies disproportionately on PNC-1 relative to the need to synthesize NAD⁺ because there are other NAD⁺ biosynthetic pathways that can contribute to the production of NAD⁺ (Wang *et al.* submitted), but there is not the same flexibility in terms of mechanisms to clear toxic buildup of NAM. This disparity may explain the importance of secreted PNC-1a or proximal intracellular PNC-1b activity to uv1 survival. Further work on the mechanism by which NAM causes uv1 necrosis may help answer resolve these different possibilities.

In summary, the picture of how NAD⁺ salvage activity is provided to different tissues in *C. elegans* is more complex than we first thought. Although we have shown that PNC-1 can function cell non-autonomously, secretion is clearly not vital to cell non-autonomous PNC-1 function. It is likely that PNC-1 functions in a variety of roles depending on the tissue in question, its metabolic needs, including its requirement for NAD⁺ and its sensitivity to

NAM, and this ability to provide function to and from a range of tissues may provide the robustness we would expect for a pathway as vital as NAD⁺ biosynthesis.

Materials and Methods

C. elegans strains and maintenance

Strains were maintained under standard conditions at 20 °C (Brenner, 1974). We used the following strains; N2 (wild type), MH968 *unc-119(ed3)* III, HV723 *pnc-1(pk9605)* IV and HV554 *unc-119(ed3)* III; *pnc-1(pk9605)* IV. To determine the expression patterns of *pnc-1* and its individual promoters we generated extrachromosomal arrays by injecting hermaphrodites with LiCl purified plasmid DNA at concentrations between 10 and 110 ng.μl⁻¹ (Table 2).

Transgene construction

The *pnc-1* genomic locus and the transgenes used in this study are shown in Figure 1. For a full list of primers and construction of transgenic animals see Table 2. Four control arrays (psEx264 and psEx273-5) in a *pnc-1(pk9605)* background were generated by injecting *pnc-1(pk9605)* hermaphrodites with 60 ng.μl⁻¹ of *sur-5::gfp* (Gu et al., 1998) and 40 ng.μl⁻¹ pBluescript KS-DNA; one control array (psEx236) in an *unc-119(ed3); pnc-1(pk9605)* background was generated by injecting *pnc-1(pk9605)* hermaphrodites with 60 ng.μl⁻¹ of *unc-119(+)* DNA (Maduro and Pilgrim, 1995). All constructs were confirmed by sequencing.

pMC1: we first added a multiple cloning site, containing Nhe I, Pst I and Not I, to pPD95.67 (Addgene plasmid 1490) using Sph I/ Xba I. We then sequentially cloned a fragment containing the upstream sequence to the end of exon 1b using *pnc-1*F1/Sph I and *pnc-1* R2/Nhe I, a second fragment containing intron1b to exon2 using *pnc-1* F2/ Nhe I and *pnc-1* R18/Pst I and a final fragment containing exon 3 to the end of exon 4 using *pnc-1* F/Pst I and *pnc-1* R/Not I. We were unable to clone the 1 kbp intron 2 using multiple strategies, possibly due to the presence of a predicted hairpin loop structure.

pMC11: we mutated the exon 1a signal sequence (Fig. 1) by extension overlap PCR, changing the hydrophobic glycine-leucine-isoleucine amino acids to polar glutamic acid-histidine-lysine amino acids to inactivate the signal sequence, and then used this to replace the original sequence by Kas I/ EcoR V restriction cloning.

pMC4 and 5: the *pnc-1a* and *-1b* cDNAs were each translationally fused by extension overlap PCR to a non-nuclear localized GFP from pPD118.20 (Addgene plasmid 1592). After replacing the *unc-54* 3'UTR of pPD95.69 (Addgene plasmid 1491) with the 185 bp long *pnc-1* 3'UTR using EcoR I/ Spe I, these cDNA::gfp fusions were inserted upstream by Xma I/ EcoR I. Finally, the 1a and 1b.1 promoters were cloned upstream of their respective cDNAs by PstI/ XbaI and PstI/ NheI, respectively.

pMC3 and 6: a 3 kbp intron between exon 1b and exon 2 was first cloned as a transcriptional fusion into pPD95.69 using *pnc-1* F2/ PstI and *pnc-1* R3/ SalI. Once the presence of a third

promoter was confirmed, it was subcloned upstream of the *pnc-1b::gfp* described above by Pst I/ Xma I to create the *pnc-1b.2* transgene.

pMC13 to 16: the *pnc-1a* and *-1b* cDNAs were each placed under the control of either 1.2kbp of the *daf-7* promoter (pMC13 and 14, respectively) or 2.1kbp of the *gcy-8* promoter (pMC15 and 16, respectively). *daf-7* is expressed solely in the ASI neurons (Ren et al., 1996; Schackwitz et al., 1996; Crook et al., 2010) and *gcy-8* is expressed solely in the AFD neurons (Yu et al., 1997). The respective promoters were cloned using SphI/ XmaI upstream of either *pnc-1a* or *pnc-1b* cDNAs translationally fused with GFP.

Phenotypic Assays

Egg-laying—For each strain 20 to 30 L4 hermaphrodites were placed onto individual plates and scored for the “bag of worms” Egl phenotype after two, three and four days at 20 °C. Sterile adults and those that died from anything other than bagging were removed from the analysis. The Egl phenotype is expressed as the percentage of the remaining egg-laying adults that had not bagged by day four.

Gonad delay—Gonad development was scored in the mid-L4 stage by examining stage of uterine development relative to the stage of vulval development as described previously (Vrablik et al., 2009). In wild-type animals, the lumen in both arms of the uterus is open and they are joined above the mid-L4 stage vulva, which has a stereotypical and easily recognizable “Christmas tree” morphology. In the *pnc-1* mutants, the uterine lumen is not open or is not connected between the two arms by the time the vulva displays the typical mid-L4 stage “Christmas tree” morphology. The phenotype is reported as the percentage of L4 animals with a fully open and connected uterine lumen.

uv1 necrosis—In *pnc-1* mutants approximately 95% of the four uv1 cells at the uterine-vulval junction die by necrosis soon after they are specified (Huang and Hanna-Rose, 2006; Vrablik et al., 2009). As it is technically challenging to accurately score the actual number of dying uv1 cells on both sides of the animal by DIC, the late L4 animals were scored for presence or absence of necrotic uv1 cells. The phenotype is reported as percentage of animals without any necrotic uv1 cells.

Muscle function—Muscle function was assessed by measuring thrashing rate, the number of body bends *per* minute, as described previously (Vrablik et al., 2011). Wild-type non-transgenic animals were used as positive controls.

qPCR analysis of *pnc-1* transgene expression

Total RNA was extracted using TRIzol® Reagent (Life Technologies) according to the manufacturer’s instructions. 2 µg total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Real-time quantitative PCR amplifications were carried out using SYBR Green (PerfeCTa SYBR Green Super Mix with ROX, Quanta Biosciences) in a StepOnePlus Real-Time PCR System (Life Technologies). Reaction profile was 95°C for 10 min then 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds, followed by a melting curve analysis was carried

out (60°C to 95°C) to verify the specificity of amplicons. *pnc-1* specific primers were: *pnc-1-F* tcaacgataataacgggcgggtcca and *pnc-1-R* ccaggcaagtaaaccgaacgcaaa. Three internal reference genes; *cdc-42*, *pmp-3* and *tba-1*, were used as described previously (Hoogewijs et al., 2008). Three technical replicates were carried out for each of three biological replicates and the Ct value for each sample was calculated by subtracting the geometric mean of the reference gene Ct values from that of *pnc-1*. The Ct values for each biological replicate were calculated by subtracting the experimental *pnc-1* Ct value from the wild-type Ct value and then transformed using 2^{-Ct} (Livak and Schmittgen, 2001). Finally, transformed Ct values were normalized using the percentage transgenic animals in each biological replicate.

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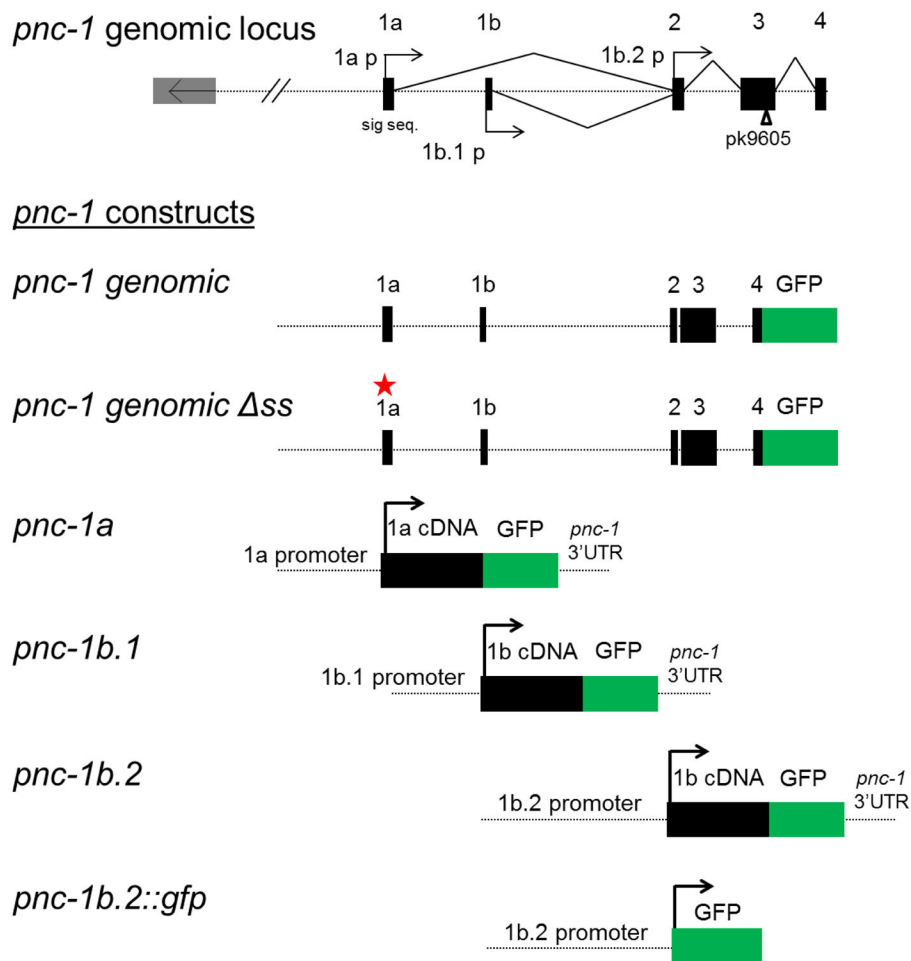
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Bullet points

- PNC-1 expression is not detected in tissues with phenotypes, suggesting non-autonomous function
- Either the secreted PNC-1a isoform or the intracellular PNC-1b isoform is sufficient to provide function *in vivo*
- The secreted PNC-1 isoform contributes to *in vivo* activity
- Intracellular and extracellular PNC-1 isoforms function cell non-autonomously in *C. elegans* development and cell survival.

**Figure 1.**

The genomic organization of the *pnc-1* locus showing intron-exon structure and promoter regions. The constructs used in this study are also diagrammed. The location of the point mutation in *pnc-1*(pk9605) is indicated by an open arrowhead. The red star indicates the mutated signal sequence in *pnc-1* Δ SS. Solid boxes and right pointing arrows represent coding regions and promoters, respectively. The *1a* exon contains the first 17 signal sequence amino acids and the *1b* exon the remaining three signal sequence amino acids and cleavage site. In all other respects, PNC-1 expressed from the three promoters is identical.

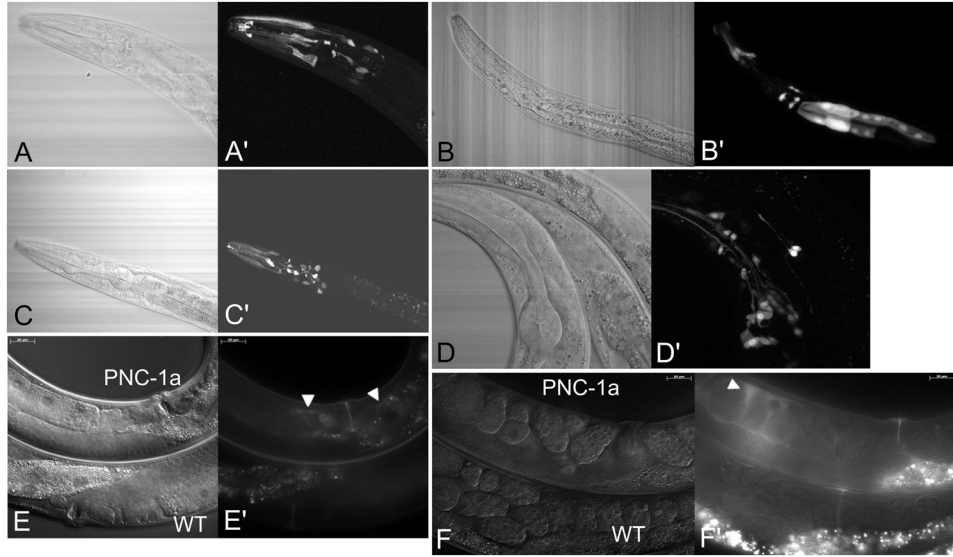
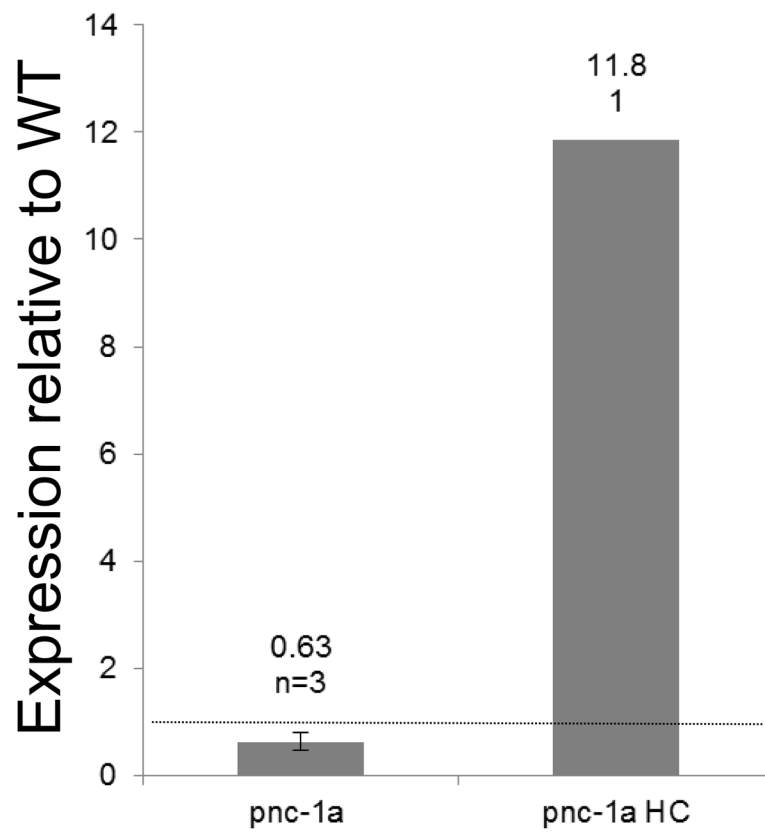
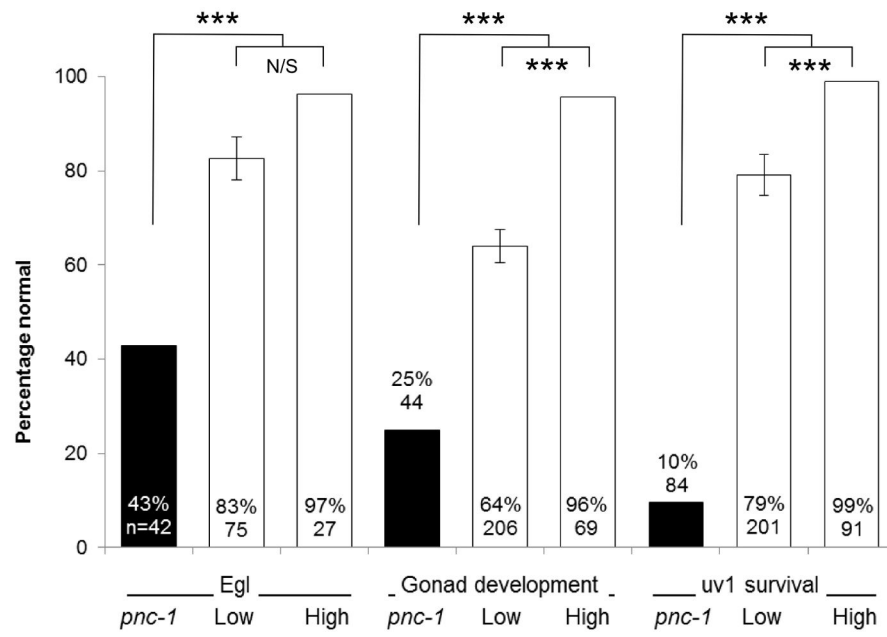


Figure 2. GFP reporter expression for *pnc-1* transgenes. A,A') *pnc-1a* transgenic L2 animal, B,B') *pnc-1b.2* transgenic L2 animal, C,C') *pnc-1b.1* transgenic L2 animal, D,D') *pnc-1* genomic transgenic L4 animal. The majority of expression for all constructs was neuronal and in the head and clearly not ubiquitous. *pnc-1b.2* expression was also seen in the intestine (B'), a previously unrecognized expression site. E and F) Expression of PNC-1a::GFP results in extracellular fluorescence. Both E and F show one *pnc-1a* transgenic animal (upper) and one wild-type animal (lower). Transgenic E) young adult hermaphrodites and F) wild-type gravid adult hermaphrodites have faint, diffuse extracellular PNC-1a::GFP in the uterus (white arrowheads in E' and F') that is distinct from the autofluorescence visible in both transgenic and non-transgenic animals. Confocal images A to D were captured using an Olympus Fluoview FV1000 microscope and 60x objective, images E and F were captured using a Zeiss Axioplan 2 microscope and x40 oil immersion DIC optic. Confocal GFP images are Z-stacks assembled from scans using ImageJ (Schneider et al., 2012) and corresponding white light images are single pass images from the white light channel of that same scan.



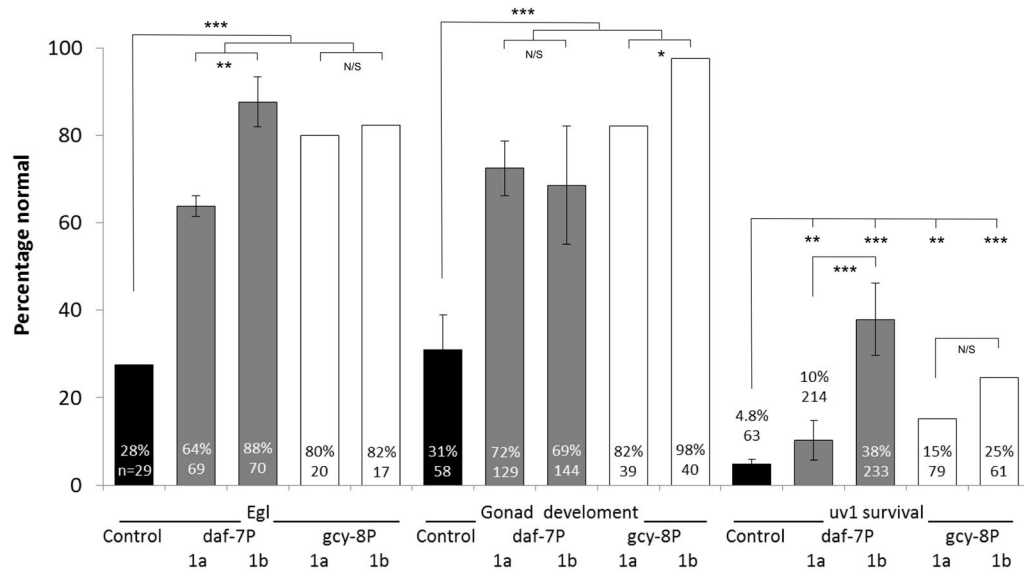
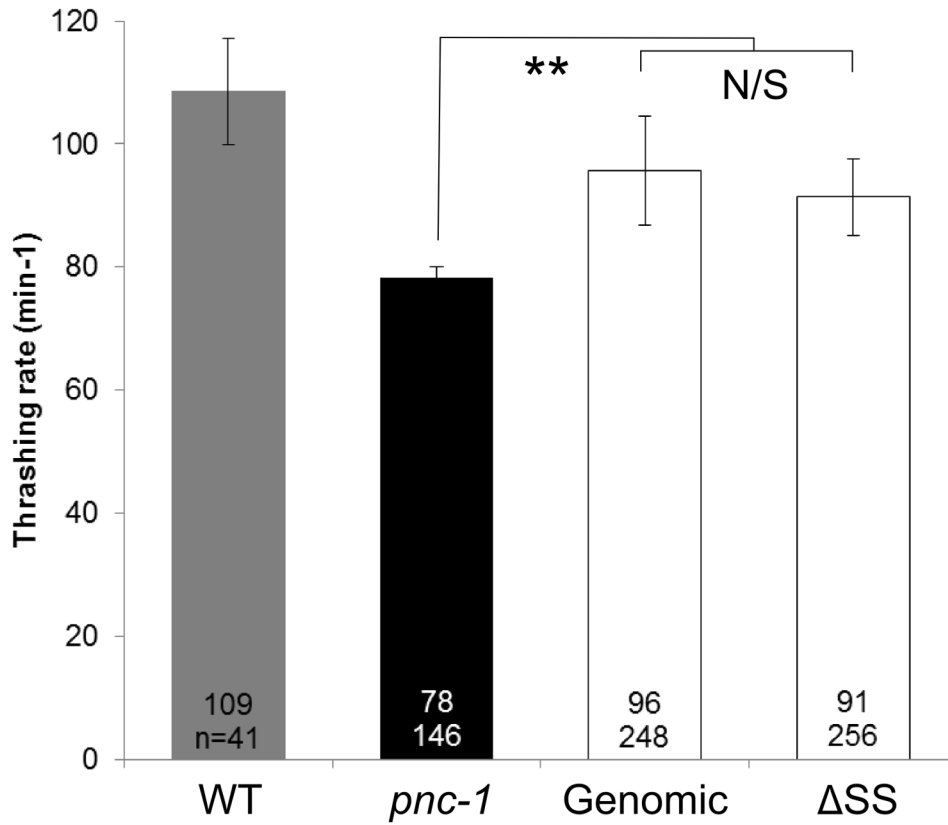
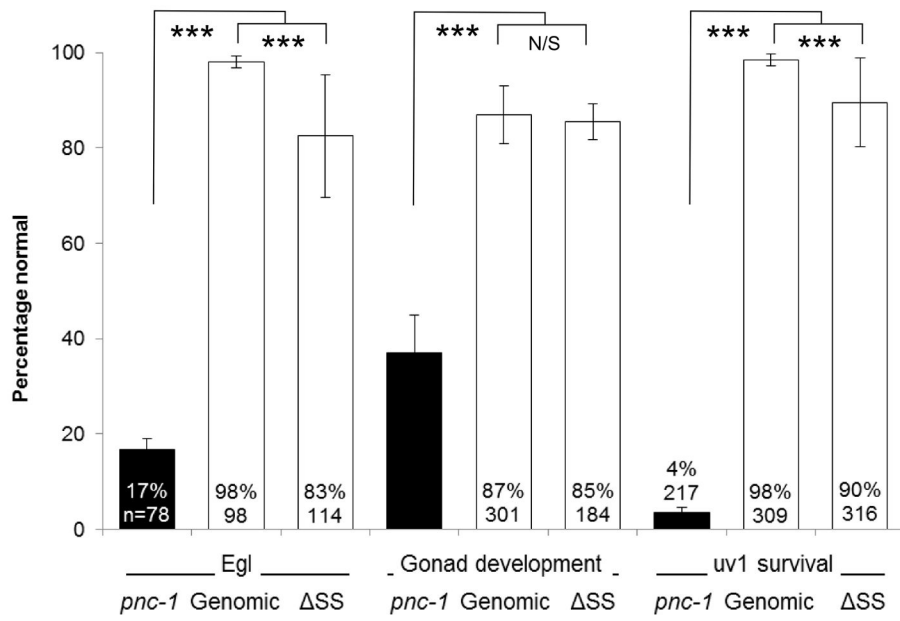


Figure 3.

Cell non-autonomous function of PNC-1 isoforms. A) Percentage of *pnc-1(pk9605)* mutant populations with normal egg-laying (Egl), gonad development and uv1 cells is compared for strains with control transgene or *pnc-1a* transgenes (Fig. 1) injected at either a low concentration (1a, 1 ng.ml⁻¹, transgenic arrays psEx213-5, 220-1 and 228 (Table 2)) or at a high concentration (1aHC, 60 ng.ml⁻¹, transgenic array psEx238 (Table 2)). Control array psEx236 was used. B) qPCR analysis comparing *pnc-1* message levels in *pnc-1a* arrays injected at two different concentrations. Arithmetic means of Ct values were calculated for each type of array from the averages of three biological replicates *per* line. Arrays assayed were *pnc-1a*: psEx213, 215 and 220; and *pnc-1a* HC: psEx238. Error bar is *S.D.* C) The secreted PNC-1a and the intracellular PNC-1b isoforms were expressed individually in the ASI or AFD neurons using the *daf-7* promoters (*pnc-1a* transgenic arrays psEx283-6, *pnc-1b* transgenic arrays psEx287-290, see Table 2) and *gcy-8* promoters (transgenic arrays psEx291 and 292 for *pnc-1a* and *pnc-1b*, respectively, see Table 2), respectively, and assayed for rescue of Egl, gonad developmental delay and uv1 necrosis. Control was *unc-119(ed3); pnc-1(pk9605)*. Number of animals assayed and average percentage of the population that were normal for each phenotype for each experiment are shown. *gcy-8p::pnc-1a* and *gcy-8p::pnc-1b* experiments were not compared to each other statistically because we had only a single transgenic line for each construct. Error bars are *s.e.m.* Percentages were analyzed using Fisher's Exact test. *, ** and *** represent P values of <0.05, <0.01 and <0.001, respectively. n.s. is not significant.



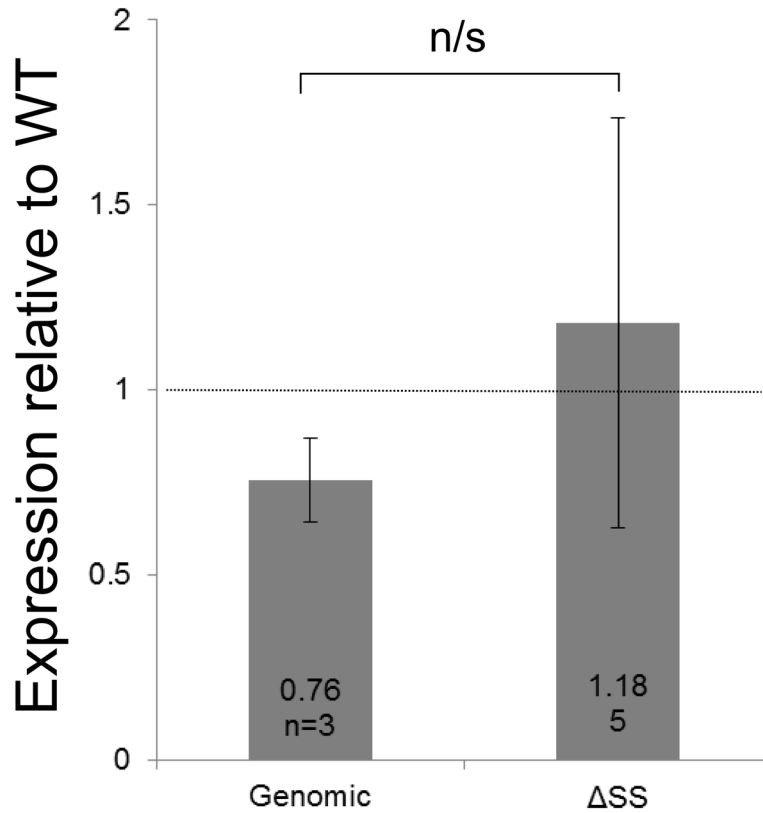


Figure 4.

Is the secreted PNC-1a isoform necessary? Percentage of *pnc-1(pk9605)* mutant populations with (A) normal egg-laying (Egl), gonad development and uv1 cells and (B) average thrashing rate (expressed as number of body bends *per* minute) is compared for strains carrying control *pnc-1* genomic transgenes (Genomic, transgenic arrays psEx251-2, 265-6, 268, see Table 2) or experimental genomic transgenes that have a mutated signal sequence (ΔSS, transgenic arrays psEx249, 253, 271, 276-8, see Table 2). Number of animals assayed and average result for each experiment are shown. Controls were arrays psEx264 and 273-5, with wild-type non-transgenic animals as positive controls for thrashing rate. Error bars are *s.e.m.* for a) and *S.D.* for b). Percentages were analyzed using Fisher's Exact test and thrashing rate was analyzed using Students T-test. *, ** and *** represent P values of <0.05, <0.01 and <0.001, respectively. n.s. is not significant. (C) qPCR analysis comparing *pnc-1* message levels in genomic and ΔSS arrays. Arithmetic means of Ct values were calculated for each type of array from the averages of three biological replicates *per* line. Arrays assayed were *pnc-1* genomic: psEx251, 252 and 266; and *pnc-1* ΔSS: psEx 249, 253, 272, 277 and 278. Error bars are *S.D.* Means were analyzed using Students T-test. n.s. is not significant.

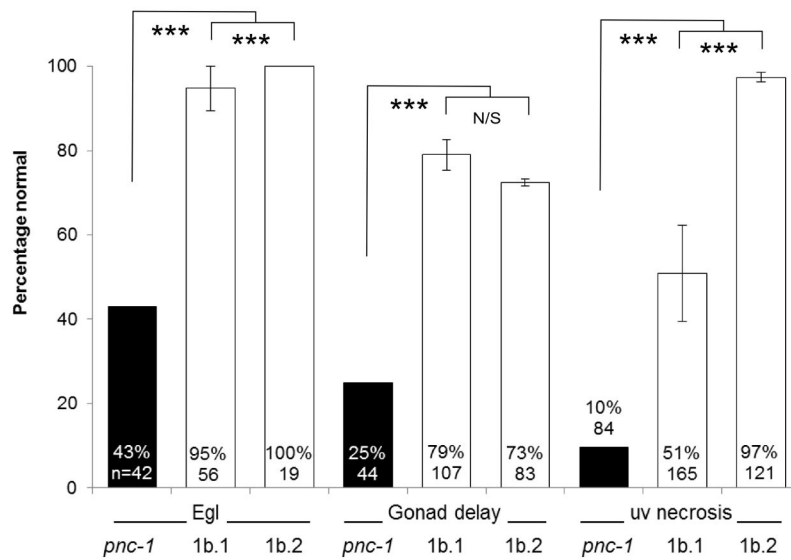


Figure 5.

Functional analysis of two PNC-1b.promoters. The intracellular PNC-1b isoform was expressed at high levels under the control of its two endogenous promoters, 1b.1 (transgenic array psEx222-5, see Table 2) and 1b.2 (transgenic array psEx217-9, see Table 2), and assayed for rescue of Egl, gonad developmental delay and uv1 necrosis. Number of animals assayed and average result for each experiment are shown. Control array psEx236 was used. Error bars are *s.e.m.* Percentages were analyzed using Fisher's Exact test. *, ** and *** represent P values of <0.05, <0.01 and <0.001, respectively. n.s. is not significant.

Table 1

pnc-1 global and promoter-specific expression patterns.

Cell type <i>l</i>	Genomic	Promoter		
		1a	1b.1	1b.2
Head				
Neurons	ADF/L, AFD, AIA, ASG/H/I/K/L, AVA, IL1/2, Retrovesicular ganglion	x	(²)	
	ASE, ASJ, AWA/B	x	x	x
	AUA		x	x
	CEP	x	x	x
	IL/OLQso (sheath cells)		x	x
	I2/3, M3			
	I4, M1		x	x
	I5, M2			x
Other	pm3	x	x	x
	pm4		x	x
	pm6VR, pm7VR/D, pm8, ventral and dorsal gland cell	x		
	pm7			x
	vpi		x	
	hyp1	x		x
	hyp2/3	x		
	hyp4			x
	hyp6	x	x	
	arcade cells	x		x
Mid-body				
Neurons	CANL, ventral cord neurons (occasional)	x		
Other	ccPL+AL, ccDR+ccDL	x		
	Intestinal cells	x		x
	vulF (occasional, diffuse)		x	
	uv1 cells	-	-	-
	Gonad	-	- ₂	-
	Body wall or vulva muscle	-	-	-

Cell type	<i>I</i>	Promoter		
		Genomic	1a	1b.1 1b.2
Tail				
Neurons	PHA/B	x	x	x
	PVC	x		
	PVT		x	
	Phso1/2 (phasmid sheath cell)		x	x
Other	RecB			x
	RecF, RecU			x

¹ Every cell observed for individual promoters is recorded; for the *pnc-1 genomic* construct some expressing cells may have been overlooked due to brighter neighboring expression sites. Expression of the *pnc-1 SS* signal sequence mutant was identical to that of the *pnc-1 genomic* with the additional expression sites of ccPR, ccAR, RecB and Phso1/2. These may correspond to sites of expression of PNC-1a because retention of previously exported GFP due to mutation of the signal sequence would cause expression sites to be revealed. The transgenic arrays used to analyze PNC-1 expression are as follows: *pnc-1 genomic*, psEx216-9; *pnc-1 SS*, psEx246-7; *pnc-1a*, psEx213-5 and 238; *pnc-1b.1*, psEx222-5; *pnc-1b.2*, psEx207-8.

² Expression previously seen in ASK, DTC, uv2 and rectal gland cells using a *pnc-1a::gfp* reporter with an *unc-54* 3' UTR (Vrablik et al., 2009).

Table 2

Constructs, primers and transgenes used in this study.

Construct	Vector	Parent plasmid	Primers (5'-3')/	Transgene psEx#	Background/coinjection marker(ng- μ l ⁻¹) ²	ng. μ l ⁻¹
<i>pnc-1 genomic</i>	pMC1	pPD95.67	Restriction linker F	216-9	<i>unc-119</i> ; <i>pnc-1</i> / <i>unc-119(+)</i> (60)	20
			GCAITGGCTAGCCTGCAGGCGGGCCGCTCTAGA			
			Restriction linker R			
			TCTAGAGCGGGCCCTGCAGGCTAGCGCATGC			
			<i>pnc-1</i> /F (Pst I)			
			AACTGCAGAAATCGCCAACTGGAICTTC			
			<i>pnc-1</i> /R (Not I)			
			AAGGGCGCCTTCTTCAGATCCTTTGAACCCATTTC			
			<i>pnc-1</i> /Fa (Sph I)			
			GCATGCCAGGCTTTCTTGACTTTCTGTTACTTTTT			
			<i>pnc-1</i> /Ra (Nhe I)			
			GCTAGCAITTTACCTCTCTAAACTCCGAAAAAC			
<i>pnc-1</i> /F9 (Nhe I)						
AAGCTAGCGTAAATGAATGTTTCCGGTGTGTTGTTGTG						
<i>pnc-1</i> /R18 (Pst I)						
AACTGCAGCCCAAGCCAAAGATTCCATAATCCTCT						
<i>pnc-1 genomic SS</i>	pMC11	pPD95.67	<i>pnc-1</i> /F17 (Kas I)	246-7	<i>unc-119</i> / <i>unc-119(+)</i> (60)	20
			GAGGGCCACATCTCTTTCCC			
			<i>pnc-1</i> /R25 (EcoR V)			
			CTGGATATCGTTTTTAAACGGTCTTAAACGAG			
<i>pnc-1</i> /F18 (mutagenesis linker)	249, 253, 271, 276-8	<i>pnc-1</i> / <i>sur-5</i> :: <i>gfpX</i> (60) and pBS KS-(39)	1			
GCATTTATTTGAGCaAagTGGGTGA ³						
<i>pnc-1</i> /R26 (mutagenesis linker)						
TCACCCActTgGcCcaATAAATGC						
<i>pnc-1a</i>	pMC4	pPD95.69	Common to pMC4/5/6	213-5, 220-1, 228	<i>unc-119</i> ; <i>pnc-1</i> / <i>unc-119(+)</i> (60)	1
			<i>pnc-1</i> /F16 (EcoR I)			
			AAGAATTCAGTAATAATAAAATATTTGTGCATAATTTTGTAAAT			
			<i>pnc-1</i> /R24 (Spe I)			
			AAACTAGTAGGAGTACACGGCGCGT			
			pDI18.20 R1			
			CCAAGCGAGGACAAATTTCTCATCG			
			GFP F7			
			ATGGTTCAAAGGATCGTGAAGAAGATGAGTAAAGGAGAAGAACTTTCACTGG			
			<i>pnc-1a/b</i> CM R4			
			CCAGTGAAAAGTTCTTCTCCCTTACTCATCTTCTTCAGGATCCTTTGAACCCAT			
			Specific to pMC4			
<i>pnc-1a/b</i> CM F8						
ATGTTCCCTGCCCAAAGCTTATATTC						
<i>pnc-1</i> /F3 (Pst I)						
AACTGCAGTTTTCCAGGCTTTTCTTGACTTTCTGTTAC						
<i>pnc-1</i> /R4 (Xba I)						
AACTAGACAAGAAATTTGATCTAAGAATGTAATCTCCAG						

Construct	Vector	Parent plasmid	Primers (5'-3') ¹	Transgene psEx#	Background/coinjection marker(ng.µl ⁻¹) ²	ng.µl ⁻¹
<i>pnc-1b.1</i>	pMC5	pPD95.69	Common to pMC5 and 6 <i>pnc-1a/b</i> CM F9 CAGCTAATCCATTTTGGCTCCTAGC Specific to pMC5 <i>pnc-1</i> F4 (Pst I) AACTGCAGGTGAGTTTGGTGGATAGGACGGT <i>pnc-1</i> R5 (Nhe I) AAGCTAGCCTTCGTTCCGCGGAAACGAGTG	222-5	<i>pnc-1/-</i>	10
<i>pnc-1b.2</i>	pMC3	pPD95.69	<i>pnc-1</i> F2 (Pst I) CTGCAGGTA AAAATGAATGTTCCGTTGTTGTTGTTG <i>pnc-1</i> R3 (Sal I) GTCGACCATCTACAACAATACTAATTTGGGAACAAC	207-8	<i>unc-119/unc-119(+)(60)</i>	20
<i>daf-7 pnc-1a/b</i>	pMC6			217-9	<i>pnc-1/-</i>	10
<i>daf-7 pnc-1a/b</i>	pMC13/14	TU#61/2	<i>daf-7</i> CF1/2 (Sph I) GCATGCGTAAACACTTTTCAGATTTTCAAATTTTCAG <i>daf-7</i> CR1/4 (Xma I) CCCCGGGAACATGGCTGAACTTCAAGC	<i>pnc-1a</i> : 283-6 <i>pnc-1b</i> : 287-290	<i>unc-119; pnc-1/unc-119(+)(60)</i>	10
<i>gcy-8 pnc-1a/b</i>	pMC15/16	TU#61/2	<i>gcy-8</i> F1 (Sph I) GCATGCCCTTCTCCTTGACGAGTCTTCC <i>gcy-8</i> R1 (Xma I) CCCCGGTTTGATGTGGAAAAGGTAGAATCG	<i>pnc-1a</i> : 291 <i>pnc-1b</i> : 292	<i>unc-119; pnc-1/unc-119(+)(60)</i>	10

¹ Restriction enzymes in parentheses refer to the enzymes used in the initial cloning step with fragments generated using these primers. Subsequent sub-cloning steps are detailed in the methods section.² Coinjection markers used were either pDP#MM016B (*unc-119(+)*) DNA (Maduro and Pilgrim, 1995) or *sur-5:gfp* DNA (pTG96_1 (Gu et al., 1998)) and pBluescript KS- to a total of 100ng.µl⁻¹.³ Nucleotides in lowercase are those changed to inactivate the *pnc-1a* signal sequence.