Paralogs of *nos-1* in *Caenorhabditis elegans* Exhibit Genetic Redundancy as Translational Regulators in Dendrite Morphogenesis

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Abstract:

Neurological disorders often present with defects in dendritic arborization, illustrating the significance of dendrites and synaptic connections for cognition. As such, it is important to the study of these disorders to understand the mechanism by which neurons establish proper dendritic morphology. Because dendrites are distantly located from the nucleus and exhibit independent changes in growth and connectivity, regulation of dendrite morphogenesis likely occurs through a localized post-transcriptional mechanism. RNA-binding proteins are important mediators of post-transcriptional regulation and several such proteins have been implicated in dendrite development. The RNA-binding protein, Nanos, has been previously characterized as important for dendrite morphogenesis in *Drosophila*. However, knockout of *nos-1*, the strongest *nanos* homolog in *C. elegans*, does not reveal significant dendrite defects. Due to the presence of two additional *nos* paralogs in the *C. elegans* genome, *nos-2* and *nos-3*, we suspected that the lack of dendrite defects upon knockout of *nos-1* indicated the possibility of genetic redundancy. Our results support the notion that the *nos* paralogs are genetically redundant and further identifies the redundant function of these proteins as important for fourth order dendrite morphogenesis in *C. elegans*. Furthermore, this study also identifies *nos-3* alone as important for the morphogenesis of second and third order dendrites. The expression pattern of the three NOS proteins in the *C. elegans* PVD neuron is also consistent with the predicted function of NOS as a translational regulator, suggesting a role for the *nos* paralogs in dendrite development and maintenance.

Introduction:

Neuronal morphogenesis

The process by which a neuron matures includes the development of dendrites that branch out and project away from the cell body. This branching allows each neuron to establish dendritic connections within an organism at varying distances and directions from the cell body (Scott and Luo, 2001). The spatial locations and connections of these dendrites are important to the overall function of the cell because dendrites are responsible for the reception and transmission of signals from their immediate environment to the cell body. This dendritic function, however, often requires a degree of neural plasticity such that new growth and connectivity can occur in response to changing environmental stimuli (Sjöström *et al.*, 2008). As such, the dendrites of a neuron are capable of rapid modifications in length and association in order to address the changing needs of the cell (McAllister, 2000). This dendritic plasticity

enables neurons to form proper dynamic neural connections and associations, which are required for normal neural function in an organism. However, flaws in dendrite morphogenesis, whether in development or maintenance of the dendrites, often lead to the manifestation of aberrant cognition and neural dysfunction (Kulkarni and Firestein, 2012; Ardent *et al.*, 1995). This suggests that dendrite morphogenesis is an important component of development in the context of neurological disease.

The neural morphology in individuals exhibiting normal brain function and in those with neurological disorders often exhibit different characteristic dendrite phenotypes. One common phenotype among individuals with neurological disorders is a decrease in the number of dendrite branches as compared with neurons from healthy individuals (Figure 1; Baloyannis, 2009; Ferrer and Gullotta, 1990). In human disorders such as Down syndrome, schizophrenia, and Alzheimer disease, this phenotype is exemplified along with impaired cognition (Kulkarni and Firestein, 2012). Because it is difficult to measure and quantify cognitive abilities in many model organisms, impairment of neuronal functions can be implied by other characteristics, like dendrite defects, in a research setting. Factors that are necessary for dendrite morphogenesis will elicit dendritic defects in their absence, and can thus be identified as having potential involvement in neurological disease (Gao *et al.*, 1999). Identification of these factors is therefore important to the study of neurological development and disorders.

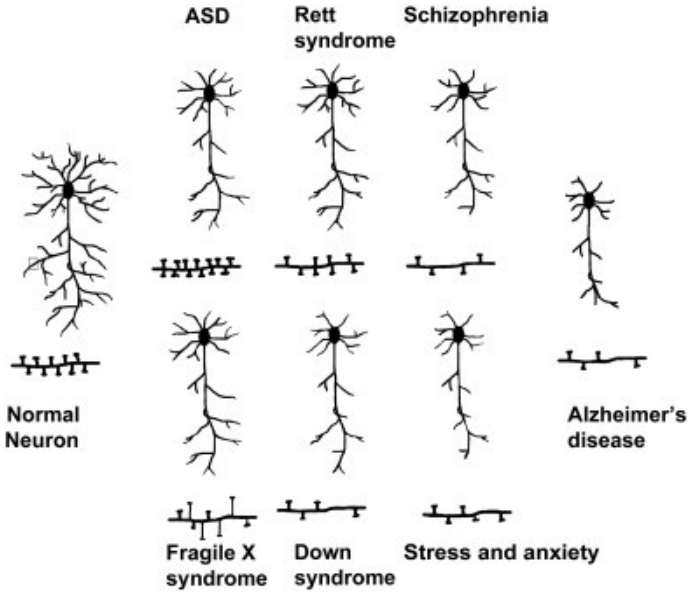

Figure 1. A representation of the dendritic defects and abnormalities associated with various neurological diseases. A decrease in dendritic branching is common among all depicted disorders (Kulkarni and Firestein, 2012).

RNA-binding proteins

One class of cellular factors that is involved in dendrite morphogenesis is the RNA-binding protein (RBP) class of post-transcriptional regulators (Nussbacher *et al.*, 2015). RNA-binding proteins can perform a variety of functions including, but not limited to, mRNA transport,

translational regulation, and splicing (Gamberi *et al.*, 2008). Because dendrites are capable of rapid changes, regulation at the post-transcriptional level is one way for dendrites to respond quickly to changing signals. Post-transcriptional regulation allows for selective transport of mRNA as well as localized translational repression and activation, which can be responsible for some of the changes that occur independently in the different dendrites of a neuron (Martin and Zukin, 2006; Gamberi *et al.*, 2006). This is especially important in circumstances where the dendritic branching occurs at a large molecular distance from the cell body, because independent and localized regulation would bypass the delay caused by transport of proteins to and from the cell body. Along these lines, studies have found that mRNA transport to and storage at branch points of dendrites allows for rapid response to signals in the form of localized translational activation and repression (Bramham and Wells, 2007; Krichevsky and Kosik, 2001).

RBPs, due to their ability to recognize and bind specific RNA sequences or secondary structures, are prime candidates for investigation into mRNA transport and translational control in the context of dendrite morphogenesis. Previous RBP knockout studies on dendrite morphogenesis revealed that the absence of certain RBPs, like mouse FMR1 and frog CPEB1, led to defects in branching and aberrant dendritic phenotypes (Nimchinsky *et al.*, 2001; Bestman and Cline, 2008). From studies like these, several RBPs have been characterized as important to the process of dendrite morphogenesis. However, not all RBPs have been investigated in this context, and thus the extent to which RBPs are required for and function in dendrite development is still unknown. A screen of RBPs in the *Drosophila* genome identified 63 RBPs as necessary to this process (Olesnicky *et al.*, 2014). Of these 63 RBPs, 54 of them have both human and *C. elegans* homologs, prompting one to hypothesize that the homologous RBPs are also likely to be involved in human and *C. elegans* dendrite morphogenesis. However, a subsequent screen of those 54 homologous RBPs in the *C. elegans* genome identified only 12 RBPs as important to dendrite morphogenesis (Antonacci *et al.*, 2015). Due to functional conservation often observed between *C. elegans* and *Drosophila RBPs*, one would expect to have seen a comparable number of neurologically important *C. elegans* and *Drosophila* RBPs. The comparatively smaller number of *C. elegans* RBPs identified in the screen suggests a lower level of conservation, which, due to the potential for underestimation by confounding factors, warrants reinvestigation of the remaining 42 proteins.

Nanos and NOS proteins

One such protein under reinvestigation is the *C. elegans* homolog of the *Drosophila* protein, Nanos. The Nanos protein contains a C-terminal CCHC zinc-binding domain, which is characteristic of RBPs (Gambieri *et al.*, 2006; Curtis *et al.*, 1997). This domain allows for Nanos to associate with RNA, but does not convey RNA sequence specificity (Curtis *et al.*, 1997). The function of Nanos as a translational regulator has been heavily characterized in the germline of several organisms, but information is still limited pertaining to its function in neurons.

In the *Drosophila* germline, Nanos functions as a translational repressor in early embryos to establish body-axis polarity (Wang & Lehmann, 1991). Rather than binding to mRNAs directly, embryonic Nanos binds to target ribonucleoprotein particles (RNPs) and prevents translation of mRNA in a spatially dependent manner. This spatial information is conveyed through the expression of Nanos across a maternally established concentration gradient in early *Drosophila* embryos (Murata & Wharton, 1995). One mechanism for the translational repression exhibited by Nanos is the recruitment of deadenylase to mRNAs in embryonic RNPs, which occurs when Nanos regulates the mRNA of cell cycle regulators, like cyclin B (Figure 2; Kadyrova *et al.*, 2007).

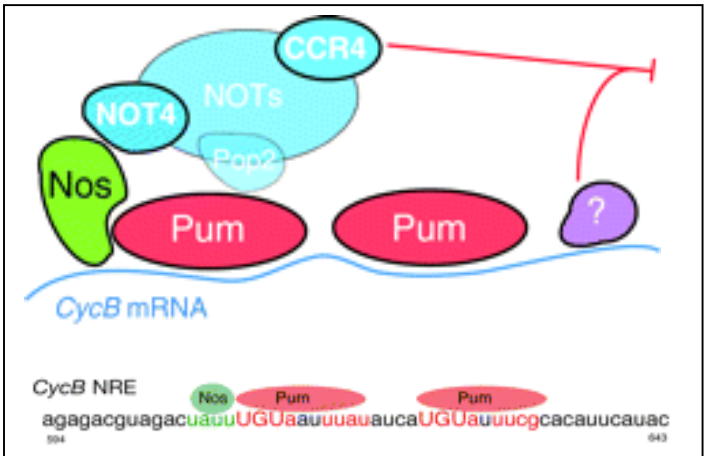

Figure 2. A model of translational repression by the *Drosophila* Nanos RBP. The RNA-binding domain of the Nanos protein recognizes target sites on mRNA and Nanos then recruits silencing factors, such as Pum, CCR4, Pop2, NOT4, and NOTs, to the target genes in order to prevent translation (Kadyrova *et al.*, 2007).

In order to promote biologically relevant expression patterns, *nanos* mRNA itself also undergoes translational regulation. Motor proteins such as dynein associate with *nanos* mRNA in order to localize the transcript and establish proper spatial distribution of the Nanos protein (Xu *et al.*, 2013). In oogenesis, *nanos* mRNA is also found to be anchored in an actin-dependent manner to the posterior germ plasm (Forrest and Gavis, 2003; Sinsimer *et al.*, 2011). In further support of regulation, *nanos* mRNA has been identified in mobile ribonucleoprotein particles in *Drosophila* da neurons (Brechbiel & Gavis, 2008). This suggests that neuronal

subcellular localization of Nanos is important to its later function, as is the case in *Drosophila* embryos where ectopic Nanos expression leads to severe developmental defects (Wang & Lehmann, 1991). The *nanos* 3' UTR also serves as a regulation point for *nanos* mRNA so as to maintain expression in the germline while downregulating somatic cell expression (Agostino *et al.*, 2006).

Some organisms contain multiple homologs of a gene in their genome formed by duplication events. These duplicated genes are known as paralogs. The *C. elegans* genome contains three paralogs of *nos*, which is the *C. elegans* homolog of D*rosophila nanos*. When paralogs act independently, they each exhibit distinct functions, whereas redundant paralogs have overlapping functions. Studies have found that *nos* paralogs often share general translational repression functions in the germline, but differ in their specific effects and targets, causing most paralogs to be only partially redundant (Kraemer *et al.*, 1999; Suzuki *et al.*, 2007). RNAi and knockout experiments in *C. elegans* suggest that the NOS-1 and NOS-2 proteins function redundantly in the germline, whereas germline NOS-3 may have a unique role distinct from the other paralogs (Subramaniam & Sedoux, 1999). The comparatively larger size of the NOS-3 protein and a unique N-terminus Q/N rich domain in NOS-3 known to modulate PAR proteins also support the idea that NOS-3 may have additional roles independent from the other paralogs (Labbe *et al.*, 2006).

Role of Nanos in neuronal morphogenesis

As an RNA-binding protein and translational repressor, Nanos is an important protein to examine in the context of dendrite morphogenesis. Investigations into somatic expression of Nanos in *Drosophila* have found *nanos* mRNA expressed in neural tissue (Haraguchi *et al.*, 2003; Ye *et al.*, 2004). Because *nanos* mRNA is transported in RNPs to dendritic termini and branch points, it is likely that Nanos may perform a role within neurons relating to dendrite morphogenesis (Brechbiel & Gavis, 2008; Xu *et al.*, 2013). Synaptic plasticity and dendrite morphogenesis are both dependent upon environmental stimuli, and can be responsible for an organism's ability to adapt to changes. As such, environmental adaptation is one way to study the role of a protein in effecting neurological changes. In order to test the effects of *nanos* null mutations on synaptic plasticity, odor adaptation experiments were performed in *C. elegans* which revealed that NOS-1 is required for odor adaptation, while NOS-3 is not (Kaye *et al.*, 2009). This study highlights the importance of Nanos proteins in neurological functions and raises questions about how extensive the redundancy of Nanos paralogs is in this role.

There are many specific ways in which Nanos proteins contribute to synaptic plasticity and dendrite morphogenesis. Nanos has been found to be a negative regulator of bouton formation in neuromuscular junctions such that excess Nanos leads to a decrease in the number of synaptic boutons (Menon *et al.*, 2009). This is important because boutons are a component of neuron morphology, and thus by affecting bouton formation, Nanos must be important to at least one aspect of neuron morphogenesis. Along this line, Nanos has also been suggested to be responsible for the maintenance of dendrites, as removal of Nanos did not show effects in the initial formation of da neuron dendrites but did show effects in the later stages of dendrite morphogenesis (Brechbiel & Gavis, 2008). In *Drosophila*, Nanos increases the stability of existing dendrites and promotes the outgrowth of new dendrites from da neurons after a certain stage in larval development (Olesnicky *et al.*, 2012). Additionally, Nanos is known to regulate postsynaptic glutamate receptors in *Drosophila* neurons, which is important because glutamate receptors mediate neurotransmission and play a key role in learning, memory, and neurological diseases (Menon *et al.*, 2009; Nakanishi, 1992).

Experiments causing Nanos to be over-expressed or under-expressed in *Drosophila* da neurons show that Nanos is important to dendrite morphogenesis. When Nanos is over-expressed in *Drosophila* da neurons, there is a decrease in higher order dendrites, and when it is under-expressed, neurons likewise exhibit dendrite abnormalities (Ye *et al.*, 2004). However, when the strongest *C. elegans* or mouse homologs, *nos-1* and *nanos1*, respectively, are removed from expression, there appear to be no PVD dendrite defects in C. elegans or behavioral abnormalities in mice (Antonacci *et al.*, 2015; Haraguchi *et al.*, 2003). This information on its own would suggest that Nanos is not important to neurological development in these organisms, but the tendency for Nanos to have a conserved role across species brings that conclusion into question (Subramaniam & Seydoux, 1999; Kanska and Frank, 2013). One explanation for this discrepancy is that both mice and *C. elegans* contain multiple paralogs of Nanos, which may be acting redundantly and diminishing the effect of a single paralog knockout. The *Drosophila* genome only contains one *nanos* gene, whereas both *C. elegans* and mice contain three *nanos*-homolog genes, which might explain why a single knockout in *Drosophila* is more severe than in other model organisms (Suzuki *et al.*, 2007; Subramaniam & Seydoux, 1999).

C. elegans and the PVD neuron as model systems

Nanos has not yet been investigated to the same extent in *C. elegans* neurons as it has been in *Drosophila* neurons. However, because the functions of RBPs are often conserved

between *Drosophila* and *C. elegans,* and the Nanos proteins have already shown conserved functions in the germline, it is possible that the *nanos*-homolog genes also play a conserved role in *C. elegans* neurons (Subramaniam and Seydoux, 1999). While RBP conservation with *Drosophila* is likely true for many organisms, there are certain advantages to using *C. elegans* for this screen.

For the purposes of our experiments, the multinucleate syncytium of the *C. elegans* germline allows for easy introduction of transgenes to future progeny through microinjection (Mello *et al.*, 1991; Mello and Fire, 1995). Additionally, the transparent cuticle of the worm allows for *in vivo* visualization of fluorescently labeled neurons, which is not possible in some other model organisms. The fixed number and position of neurons in a wild-type organism also makes it possible to consistently identify the same neuron for experimental assessment (Sulston and Horvitz, 1976).

We chose to investigate the PVD neuron, which is a multidendritic neuron that acts as a mechanoreceptor, proprioceptor, and nociceptor throughout the *C. elegans* body (Albeg *et al.*, 2011). There are two posteriorly localized neural cell bodies corresponding the left PVD and the right PVD (PVDL and PVDR, respectively). Each PVD contains a regular pattern of dendritic branching with primary dendrites extending from the cell body along the anterior/posterior axis and subsequent branch orders extending orthogonally from the prior branch order (White *et al.*, 1984). Because of this regular branching pattern, dendrite abnormalities can be easily quantified by comparing the number of dendrites on PVD neurons (Figure 3).

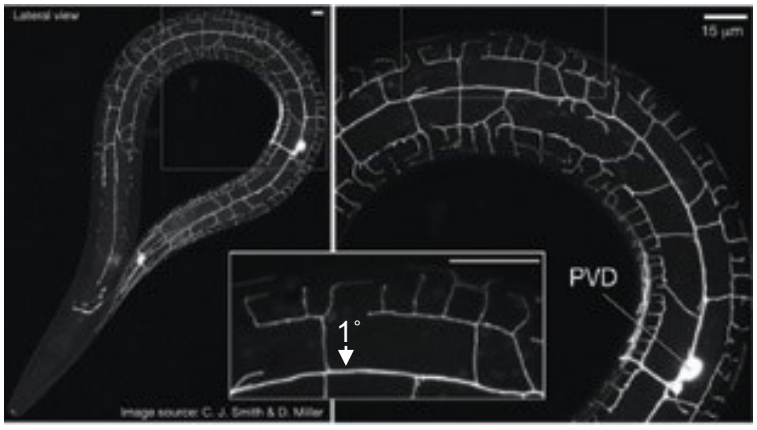

Figure 3. A fluorescently labeled PVD neuron, which highlights the structure and branching pattern of the neuron. Centered is a section of the PVD dendritic tree with numbers to indicate the branch orders of the dendrites (Image by C. J. Smith and D. Miller, WormAtlas).

Results:

Single knockout of nos paralogs show no fourth order dendrite defects

Despite homology with *Drosophila nanos* and a conserved role of germline Nanos, single knockout of *C. elegans nos* paralogs show no decrease in terminal dendrites, as it does with *Drosophila.* The *nanos* homolog with the most conserved amino acid sequence, *nos-1*, was tested in our original RBP screen (Antonacci *et al.*, 2015), but the *nos-1* mutant did not show significant abnormalities in the PVD neuron. Using green fluorescent protein (GFP) driven by a PVD-specific promoter, we subsequently looked at the PVD neurons of *nos-2* mutants and *nos-3* mutants and found no noticeable difference in the fourth order dendrites as compared with control worms (Figure 4).

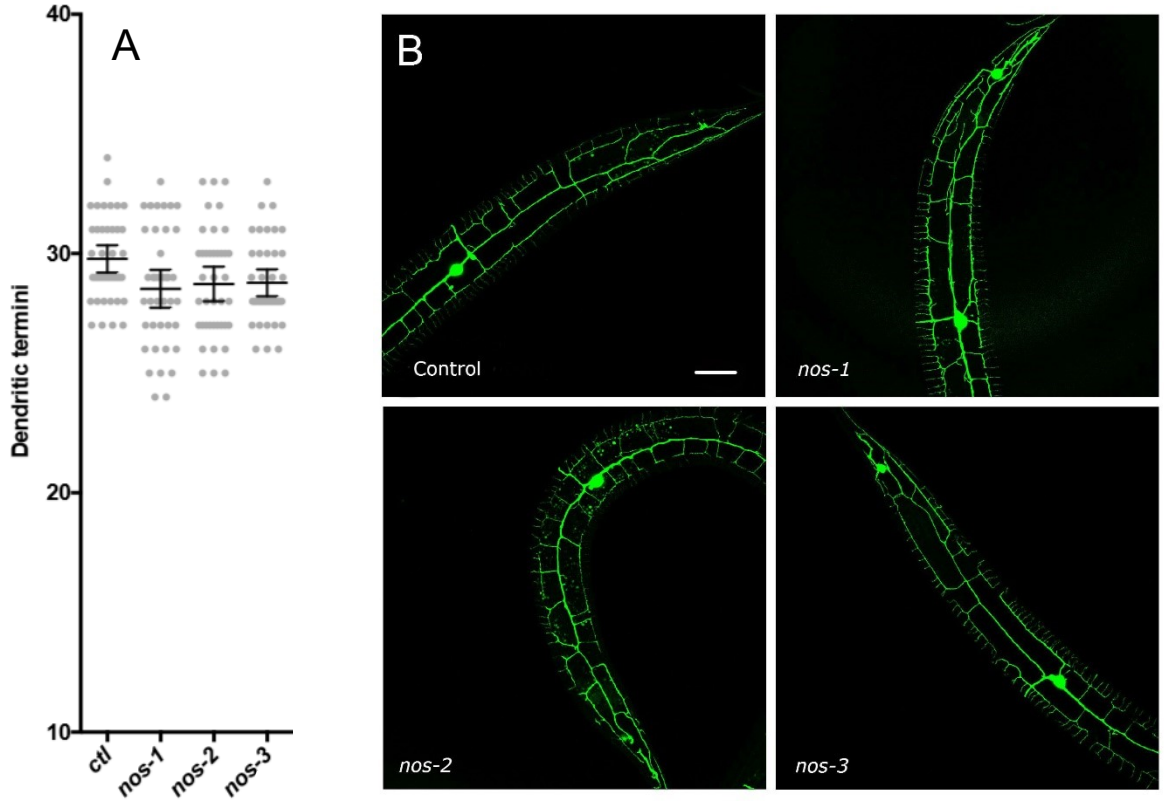

Figure 4. Single knockout of *nos* paralogs did not lead to any significant decrease or apparent abnormality in terminal dendrites as compared with control worms. All strains also carried the *wdIs51* and *uIs69* transgenes for GFP labeling of the PVD and sensitization to RNAi, respectively. Significance was determined using a p-value of 0.05 and a one way ANOVA with Tukey multiple comparison test for n=40 samples. **(A)** Graphical depiction of individual dendrite counts and average number of terminal dendrites for each strain. **(B)** Fluorescently labeled PVD neurons of control worms and each single *nos* knockout mutant. Scale Bar = 25 μm.

Disruption of multiple nos paralogs does lead to fourth order dendrite defects

The possibility of genetic redundancy due to the existence of three *nos* paralogs in the *C. elegans* genome prompted the investigation into multiple *nos* knockout mutants. Unlike the single *nos* mutants, the *nos-2 nos-1* double knockout mutant showed a notable decrease in fourth order dendrites. Because the three *nos* genes are all contained within 2.75 centiMorgans on chromosome II, recombination is very infrequent. As a result, we decided to use RNAi

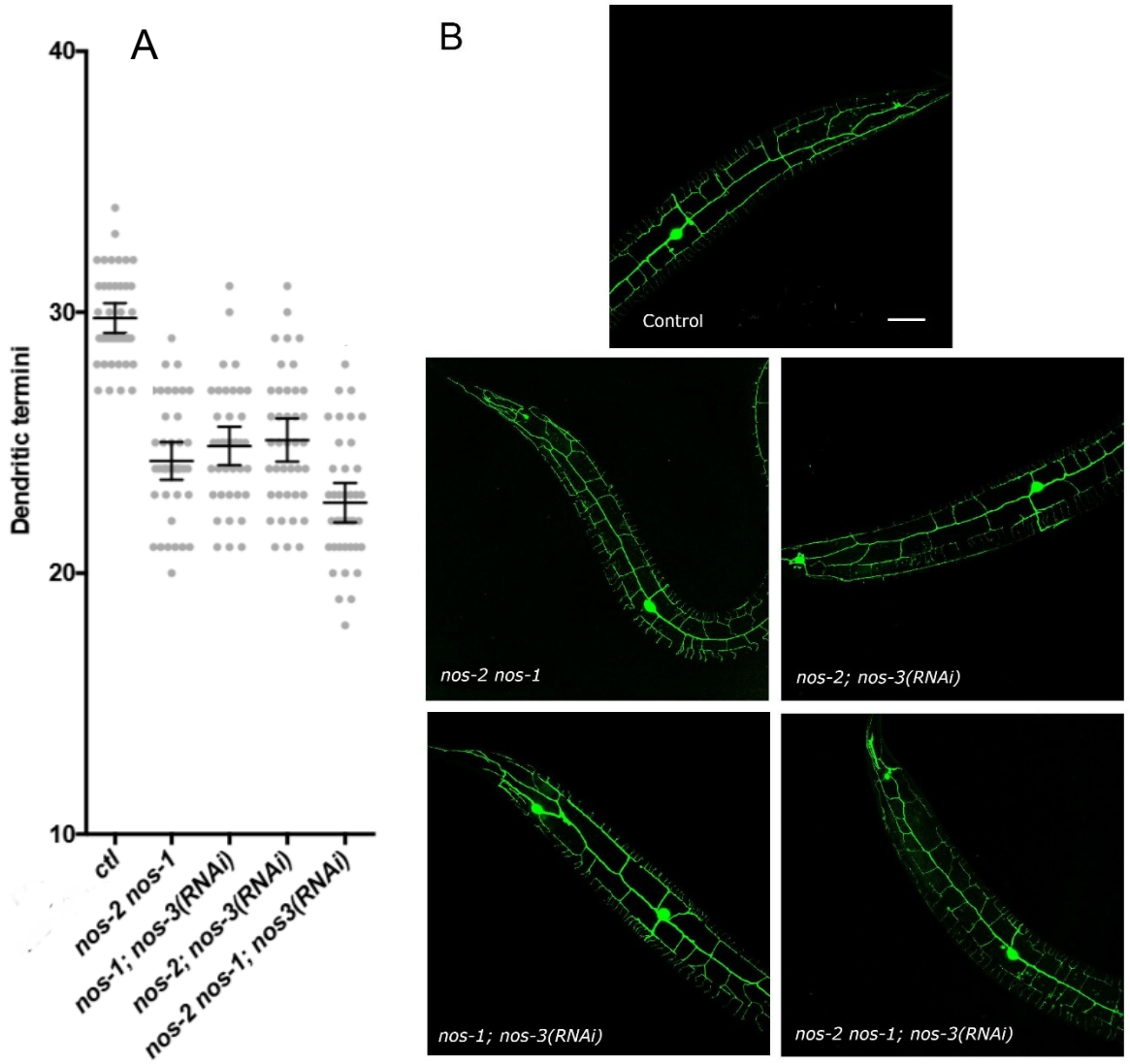

Figure 5. Double and triple *nos* mutants all display significant decreases in the number of terminal dendrites as compared with the control worms. All strains also carried the *wdIs51* and *uIs69* transgenes for GFP labeling of the PVD and sensitization to RNAi, respectively. Significance was determined using a p-value of 0.05 and a one way ANOVA with Tukey multiple comparison test for n=40 samples. **(A)** Graphical depiction of individual dendrite counts and average number of terminal dendrites for each strain. **(B)** Fluorescently labeled PVD neurons of control worms and each double and triple *nos* mutant. Scale Bar = 25 μm.

knockdown of *nos-3* to investigate dendritic phenotypes resulting from the disruption of two or three *nos* paralogs. The simultaneous disruption of two or three paralogs, made by RNAi knockdown on single or double mutants, exhibited a strong phenotype of fourth order dendrite reduction. Disruption of two paralogs led to significantly fewer fourth order dendrites than the control worms, while disruption of all three led to significantly fewer dendrites than both the control and the double mutants (Figure 5).

Paralogs of nos are redundantly required for proper fourth order dendrite morphology

Because a reduction in dendrite numbers was not evident upon removal of only one *nos* paralog but defects were present upon the removal of two or three paralogs, the results suggest that the *nos* paralogs act redundantly to establish and maintain proper dendritic phenotypes. Removal of any two paralogs produced the same statistically significant decrease in fourth order dendrites, which suggests that there are no major discrepancies between the *nos* paralogs in the extent to which each paralog is involved in terminal dendrite morphology. At least two *nos* paralogs are necessary to maintain the proper number of fourth order dendrites. The triple mutant showed an even more significant decrease in dendrites, suggesting that the number of defective *nos* genes correlates with the severity of dendrite defects.

Knockout mutants of nos-3 show second and third order dendrite defects

Considering that the single *nos* knockout mutants did not show fourth order dendrite defects, we investigated the possibility of lower order dendrites being affected by single *nos* knockout. The *nos-3* single knockout mutant displayed a 19.4% decrease in third order dendrites and an 18.9% decrease in second order dendrites. The other single knockout mutants did not show significant reductions in secondary or tertiary dendrites (Figure 6).

Paralogs of nos are expressed in the PVD neuron

Functional studies suggest that *nos* paralogs regulate dendrite morphology in the PVD neuron. Therefore, it is important to determine the *nos* expression patterns to see if *nos* genes are expressed within the PVD. When GFP was expressed under the control of the *nos-1* and *nos-3* promoters, fluorescence was observed in the PVD, suggesting that these genes are natively expressed in the PVD. There were also many other areas of the *C. elegans* body exhibiting *nos* expression, including the spermatheca, muscle cells, and other neurons. The indication of *nos-1* and *nos-3* expression in the PVD, coupled with the importance of NOS proteins for PVD dendrite morphogenesis, suggests that the NOS-1 and NOS-3 proteins

function in a cell-autonomous manner to ensure proper PVD morphology (Figure 7). The expression pattern for *nos-2* has not yet been determined in this manner.

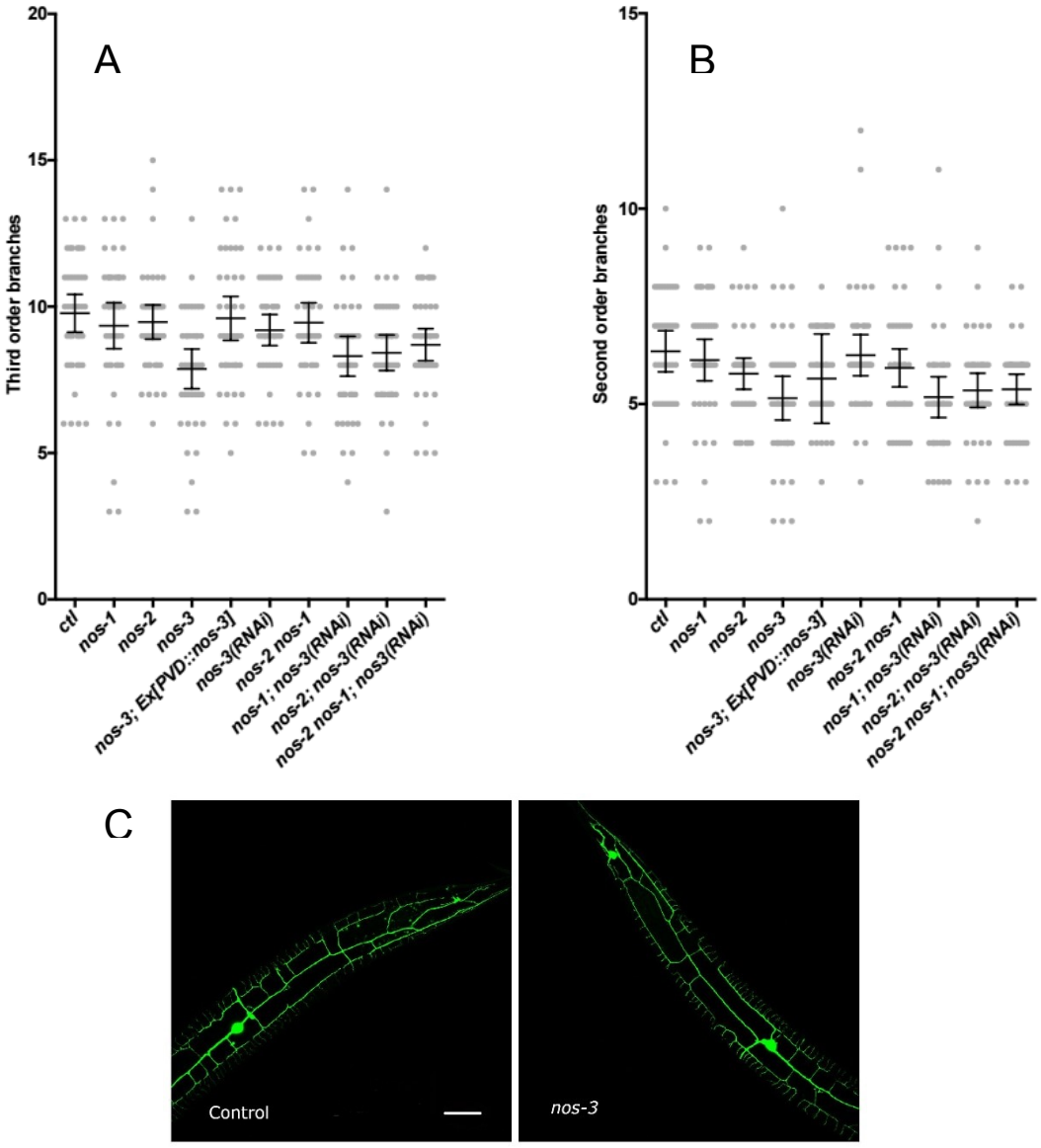

Figure 6. The *nos-3* mutant exhibited decreases in both secondary and tertiary dendrites as compared with control worms. All strains also carried the *wdIs51* and *uIs69* transgenes for GFP labeling of the PVD and sensitization to RNAi, respectively. Significance was determined using a p-value of 0.05 and a one way ANOVA with Tukey multiple comparison test for n=40 samples. **(A)** and **(B)** Graphical depiction of individual dendrite counts and average number of secondary and tertiary dendrites for each strain. **(C)** Fluorescently labeled PVD neurons of control worms and *nos-3* mutants. Scale Bar = 25 µm.

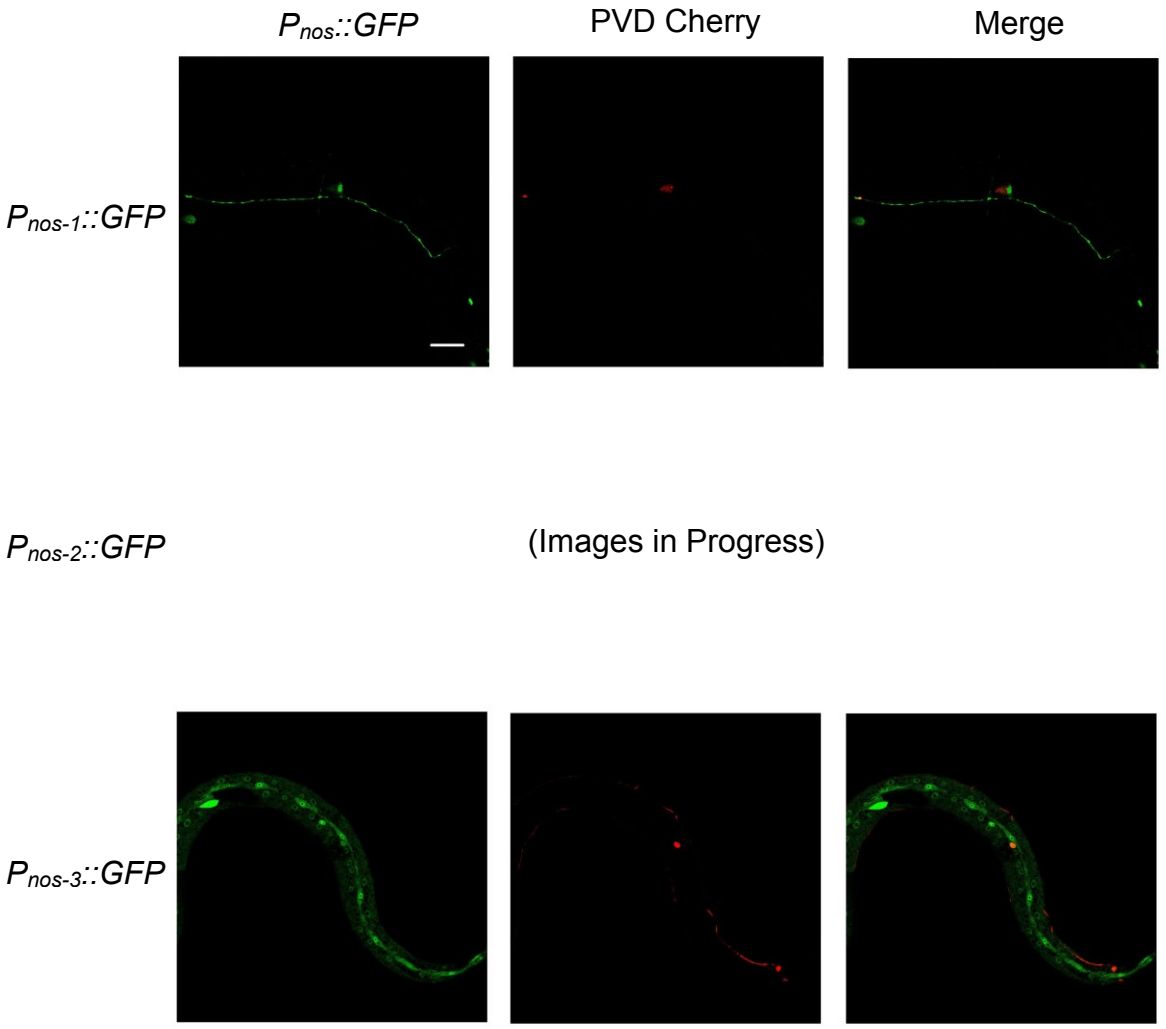

Figure 7. Transcriptional fusions of the *nos* paralog promoters drive GFP expression in the PVD neuron. Areas displaying green fluorescence represent areas with endogenous *nos* expression. Areas displaying red fluorescence represent areas under the control of the *mec-7* promoter, most notably the PVD neuron. Areas where red and green fluorescence overlap appear yellow and indicate *nos* expression in the PVD neuron. Scale Bar = 25 μm.

All three NOS proteins localize as particles in the cytoplasm of the PVD neuron

After determining the expression pattern of the *nos* paralogs, we felt it was important to address the subcellular localization of NOS proteins in the PVD because a detailed characterization of neuronal NOS proteins may offer insight into their molecular functions in dendrite morphogenesis. Using animals expressing a fusion of each NOS protein with GFP

driven by a PVD-specific promoter, we visualized the subcellular localization of the NOS proteins in the PVD. We found that each NOS protein is localized to the cytoplasm of the PVD and is also present in a small number of distinct particles or granules, which are visible in the cell body and primary dendrites (Figure 8). This localization pattern is consistent with that of *Drosophila* Nanos in da neurons because Nanos also presents as cytoplasmic granules in the cell body and dendrites (Ye *et al.*, 2004).

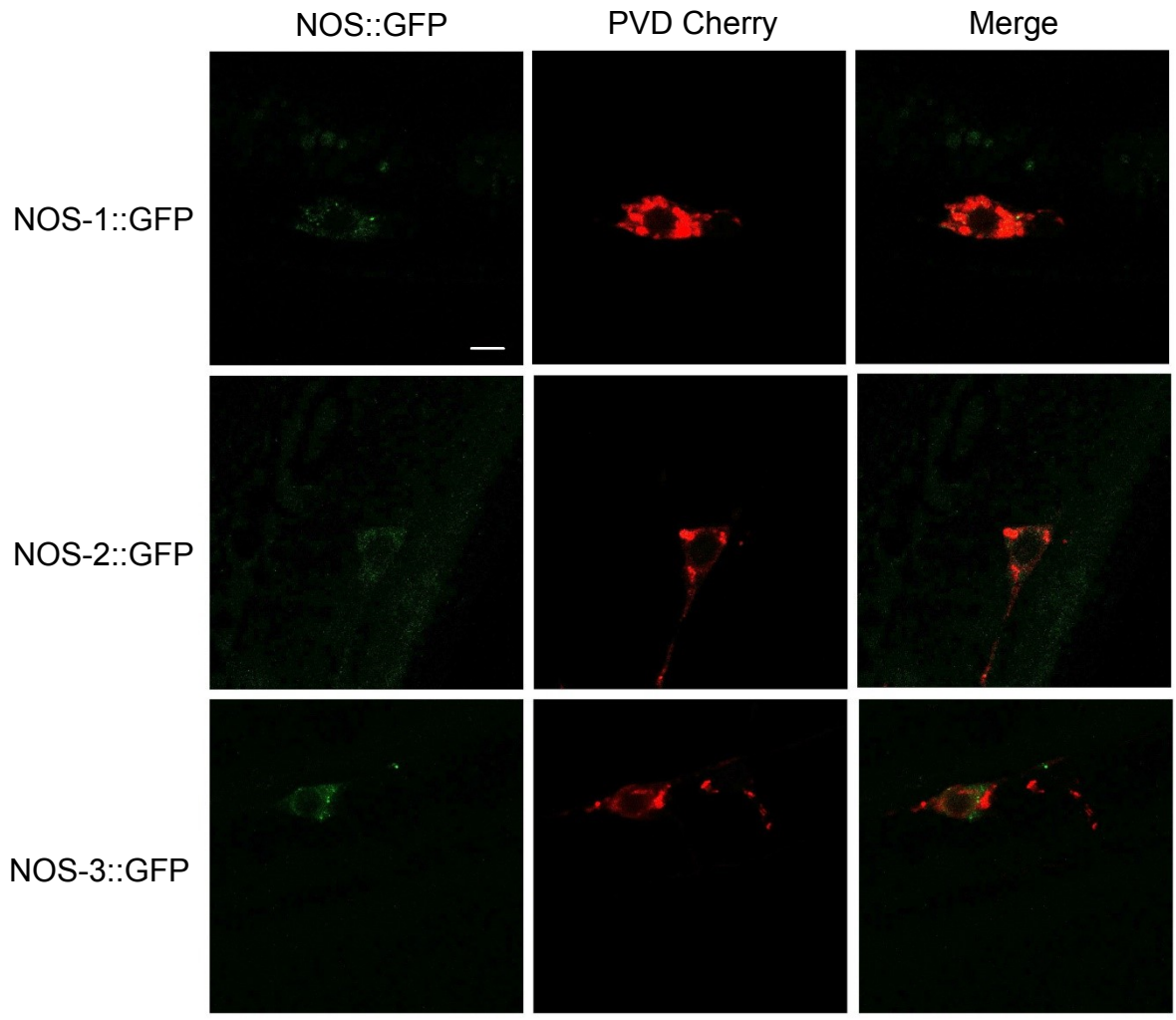

Figure 8. Translational fusions of the NOS proteins with GFP driven by *ser-2prom3.* The NOS proteins appear to express as small particles in the cytoplasm of the PVD neuron. Areas of green fluorescence indicate the subcellular localization of each NOS protein. The PVD neuron is labeled with mCherry. Scale Bar = 25 μm.

Exogenous nos-3 partially rescues second order and fully rescues third order dendrite morphology in nos-3 single knockout mutant

When dendrite defects are observed in response to a single knockout mutation, the knocked out gene is suggested to be necessary to the process of dendrite morphogenesis. In order to further support its role in dendrite morphogenesis, rescue experiments can also be performed to see if the respective proteins act cell-autonomously to restore proper dendrite morphology. A rescue experiment was performed on the *nos-3* mutant, which revealed that exogenous *nos-3* did have restorative effects on second and third order dendrites. For third order dendrites, the rescued mutants had dendrite counts which were significantly different from the nos-3 mutants, but not significantly different from the control, indicating a full phenotypic rescue (Figure 6A). For second order dendrites, the rescued mutants had dendrite counts which were not significantly different from either nos-3 mutants or the control, indicating only partial rescue (Figure 6B). Rescue experiments are still in progress to test the effects of exogenous *nos-1* or *nos-2* on the *nos-2 nos-1* double knockout mutant.

Discussion:

Paralogs of nos-1 function in a partially redundant manner in dendrites

In our previous screen of *C. elegans* RBPs, we identified only 12 RBPs important to the process of dendrite morphogenesis out of 54 gene candidates (Antonacci *et al.*, 2015). The gene candidates for the original study were selected based on homology to *Drosophila* RBPs known to function in neuron morphogenesis. All 54 of the *Drosophila* homologs were important to this process, whereas only a fraction of the 54 *C. elegans* candidates returned positive results for involvement in neuron morphology. This suggests that the number of genes identified in the original study could be an underestimate. There are many reasons why the screen may have underestimated the number of neurologically important gene candidates, including false negatives due to RNAi inefficiency and maternal rescue in null mutants. In the case of *nos-1*, we suspected that the presence of multiple *nos* paralogs in the *C. elegans* genome masked the effects of the *nos-1* mutation due to genetic redundancy between paralogs.

Drosophila Nanos was previously identified as important to both neuron development and body axis determination in early embryos (Olesnicky *et al.*, 2012; Wang and Lehmann, 1991; Ye *et al.*, 2004). Its mRNA localization and its function as a translational repressor are crucial to both of these processes. Not only is the Nanos protein an RBP, but its mRNA is also an important RBP target for transportation and spatial determination (Brechbiel and Gavis,

2008). This information, coupled with the fact that *nanos* mRNA has been found in dendrites, makes it unsurprising that Nanos is important to dendrite morphogenesis, as this is a process that relies heavily upon post-transcriptional regulation. It was surprising, however, that *nos-1*, the strongest *nanos* homolog in *C. elegans*, was not initially identified as an important RBP gene in our original *C. elegans* RBP screen despite the many ways in which NOS proteins can participate in post-transcriptional regulation. Because Nanos has similar properties to other important translational regulators in neurons, *nos-1* was the first gene we wanted to reexamine to understand why it presented as a negative hit in our initial screen.

In order to identify why Nanos was necessary to *Drosophila* neuron morphogenesis while NOS-1 was not to *C. elegans*, it was important for us to look for differences in the two organisms with respect to the *nanos* and *nos* genes. The most noticeable difference was that *Drosophila* only contains one copy of the *nanos* gene, whereas there are three *nos* paralogs in the *C. elegans* genome which could be contributing to genetic redundancy. This difference turned out to be the one primarily responsible for the missing phenotype in the *nos-1* knockout mutant. In the presence of additional *nos* paralogs, the *nos-1* null mutation had incomplete penetrance because the overlapping functions between paralogs reduced the phenotype of the single mutant. Mutant phenotypes only became noticeable when at least two of the three paralogs were non-functional or absent. However, the presence of aberrant dendritic phenotypes in the multiple *nos* mutants indicates that the *nos* paralogs are necessary to dendrite morphogenesis as we initially suspected. Our results may be applicable to other organisms with multiple paralogs of this gene, and it could be the case that genetic redundancy was responsible for the decreased penetrance of the *nanos1* mutation in mice, an organism that also has three *nanos* paralogs (Haraguchi *et al.,* 2003).

The genetic redundancy of the *nos* paralogs includes sufficient functional overlap between all three paralogs such that any two paralogs could carry out a sufficient number of common functions to buffer against fourth order dendrite defects in the *C. elegans* PVD neuron. However, the paralogs did not exhibit complete redundancy due to unique roles of the *nos-3* paralog, which, on its own, is necessary to the development and maintenance of second and third order dendrites. The presence of functional NOS-1 and NOS-2 could not prevent second and third order dendrite defects in the *nos-3* mutant. The observation of additional roles for the NOS-3 protein is consistent with prior studies indicating that there are additional binding domains and interactions unique to NOS-3 due to the 2 Kb larger size of the *nos-3* gene than *nos-1* and *nos-2* (Kraemer *et al.*, 1999; Labbe *et al.*, 2006). Our results suggest that the three

nos paralogs are genetically redundant in the roles necessary for proper terminal dendritic branching in the PVD, but overall the paralogs are only partially redundant in neurons due to unique roles held by *nos-3* in the context of lower order dendritic branching.

Translational repression by nos paralogs and localization of nos mRNA contribute to dendrite morphogenesis

Transport of mRNA into dendrites is a major mechanism involved in dendrite morphogenesis due to the resulting localization and translational control of transcripts relevant to the morphogenic process (Brahman and Wells, 2007; Xu *et al.*, 2013; Di Liegro *et al.*, 2014). One such transcript known to exist in mobile neuronal ribonucleoprotein particles is *nanos* mRNA, and thus the importance of Nanos in maintaining dendrites is consistent with the idea that mRNA localization aids in morphogenesis (Brechbiel and Gavis, 2008; Xu *et al.*, 2013). Additionally, we found that the three NOS RBPs exhibit cytoplasmic expression in distinct granules, relating to the previously described function of NOS as a translational repressor within RNPs (Gambieri *et al.* 2006; Kadyrova *et al.*, 2007). This expression pattern and subcellular localization resembles that of other RBPs thought to be involved in mRNA transport to dendrites and translational regulation, such as CPEBs and CGH-1 (Antonacci *et al.*, 2015). Because of this resemblance and the presence of NOS particles in primary dendrites, it can be speculated that the NOS proteins have additional functions relevant to mRNA transport.

Future directions

In order to address the possibility of *nos* paralogs being involved in dendritic mRNA transport, it would be important to assess whether or not the NOS particles are mobile. A total internal refection fluorescence (TIRF) microscope would be appropriate for this experiment because of its high resolution and ability to visualize fluorescent particle motion *in vivo*. It would also be helpful to further identify specific roles of the NOS RBPs in order to see how they interact with other neuronal components and identify the degree to which the paralog functions overlap. This could be accomplished through immunoprecipitation studies which would identify proteins and mRNAs that associate with the NOS RBPs. The identification of associated proteins and mRNAs could provide insight on which specific pathways are regulated by NOS-mediated translational repression and how those interactions lead to proper neuron morphology. The results of these studies could also reveal which targets overlap between the three NOS proteins and provide additional support for the conclusion of partial genetic redundancy between

the paralogs. Additionally, the identification of these targets could reveal new candidates for the regulation of dendrite morphogenesis and of specific branch orders.

Like *C. elegans*, humans also contain three *NANOS* paralogs, *NANOS1*, *NANOS2*, and *NANOS3*. There is confirmed expression of NANOS1 in human brains, and while NANOS3 does not show brain expression, it is still expressed in ocular tissue, which is home to many sensory neurons. The expression pattern of NANOS2 has not yet been analyzed, but because it is the strongest human homolog of both *nos-1* and *nos-2*, it would not be surprising to find brain expression of NANOS2 in humans (Table 1). Information on the human *nanos*-homolog expression pattern was obtained through the TiGER database (Liu *et al.*, 2008; Antonacci *et al.*, 2015). It would be interesting to determine whether the three *NANOS* paralogs in the human genome share partial redundancy similar to that of the nematode paralogs. With the knowledge gained through these experiments, and the suggested experiments above, we will hopefully be able to suggest a role for the human *NANOS* proteins in dendrite morphogenesis. Ultimately the goal of this study is to characterize neuronal RBPs so future researchers can identify their possible contributions to aberrant neuronal morphogenesis and observed neurological diseases in humans.

Table 1.

Brain expression of NOS-1 human orthologs.

OrthoDB ID	Human Ortholog	E Value	Brain Expression
EOG7PGDT1	NANOS1	7e-05	Yes
	NANOS2	5e-05	Not assessed
	NANOS3	0.007	No

Note: The NOS-1 protein was aligned to each human NANOS protein sequence from the RefSeq database using BLASTp to generate E values. Brain expression was determined using the TiGER Database (Liu *et al.*, 2008).

Materials and Methods:

C. elegans strains

Strains used during the course of this study can be found in Table 2.

Table 2.

C. elegans strains.

Strain	Genotype	Reference
DR96	[*unc-76*(e911)-V]	(Brenner, 1974)
DR466	[*him-5*(e1490)]	(Hodgkin *et al.*, 1979)
TV15709	[*wyIs5787*(*ser-2prom3*::*myr-mcherry*)-II]	(Liu and Shen, 2012; Dong *et al.*, 2013)
NC1686	[*wdIs51*($P_{F49H12.4}$::*GFP unc-119*(+))-X]	(Watson *et al.*, 2008; Smith *et al*., 2010)
NC1687	[*wdIs52*($P_{F49H12.4}$::*GFP unc-119*(+))-II]	(Watson *et al.*, 2008; Smith *et al.*, 2010)
TU3401	[sid-1(pk3321); uIs69(P_{myo-2}::*mCherry* + $P_{unc-119}$::*sid-1*)]	(Calixto *et al.*, 2010)
RB518	[*nos-1*(ok250)-II]	(OMRF)
JH1463	[(*nos-2*(ok230)-II, *unc-4*(e120)-II)/*mnC1*(*dpy-10*(e128), *unc-55*(e444))-II]	(Subramaniam and Seydoux, 1999)
JK2589	[*nos-3*(q650)-II]	(Kraemer *et al.*, 1999)
BS5351	[(*nos-2*(ok230)-II, *nos-1*(gv5)-II)/*mIn1*(*dpy-10*(e128), *mIs14*)-II]	(Hansen *et al.*, 2004)
DJK110	[*unc-76*(e911)-V; *rwIs1*(P_{mec-7}::*RFP*)]	
DJK177	[*unc-76*(e911)-V; *wyIs587*-II]	
DJK68	[*wdIs52*-II; *sid-1*(pk3321)-IV; *uIs69*-V]	
DJK194	[(*nos-2*(ok230)-II, *unc-4*(e120)-II)/*mIn1*-II; *wdIs51*-X]	
DJK203	[*nos-3*(q650)-II; *wdIs51*-X]	
DJK204	[(*nos-2*(ok230)-II, *nos-1*(gv5)-II)/*mIn1*-II; *wdIs51*-X]	
DJK93	[*nos-1*(ok250)-II, *wdIs51*-X]	
DJK228	[*unc-76*(e911)-V; *rwIs1*; Ex[P_{nos-3}::*GFP*, *unc-76* (+)]]	
DJK227	[*unc-76*(e911)-V; *wyIs587*-II; Ex[*ser-2prom3*::*GFP*::*nos-3* w/ 3' UTR, *unc-76*(+)]]	
DJK261	[*unc-76*(e911)-V; *rwIs1*; Ex[P_{nos-2}::*GFP*, *unc-76*(+)]]	
DJK256	[*unc-76*(e911)-V; *wyIs587*-II; Ex[*ser-2prom3*::*GFP*::*nos-2* w/ 3'UTR, *unc-76*(+)]]	
DJK269	[*unc-76*(e911)-V; *rwIs1*; Ex[P_{nos-1}::*GFP*, *unc-76* (+)]]	

DJK270	[*unc-76*(e911)-V; *wyIs587*-II; Ex[*ser-2prom3*::*GFP*::*nos-1* w/ 3'UTR, *unc-76*(+)]]
DJK254	[*nos-1*(ok250)-II; *uIs69*-V; *wdIs51*-X]
DJK257	[(*nos-2*(ok230)-II, *unc-4*(e120)-II)/*mIn1*-II; *uIs69*-V; *wdIs51*-X]
DJK255	[*nos-3*(q650)-II; *uIs69*-V; *wdIs51*-X]
DJK256	[(*nos-2*(ok230)-II, *nos-1*(gv5)-II)/*mIn1*-II; *uIs69*-V; *wdIs51*-X]
DJK271	[*uIs69*-V; *wdIs51*-X]
DJK (TBD)	[*nos-3*(q650)-II; *unc-76*(e911)-V; *wdIs51*-X]
DJK (TBD)	[(*nos-2*(ok230)-II, *nos-1*(gv5)-II)/*mIn1*-II; *unc-76*(e911)-V; *wdIs51*-X]
DJK (TBD)	[*nos-3*(q650)-II; *unc-76*(e911)-V; *wdIs51*-X Ex[*ser-2prom3*::*GFP*::*nos-3* w/ 3' UTR, *unc-76*(+)]]
DJK (TBD)	[(*nos-2*(ok230)-II, *nos-1*(gv5)-II)/*mIn1*-II; *unc-76*(e911)-V; *wdIs51*-X Ex[*ser-2prom3*::*GFP*::*nos-1* w/ 3'UTR, *unc-76*(+)]]
DJK (TBD)	[(*nos-2*(ok230)-II, *nos-1*(gv5)-II)/*mIn1*-II; *unc-76*(e911)-V; *wdIs51*-X Ex[*ser-2prom3*::*GFP*::*nos-2* w/ 3'UTR, *unc-76*(+)]]

Note: (OMRF) *C. elegans* Reverse Genetics Core Facility at the Oklahoma Medical Research Foundation. Strain names listed as DJK (TBD) are yet to be determined.

Transgene construction and microinjection

The presumptive promoter regions for each of the three Nanos paralogs were amplified using polymerase chain reaction (PCR). The primers used for the promoter amplifications flank the entire upstream intragenic region containing the presumptive promoters (their sequences are listed in Table 3). The amplified promoters were subcloned into the plasmid, pDONR221, through a BP reaction using Gateway BP Clonase II (Invitrogen). These new plasmids were then used in LR reactions with Gateway LR Clonase II Plus (Invitrogen) in order to subclone the promoters into a promoterless gateway cassette on plasmid, pDJK237. The cassette on

pDJK237 lies upstream of a GFP coding sequence with a *let-858* 3' UTR that was derived from pPD117.01.

We were unable to amplify *nos-1* cDNA, so we chose to instead amplify *nos-1* genomic DNA (gDNA) with a native 3' UTR. This PCR amplification used genomic DNA from lysates of *him-5*(e1490) as template DNA. The cDNAs from *nos-2* and *nos-3* were also amplified with native 3' UTRs using PCR. The primers for these three reactions are listed in Table 3 and a cDNA library derived from *him-5*(e1490) was used as template DNA for the amplifications of *nos-2* and *nos-3*. This cDNA library was created using Trizol/chloroform, oligo dT primers, and first-strand synthesis by Superscript Reverse Transcriptase III (Invirogen). Following amplification, the cDNA (and gDNA) fragments with 3' UTRs were cloned into pDONR P2r-P3 using Gateway BP Clonase II (Invitrogen). The resulting plasmids were then cloned into pDEST R4-R3 along with pDJK241 (pDONR P4-P1R with ser2prom3) and pDJK294 (pDONR221 with GFP) using Gateway LR Clonase II Plus (Invitrogen).

The transgenes were introduced into compatible strains through DNA microinjection using previously described procedures (Mello and Fire, 1995). The plasmids containing transcriptional fusions between Nanos promoters and GFP were injected into *unc-76*(e911); *rwIs1* hermaphrodites at a concentration of 10-20 ng/μL along with 60 ng/μL of *unc-76*(+) plasmid. The resulting strains, DJK269, DJK261, and DJK228, were used to assess expression patterns for the three Nanos paralogs. The plasmids containing translational fusions between GFP and Nanos cDNAs were injected into *unc-76*(e911); *wyIs587* hermaphrodites at a concentration of 10-20 ng/μL along with 60 ng/μL of *unc-76*(+) plasmid. The resulting strains, DJK270, DJK256, and DJK227, were used to assess the subcellular localization of each Nanos protein in the PVD.

Table 3.

Promoters used during transgene construction.

Promoter	Forward Primer	Reverse Primer
nos-1	5'GGGGACAAGTTTGTACAAAAAAG CAGGCTATTGATGAAAATTAACTAG AGATTT3'	5'GGGGACCACTTTGTACAAGAAAG CTGGGTGTTTGTAGGATTTCGAAGT TAAAATT3'
nos-2	5'GGGGACAAGTTTGTACAAAAAAG CAGGCTTATTTTATGTTTTTTGTAAC CTGAA3'	5'GGGGACCACTTTGTACAAGAAAG CTGGGTGCTTTCAAGAAGAACAAAA ACTCAAA3'
nos-3	5'GGGGACAAGTTTGTACAAAAAAG CAGGCTTTCACCTTAAAATGTTTTA GTAACG3'	5'GGGGACCACTTTGTACAAGAAAG CTGGGTGTACCAAGTGAACGTTGA CTGTAAAT3'

cDNA	Forward Primer	Reverse Primer
nos-1	5'GGGGACAGCTTTCTTGTACAAAGTGGTAATGTTGATTTTCAGGACTTCTCCG3'	5'GGGGACAACTTTGTATAATAAAGTTGGTTGACATGTTGATAAACTGAATTT3'
nos-2	5'GGGGACAGCTTTCTTGTACAAAGTGGTAATGTCTCTGGGTACTCCAAGTGAAC3'	5'GGGGACAACTTTGTATAATAAAGTTGCGAATTGTAAATTTTTATTTTCAGA3'
nos-3	5'GGGGACAGCTTTCTTGTACAAAGTGGTAATGTCTGGACAGCAGTTCCAGCAAC3'	5'GGGGACAACTTTGTATAATAAAGTTGTCGGAAACATAAATATTTGAAGATC3'
RNAi	**Forward Primer**	**Reverse Primer**
nos-1	5'GGGGACAAGTTTGTACAAAAAAGCAGGCTATCGATCCAGTTGTAAATTCCGTCG3'	5'GGGGACCACTTTGTACAAGAAAGCTGGGTACCTTGTAAGTATTGTCAGTGCTGC3'
nos-2	5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTGTATTCCTTTCCGCCACAACGCCG3'	5'GGGGACCACTTTGTACAAGAAAGCTGGGTTTCACGCCTTTTGAACAATGATGGC3'
nos-3	5'GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGTTGAGCCGCTCATGGGAGGCCC3'	5'GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTCTTTGGGGTCTTCTTCCGATG3'

Imaging and fluorescent microscopy

Young adult worms from each relevant strain were transferred to microscope slides with 2% agarose pads, then immobilized with 5 mM levamisole. Dendrite quantification experiments were conducted on a Zeiss Axioskop using the 40x objective. A Leica SP5 spectral confocal microscope was used to obtain images of mutant dendrite phenotypes using Leica LAS software and a 63x objective at 0.5 μm per step. Images of expression patterns and subcellular localization were also created using this method. A Leica M205 FA was used during strain construction in order to verify strain phenotypes and genotypes with GFP or Cherry fluorescent markers.

Quantification of PVD dendrite morphology

Fluorescently labeled PVDs from *nos* mutant strains at 20 ˚C were identified in young adults and positioned for scoring. In each organism, one PVD neuron (either the PVDL or PVDR) was selected for assessment and the dorsal and ventral branches of selected neurons were quantified separately. In order to quantify the dendrite phenotypes, dendrites from each branch order (2˚, 3˚, and 4˚) were counted from the PVD cell body to the worm posterior. Each experiment contained 20 animals for which the ventral and dorsal sides of one PVD were counted, and thus the experiments contained 40 samples of dendrite counts from cell body to

posterior. Dendrite counts were then processed with Prism6.0f software to yield graphical data representations and the results of one way ANOVA with Tukey multiple comparison tests (GraphPad Software, Inc).

RNAi

RNAi knockdown of *nos-3* was accomplished through a previously described feeding procedure (Kamath and Ahringer, 2003). *E. coli* expressing a *nos-3* dsRNA fragment were introduced to *nos* mutant strains that were hypersensitive to neuronal RNAi by feeding due to the *uIs69* transgene. To construct the active plasmids in these *E. coli*, a fragment of the *nos-3* gene was first amplified by PCR using the primers listed in Table 3. The PCR product was then subcloned into pDONR221 using Gateway BP Clonase II (Invitrogen), and the resulting plasmid was inserted into the pGC31 vector using Gateway LR Clonase II Plus (Invitrogen). The *E. coli* were seeded onto RNAi plates containing nematode growth medium, ampicillin, and IPTG. L4 hermaphrodites were first transferred to unseeded RNAi plates for 15 minutes, followed by another 15 minutes on a fresh unseeded RNAi plate, then the worms were placed onto to a seeded RNAi plate. After an hour, the original worms were then transferred to a final seeded RNAi plate and grown at 20 ˚C until progeny reached the young adult stage, at which time the progeny were scored.

Rescue experiments

The *unc-76*(e911) mutation was crossed into the *nos* mutants to create strains of the genotypes [*nos-3*(q650)-II; *unc76*(e911)-V; *wdIs51*-X] and [(*nos-2*(ok230)-II, *nos-1*(gv5)-II)/*mIn1*-II; *unc-76*(e911)-V; *wdIs51*-X]. In order to see if the fourth order dendrite defects of the *nos-2 nos-1* double mutant could be rescued with exogenous *nos-1*, we plan to inject the above mentioned *nos-2 nos-1; unc-76* strain with the previously described *nos-1* translational fusion plasmid. Transgenic progeny from the resulting lines will then be scored for fourth order dendrite defects at the young adult stage. The same process will be used with the *nos-2* translational fusion plasmid to determine whether exogenous *nos-2* can rescue fourth order dendrite defects in the *nos-2 nos-1* double mutant. To see if the second and third order dendrite defects in the *nos-3* single mutant could be rescued with exogenous *nos-3*, we injected the above mentioned *nos-3; unc-76* strain with the previously described *nos-3* translational fusion plasmid. The second and third order dendrites were scored in transgenic progeny from the resulting lines.

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