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Vitamin B12 Regulates Glial Migration and Synapse Formation through Isoform-Specific Control of PTP-3/LAR PRTP Expression

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SUMMARY

Vitamin B12 is known to play critical roles during the development and aging of the brain, and vitamin B12 deficiency has been linked to neurodevelopmental and degenerative disorders. However, the underlying molecular mechanisms of how vitamin B12 affects the development and maintenance of the nervous system are still unclear. Here, we report that vitamin B12 can regulate glial migration and synapse formation through control of isoform-specific expression of PTP-3/LAR PRTP (leukocyte-common antigen-related receptor-type tyrosine-protein phosphatase). We found the uptake of diet-supplied vitamin B12 in the intestine to be critical for the expression of a long isoform of PTP-3 (PTP-3A) in neuronal and glial cells. The expression of PTP-3A cell autonomously regulates glial migration and synapse formation through interaction with an extracellular matrix protein NID-1/nidogen 1. Together, our findings demonstrate that isoform-specific regulation of PTP-3/ LAR PRTP expression is a key molecular mechanism that mediates vitamin-B12-dependent neuronal and glial development.

In Brief

Zhang et al. describe glial migration and synapse formation defects stemming from vitamin B12 deficiency. Their study identifies a cell-autonomous mechanism regulating glial migration, where

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AUTHOR CONTRIBUTIONS

A.Z. and D.Y. devised the whole project. A.Z. characterized *mrp-5, ptp-3,* and *nid-1* phenotypes and performed all molecular, genetic, and imaging experiments. B.D.A. provided reagents and shared unpublished results. A.Z. and D.Y. wrote the manuscript with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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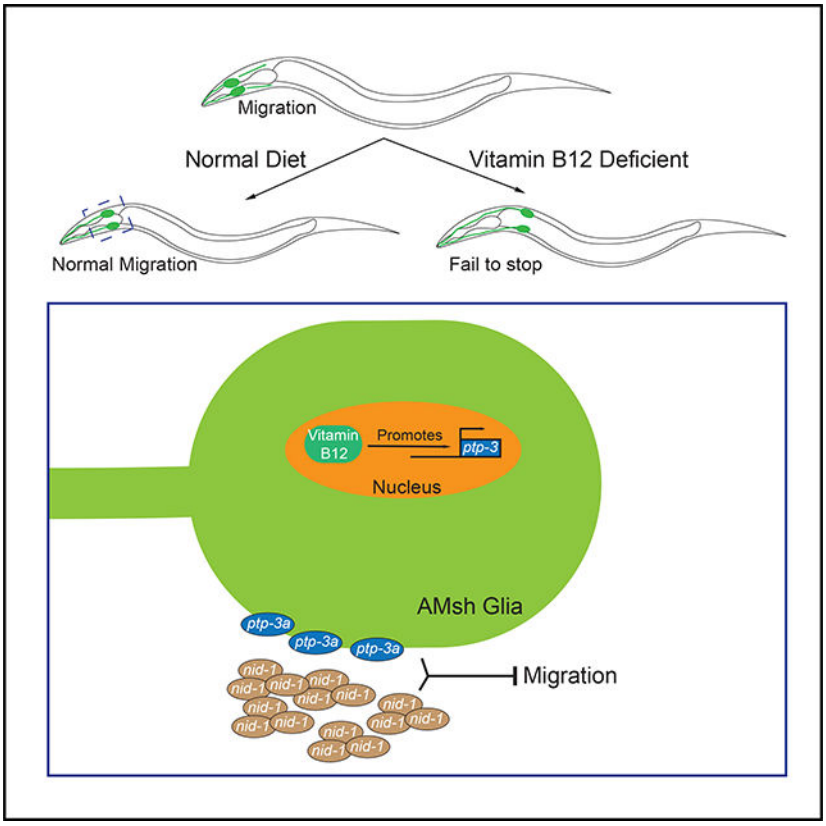
vitamin B12 regulates expression of the cell adhesion molecule PTP-3/LAR PRTP in an isoform-specific manner.

Graphical Abstract



INTRODUCTION

Vitamin B12 deficiency has long been associated with a range of neurological complications, including neurodevelopmental disorders, subacute combined degeneration, behavioral changes, and cognitive decline (Black, 2008; Douaud et al., 2013; Healton et al., 1991; Lindenbaum et al., 1988; Reynolds, 2006). Vitamin B12 functions as a coenzyme in two important biochemical reactions that both may be relevant to the symptoms. The first is during the conversion methylmalonyl coenzyme A (CoA) to succinyl CoA, which is important for fatty acid metabolism, and reduced function may lead to incorporation of abnormal odd-numbered and branched fatty acids into the myelin, resulting in increased fragility (Cardinale et al., 1970; Dror and Allen, 2008; Green and Kinsella, 1995; Stollhoff and Schulte, 1987). The other reaction mediated by vitamin B12 is the conversion of homocysteine into methionine. This is important for the generation of S-adeno-sylmethionine, a methyl donor important for the methylation of DNA and the synthesis of lipids, proteins, and neurotransmitters in the nervous system (Briddon, 2003; Dror and Allen, 2008; Green et al., 2017; Reynolds, 2006). Although the coenzyme function of vitamin B12 has been well documented in the development and aging of the brain, other

studies have also highlighted potential non-coenzyme functions of vitamin B12 in the nervous system (Scalabrino, 2009; Scalabrino et al., 1995), and there is still much to know about whether and how vitamin B12 affects the nervous system through its non-coenzyme functions.

Glial cells perform a wide range of vital roles in the nervous system, ranging from modulating development to actively playing a role in neuronal function (Barres, 2008; Fields et al., 2015; Mori et al., 2005). They need to migrate over what are often long distances from their birthplace to the appropriate regions to form functional units with neurons or perform other roles (Gilmour et al., 2002; Jarjour and Kennedy, 2004; Kinrade et al., 2001; Klämbt, 2009). The migration of glial cells can be divided into three steps: initiation; movement; and termination. Guidance molecules, including those from the netrin, slit, and semaphorin families can initiate and direct the movement of glial cells through interactions with their receptors that they express (Jarjour et al., 2003; Kinrade et al., 2001; Sasse and Klämbt, 2016; Spassky et al., 2002; Unni et al., 2012). However, it is still unclear what controls the termination of migration after glial cells reach their final positions.

C. elegans possess 56 glial cells in their body, which include 50 neuroepithelial glia that ensheath sensory neuron receptive endings (Oikonomou and Shaham, 2011; Shaham, 2015). The largest sensory organ of the worm, called the amphid sensilla, is a pair of sensilla composed of 12 neurons and 2 glial cells each (Ward et al., 1975). These glia, called the amphid sheath (AMsh) and amphid socket (AMso) cells, form channels that ensheath the dendrites of sensory neurons in the amphid sensilla (Oikonomou and Shaham, 2011; Perkins et al., 1986; Ward et al., 1975). Furthermore, these glial cells are vital for the proper functioning of the neurons that they ensheath, where they can modulate neural activity through secreted molecules at the receptive endings and control neuron receptive ending shape (Bacaj et al., 2008; Shaham, 2010; Singhvi et al., 2016).

Here, we describe a function of vitamin B12 in the regulation of glial migration and synapse formation. We found that either dietary vitamin B12 deficiency or reduced uptake in the intestine due to a mutation in the ABC transporter *mrp-5* (multidrug resistance protein 5) leads to an over-migration of AMsh glia. Furthermore, vitamin B12 functions during glial migration and synapse formation by regulating the expression of the protein tyrosine phosphatase PTP-3/LAR PRTP in an isoform-specific manner. PTP-3A interacts with the extracellular matrix protein NID-1/nidogen 1 near the pharynx to regulate glial migration. Our findings reveal a vitamin-B12-dependent pathway regulating both glial and neuronal development that may inform our understanding of neuropathies derived from vitamin B12 deficiency.

RESULTS

To study mechanisms regulating glial migration, we used *C. elegans* AMsh glial cells as a model. AMsh glia are born close to the nose of *C. elegans* and then their processes anchor to the surface of embryos while the cell bodies migrate toward the nerve ring (Heiman and Shaham, 2009). In day 1 (D1) young adults, AMsh cell bodies reside at the side of and align with the center of pharyngeal terminal bulbs (Figures 1A and 1B). In a genetic screen, we

isolated a mutant *yad138*, whose AMsh cell body positions were indistinguishable from those of control animals in L1 larvae but possess AMsh cell bodies that fail to stop at the pharyngeal terminal bulbs and migrate further down the body in D1 adults (Figures 1A, 1B, and S1A–S1C). To further confirm these observations, we visualized single animals at different developmental stages and found all mutants examined in this study have similar AMsh cell body positions at the L1 stage when compared to control animals, but their AMsh cell bodies migrate further down the body and fail to stop at the correct place in the L4 and adult stages (Figure S1D). These results suggest that *yad138* likely affects a molecule involved in terminating AMsh migration. *yad138* causes a missense mutation in the *C. elegans* ABC transporter *mrp-5,* which has been shown to play essential roles in the export of vitamin B12 and heme from the intestine to other tissues (Korolnek et al., 2014; Na et al., 2018; Figure S1E). We found that driving expression of *mrp-5* by its own promoter or an intestine-specific promoter was able to fully rescue *yad138* phenotypes, although expression of *mrp-5* in AMsh glia or pharyngeal muscles did not change the phenotypes (Figure 1C). These results show that *yad138* is a loss-of-function allele of *mrp-5,* and *mrp-5* functions in the intestine to affect AMsh migration.

To determine whether the role of *mrp-5* in AMsh glial migration is related to its function in the uptake of vitamin B12 or heme in the intestine, we directly injected vitamin B12 or heme to *mrp-5(yad138)* L1 larvae and observed full rescue of AMsh migration defects in vitamin B12-injected, but not heme-injected, animals (Figure 1D). Vitamin B12 also appears to function during early developmental stages, as injection of the same concentration of vitamin B12 in L4 larvae did not show any rescue ability on *mrp-5(yad138)* animals when they were scored as D1 adults (Figure 1D). To further confirm the importance of vitamin B12 in regulating AMsh glial migration, we cultured control animals in vitamin-B12-deficient conditions as outlined by Bito et al. (2013); Figure 1E. Similar to what they reported, we observed a reduction in brood size when animals were grown for five generations in vitamin-B12-deficient conditions (Bito et al., 2013; Figure S1F). Vitamin-B12-deficient animals displayed similar AMsh migration defects as those observed in *mrp-5* mutants (Figure 1F). Dietary supplementation of vitamin B12, but not heme, was able to prevent the AMsh migration defects observed in vitamin-B12-deficient conditions (Figure 1G). We also found that feeding animals a vitamin-B12-enriched diet did not cause any observable abnormalities in AMsh development (Figure S1G).

In the same genetic screen, we isolated other mutants with similar phenotypes as those in *mrp-5* mutants, and further analyses showed that two of them, *yad122* and *yad139,* belong to a same complementation group and both affect *cubn-1* (Figures S2A–S2D), the only *C. elegans* homolog of the intrinsic factor-vitamin B12 receptor cubilin (Kozyraki et al., 1998; Seetharam et al., 1997). Expression of *cubn-1* under its own promoter fully rescued *yad122* phenotypes, and *yad122* exhibits a similar degree of AMsh migration defects as presumed null alleles (Figure S2C). These results support the conclusion that *yad122* and *yad139* are loss-of-function alleles of *cubn-1,* and *yad122* is likely a null allele. Cubilin is a giant secreted protein and functions together with a receptor called megalin to uptake intrinsic factor-vitamin B12 (Ahuja et al., 2008; Moestrup et al., 1998; Nielsen et al., 2016). To understand how *cubn-1* affects AMsh migration, we examined the function of *C. elegans* megalin *lrp-1,* and the results showed that knockdown of *lrp-1* caused a similar degree of

AMsh migration defects as was found in *cubn-1* mutants, but knockdown of *lrp-1* in a *cubn-1(lf)* background did not further enhance *cubn-1* mutant phenotypes (Figures S3A–S3C). These results suggest that the function of *cubn-1* likely relies on its binding with *lrp-1* to uptake vitamin B12, similar to what was previously reported in mammalian systems (Ahuja et al., 2008; Moestrup et al., 1998; Nielsen et al., 2016). Further experiments showed that knockdown of *lrp-1* expression only in AMsh glia caused the same degree of migration defects as those in global knockdown animals (Figure S3C), suggesting *lrp-1* cell autonomously regulates AMsh migration. We also found that double mutants of *cubn-1;mrp-5* exhibited similar defects as those in *mrp-5* single mutants, and knockdown of *lrp-1* did not further enhance *mrp-5* mutant phenotypes (Figures S3D–S3F). These results suggest that CUBN-1 and LRP-1 likely function as co-receptors in AMsh glia to uptake vitamin B complex into AMsh glia to terminate AMsh migration. Taken together, these results demonstrate the important role of vitamin B12 in regulating glial migration. To understand how vitamin B12 regulates AMsh migration, we tested three important enzymes involved in *C. elegans* vitamin-B12-dependent metabolic pathways—*pcca-1*/propionyl-CoA carboxylase alpha chain, *mmcm-1*/methylmalonyl-CoA mutase, and *metr-1*/5-methyltetrahydrofolate-homocysteine methyltransferase (Watson et al., 2014), but we did not notice any abnormalities in AMsh migration in loss-of-function mutants of these three genes (Figure S3G), suggesting that vitamin B12 may regulate AMsh glial migration through other means.

To further illuminate the molecular mechanisms underlying AMsh migration mediated by vitamin B12, we analyzed two other mutants with AMsh over-migration defects that were isolated in the initial screen, *yad120* and *yad121* (Figures 2A, 2B, and S1A). They failed to complement each other, and both alter the *C. elegans* homolog of LAR PRTP (leukocyte-common antigen-related receptor-type tyrosine-protein phosphatase) *ptp-3* (Figure S4A). Interestingly, *yad120* has stronger defects than *yad121,* but it only affects the long isoform *ptp-3a,* although *yad121* affects both long and short isoforms *ptp-3a* and *ptp-3b* (Figures 2A, 2B, and S4A). Like what we observed in *mrp-5* mutants, loss of function of *ptp-3* did not affect the position of AMsh cells in L1 larvae, suggesting that it may play a role in terminating glial migration (Figure S1B). The two isoforms *ptp-3a* and *ptp-3b* have been shown to play distinct functions in synapse formation, axon guidance, and neuronal migration (Ackley et al., 2005; Sundararajan and Lundquist, 2012). To determine whether both isoforms are important for AMsh migration, we carried out rescue experiments and found that expression of *ptp-3a* under either its own promoter or an AMsh-specific promoter was able to fully rescue *yad121* phenotypes, although expression of *ptp-3b* did not show any rescue ability (Figure 2B). Similar results were obtained from *yad120* rescue experiments that *ptp-3a,* but not *ptp-3b,* can rescue AMsh migration defects (Figure 2B). We also examined a null allele of *ptp-3a(ok244)* and showed that it had the same degree of AMsh migration defects as observed in *yad120* (Figure 2B). Using both transcription and translational reporters, we confirmed that *ptp-3a* is expressed in AMsh glial cells (Figures S4B and S4C). These results support the conclusion that *ptp-3a,* but not *ptp-3b,* cell autonomously regulates AMsh migration.

Because both *mrp-5(lf), cubn-1(lf),* and *ptp-3a(lf)* cause over-migration of AMsh glia, we decided to test whether they might function in the same genetic pathway, and we found that

double mutants of *mrp-5;ptp-3a* and *cubn-1;ptp-3a* showed a similar degree of phenotypes as was found in *ptp-3a* single mutants, supporting the conclusion that *ptp-3a* likely lies in the same pathway as *mrp-5* and *cubn-1* (Figures 2C, 2D, and S4D). Because *ptp-3a* functions in AMsh cells to affect migration, it is possible that its expression or functions are regulated by *mrp-5* through vitamin B12. To test this hypothesis, we first examined whether loss of function of *mrp-5* may affect *ptp-3a* expression or localization using a well-established *ptp-3a* translation reporter (Ackley et al., 2005). As shown in Figure 3A, in control animals, we observed puncta expression of PTP-3A in the nerve ring as well as in AMsh glial cells. Loss of function of *mrp-5* reduced the overall expression of PTP-3A to less than 30% of that in control, and the PTP-3A expression reporter becomes invisible in the AMsh glia of *mrp-5* mutants (Figures 3A and 3B). In contrast, mutations of *mrp-5* did not alter PTP-3B expression (Figures 3A and 3C). Consistent with the observations in *mrp-5(lf)* animals, we showed that animals cultured in vitamin-B12-deficient conditions exhibited a similar decrease of PTP-3A expression, although the expression of PTP-3B was comparable to that in control conditions (Figures 3D–3F). The regulation of *ptp-3a* expression by *mrp-5* and vitamin B12 has also been confirmed on the mRNA level by real-time PCR (Figures 3G and 3H). These results suggest that the uptake of vitamin B12 mediated by *mrp-5* may regulate AMsh migration through transcriptional control of *ptp-3a* expression in AMsh cells. To further confirm this conclusion, we overex-pressed *ptp-3a* or *ptp-3b* only in AMsh cells and tested whether those transgenes were able to bypass the requirement of *mrp-5* in the termination of AMsh migration. In support of our hypothesis, overexpression of *ptp-3a,* but not *ptp-3b,* in AMsh glia nearly completely suppressed AMsh migration defects in *mrp-5* mutants (Figures 3I and S4E). We also found that overexpression of *ptp-3a,* but not *ptp-3b*, was able to eliminate the AMsh migration defects caused by vitamin B12 deficiency (Figure 3J). In conclusion, these results demonstrate that the uptake of vitamin B12 from the diet mediated by *mrp-5* in the intestine can control the expression level of *ptp-3a,* and the expression of *ptp-3a* in AMsh glia has a critical role in terminating cell migration at the appropriate place. As AMsh glia are essential for the function of sensory neurons they ensheath, we used the classic dye filling assay to examine whether the AMsh migration defects we described in this study could affect sensory neuron function (Oikonomou and Shaham, 2011; Shaham, 2015). We found that neither *mrp-5(lf)* nor *ptp-3a(lf)* caused dye filling defects in D1 young adults (Figure S4F). However, we observed a significant reduction in the number of neurons that uptake Dil in D7 *mrp-5(lf)* and *ptp-3a(lf)* animals when compared with the same age control animals (Figure S4G). The dye filling defects displayed in aged *mrp-5(lf)* and *ptp-3a(lf)* animals were caused by abnormal migration of AMsh, as expression of *ptp-3a* only in AMsh glia could fully rescue their dye filling defects (Figure S4G). Taken together, these results suggest that the correct localization of AMsh cell bodies is critical for maintaining the function of sensory neurons they ensheath during aging.

Because *ptp-3a* plays a critical role in regulating synapse formation (Ackley et al., 2005), we tested whether *mrp-5* and vitamin B12 have a role in synapse formation as well. As shown in Figures 4A and 4B, loss of function of *mrp-5* caused enlarged synapses and decreased synapse number, similar to what has been shown in *ptp-3a* mutants. *mrp-5(lf)* phenotypes were caused by defects in vitamin B12 uptake, as injection of vitamin B12 was able to completely rescue *mrp-5(lf)* phenotypes (Figures 4A and 4B). We also found that

overexpression of *ptp-3a* can cell autonomously suppress *mrp-5(lf)* defects, supporting the conclusion that lack of vitamin B12 caused by a mutant form of *mrp-5* disrupts synapse formation through downregulating *ptp-3a* expression. We further confirmed this by showing that culturing animals in vitamin-B12-deficient conditions caused similar synapse formation defects as those displayed in *mrp-5(lf)* or *ptp-3a(lf)* animals, and overexpression of *ptp-3a* prevented synapse formation defects caused by vitamin B12 deficiency (Figures 4C and 4D).

In synapse formation, PTP-3A functions through interactions with an extracellular matrix protein NID-1, an ortholog of human nidogen 1 and nidogen 2 (Ackley et al., 2005). We found that loss of function of *nid-1* caused AMsh over-migration defects, and double mutants of *nid-1;ptp-3a* exhibited a similar degree of defects as those found in *ptp-3a* single mutants (Figures 4E, 4F, and S1A–S1D), confirming that *nid-1* is likely also a functional partner of *ptp-3a* in regulating glial migration. More interestingly, expression of *nid-1* only in the pharyngeal muscle was able to fully rescue *nid-1(lf)* phenotypes, which is consistent with the strong expression of *nid-1* in the pharynx, as previously shown (Figure 4F; Ackley et al., 2005; Kang and Kramer, 2000). Expression of NID-1::GFP suggested potential accumulation of NID-1 on the surface of AMsh cell bodies (Figure S4H). Therefore, it is likely that the interactions between AMsh-expressed PTP-3A and pharynx-expressed NID-1 provide the signals to terminate AMsh migration at the pharyngeal terminal bulbs. In conclusion, our study provides a common mechanism for regulation of both neuronal and glial development by vitamin B12, in which the uptake of vitamin B12 by the intestine can regulate the expression of PTP-3 in an isoform-specific manner, and this regulation also relies on the interactions between PTP-3 and the extracellular matrix protein NID-1 (Figure S5).

DISCUSSION

In our study, we uncover a role of vitamin B12 in glial migration and synapse formation, and we show that vitamin B12 functions by regulating the expression of *ptp-3a,* the *C. elegans* homolog of mammalian LAR PRTP. *C. elegans ptp-3a* and mammalian LAR PRTPs are highly expressed in the nervous system and have been shown to be important for synapse formation, axon development, and axon regeneration in neurons (Ackley et al., 2005; Chagnon et al., 2004; Chen et al., 2011; Stoker, 2015; Takahashi and Craig, 2013). Our findings further reveal a function of LAR PRTPs in regulating glial migration and show *ptp-3a*/LAR PRTP expression to be connected to vitamin B12 uptake in the intestine. As vitamin B12 deficiency causes many neurodevelopmental and degenerative disorders that are associated with abnormality in both neurons and glial cells (Cardinale et al., 1970; Douaud et al., 2013; Healton et al., 1991; Reynolds, 2006; Scalabrino et al., 1995; Stollhoff and Schulte, 1987), it is possible that ptp-3a/LAR PRTP as a target for vitamin B12 is associated with multiple neurological symptoms of vitamin B12 deficiency.

Besides the nervous system, LAR PRTPs are also expressed in a wide range of cell types, including T lymphocytes, kidney cells, prostate cells, and intestinal epithelial cells (Murphy et al., 2005; Pulido et al., 1995; Streuli et al., 1988) and are known to be involved in the regulation of insulin signaling, epithelial integrity, and cancer progression (Mooney and LeVea, 2003; Müller et al., 1999; Murchie et al., 2014; Xu and Fisher, 2012). Furthermore,

mutations in LAR PRTPs have been linked to hyperinsulinism and obesity in human patients (Alliance of Genome Resources). A major symptom of vitamin B12 deficiency is anemia, which may or may not coincide with neurological symptoms (Green et al., 2017; Lindenbaum et al., 1988; Reynolds, 2006). Other symptoms may include fatigue, decreased appetite, glossitis, and reduced gastrointestinal function (Briani et al., 2013; Green et al., 2017; Hunt et al., 2014). As some of these symptoms appear to relate to either insulin signaling or epithelial integrity, where LAR PRTPs are known to play important roles, the regulation of *ptp-3a* by vitamin B12 may also be relevant.

In this study, we found that vitamin B12 controls glial migration and synapse formation through regulating *ptp-3a* transcription. However, the question of how this regulation happens is still unclear. Different levels of vitamin B12/folic acid have been shown to affect specific DNA methylation patterns, and maternal vitamin B12/folic acid levels during pregnancy were also shown to affect DNA methylation patterns in infants (Hoyo et al., 2011; Kok et al., 2015; McKay et al., 2012; Rampersaud et al., 2000). In our experiments, we found that injection of vitamin B12 at the early, but not late, larval stages is sufficient for normal glial and neuronal development, suggesting that this regulation of *ptp-3a* by vitamin B12 occurs during early developmental stages. Therefore, one possibility is that, during development, vitamin B12 can affect the methylation of DNA in the regulatory elements of the *ptp-3a* promoter to facilitate its transcription. A second possibility is that vitamin B12 may regulate *ptp-3a* on the mRNA level, as previous studies show that vitamin B12 can bind to regulatory elements of messenger RNAs to control gene expression (Mandal and Breaker, 2004; Nahvi et al., 2004; Padmanabhan et al., 2017). It is also possible that vitamin B12 may regulate the expression or activation of certain transcription factors that are important for *ptp-3a* expression. Further studies are needed to uncover the detailed mechanism of how vitamin B12 regulates *ptp-3a* transcription.

STAR★METHODS

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Dong Yan (dong.yan@duke.edu). *C. elegans* strains and plasmids generated in this study are available from the lead contact without restriction.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

C. elegans* genetics—C. elegans* strains were maintained on nematode growth media (NGM) plates using *E. coli* OP50 as a food source. Animals were grown according to standard methods (Brenner, 1974) at 20°C unless otherwise stated. Wild-type worms were of the Bristol N2 strain. Only hermaphrodite animals were used for experiments. All transgenes and strains are described in Table S1. *yadIs48* (*Pf53f4.13::GFP)* was used to visualize AMsh cells. *juIs1 (Punc-25::SNB-1::GFP)* was used to visualize GABA synapses. The alleles used in genetic analyses were *mrp-5(yad138), cubn-1(yad122), ptp-3a(yad120),* and *nid-1(cg119)* unless specific alleles were mentioned.

METHOD DETAILS

Cloning and constructs—All DNA expression constructs were generated using Gateway cloning technology (Invitrogen, Carlsbad, CA) and subsequently sequenced. *mrp-5* and *nid-1* were amplified from a homemade genomic pool. A 2kb fragment before the start codon of *mrp-5* was used as its promoter. A 2.5 kb fragment before the start codon of *nid-1* was used as its promoter. A complete list of DNA constructs used is included in Table S1. In general, plasmid and PCR DNA used in this study were injected at a concentration of 1–50ng/μL with a *Pttx-3::RFP* co-injection marker injected at a concentration of 50ng/μL.

RNA Interference—RNA interference (RNAi) experiments were carried out by feeding anaimls with *E. coli* strain HT115 (DE3) expressing double-stranded RNA against target genes for at least two generations. All *lrp-1* RNAi clones were made by inserting 1kb cDNA fragments into the L4440 vector. RNAi clones were first cultured overnight using LB broth containing ampicillin (100 μg/ml) at 37°C, and then concentrated bacteria were seeded on NGM plates containing 100μg/ml ampicillin and 5mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) and cultured overnight at 37°C. Animals were grown on RNAi plates for at least two generations. In all experiments, either GFP RNAi or *unc-22* RNAi was included as a control to account for RNAi efficiency. Tissue-specific RNAi was carrired out as described (Calixto et al., 2010; Feinberg and Hunter, 2003). In brief, to knock down gene expression only in pharyngeal muscles, we expressed the sid-1(cDNA) under the control of the *Pf53f4.13* promoter, a AMsh specific promoter in an *sid-1(pk3321)* mutant background. *sid-1* mutants are resistant to RNAi, and transgenic animals *(yadEx760;sid- 1(pk3321))* are resistant to RNAi in all tissues except in the AMsh glial cells which express *sid-1* cDNA.

Microscopy—Representative images were acquired with a Zeiss LSM700 confocal microscope using a Plan-Apochromat 40×/1.4 objective. Worms were immobilized using 1.5% 1-phenoxy-2-propanol (TCI America, Portland, OR) in M9 buffer and mounted on 5% agar slides. 3D reconstructions were done using Zeiss Zen software. Images of GABA neuron synapses show the dorsal cord at the middle part of animals. A Zeiss Axio Imager 2 microscope equipped with Chroma HQ filters was used to score AMsh migration defects and take images to measure Migration Index. For GABA neuron synapse number quantification, a Zeiss Axio Imager 2 microscope equipped with Chroma HQ filters was used to quantify the total number of SNB-1::GFP puncta at the dorsal cord. Each condition represented 3 experiments of at least 50 Day 1 adult animals each unless otherwise noted.

Brood size assays—Brood size assays were performed by allowing gravid adults to lay eggs for 3 days on plates seeded with vitamin B12-deficient OP50 before the adults were removed. One day later, the total number of embryos or hatched animals were counted. Three experiments of at least 20 animals were quantified for each condition.

Vitamin B12 deficiency assays—Vitamin B12 deficiency assays were performed as previously described (Bito et al., 2013). In brief, vitamin B12-deficient OP 50 was cultured as a food source: OP50 was grown in M9 medium at 37°C for 3 days. Animals were then cultured using M9 agar (2%) plates seeded with the vitamin B12-deficient OP50 as their food source. All animals were grown in this condition for five generations with or without

vitamin B12 (cyanocobalamin) (Sigma) (100 μg/L) or heme (Sigma) (500 μM) dietary supplementation.

Vitamin B12/heme injection—Vitamin B12 (3.2 mM) or heme (500 μM) were prepared using autoclaved water and were injected into the middle part of animals. We followed the standard protocol for all injections. For L1 injections, we cultured newly hatched animals in 20°C for 12 hours to reach the late L1 stage. The injection was performed by placing animals on 10% agar pads, which were newly made and air-dried for 30–45 mins, with lower injection air pressure (1/3 of pressure for L4 animal injection). The L1 injection needle tips also had to be thinner than regular L4 injection ones. When injections were performed in L1 animals, about 40%–60% animals failed to recover, and all data were collected from day 1 adults. Injected L4 animals had over a 90% recovery rate, and data were also collected in day 1 adults

Dye filling—Dil is purchased from Molecular Probes (Eugene, OR), and the stock solution is 2 mg/ml in dimethylformamide. We performed the experiments following the standard protocol. In brief, D1 and D7 adult animals were collected and washed three times in M9 buffer, and then incubated in 10 ng/ml Dil solution (in M9) for 2 hours at 20°C. After 2 hours incubation with Dil solution, animals were washed 3 times (20 min/each) in M9 buffer before visualization using 63X lens.

Real-time Reverse Transcription PCR—To measure changes in *ptp-3a* mRNA levels in *mrp-5* mutants and N2 animals with or without vitamin B12, total RNA was isolated from mixed population plates. Animals were washed with M9 buffer three times for 90 minutes in total, and TRI Reagent® (Invitrogen) was subsequently added to isolate the RNA. Animals were independently cultured and collected three times for each condition. Reverse transcription was performed on total RNA (1 μg) using an iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories). Quantitative real-time, reverse-transcription PCR (qPCR) was performed using iTaq Universal Probes Supermix (Bio-Rad Laboratories) and ready-to-use TaqMan Gene Expression Assays (Invitrogen), and mRNA levels of *ptp-3a* and *cdc-42* were quantified, with *cdc-42/cell* division control protein 42 serving as an internal control. qPCR amplifications were performed using an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems). Relative mRNA levels were quantified using the StepOnePlus Software v2.1 based on the comparative ΔΔCT method.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis—Data was analyzed using one-way or two-way ANOVA followed by Tukey's post hoc test in Graphpad Prism (Graphpad Software, La Jolla, CA). Statistical details are included in the figure legends. In general, a p value cutoff of 0.05 was considered statistically significant. The data are represented by mean ± SEM. For phenotype penetrance experiments, three biological replicates of at least 50 animals each were used unless otherwise stated.

DATA AND CODE AVAILABILITY

This study did not generate any unique datasets or code.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Vitamin B12 plays a role in regulating glial migration through PTP-3/LAR PRTP
- PTP-3 functions cell autonomously in an isoform-specific manner
- Vitamin B12 deficiency leads disruption of synapse formation
- PTP-3 functions by interacting with the extracellular matrix protein NID-1

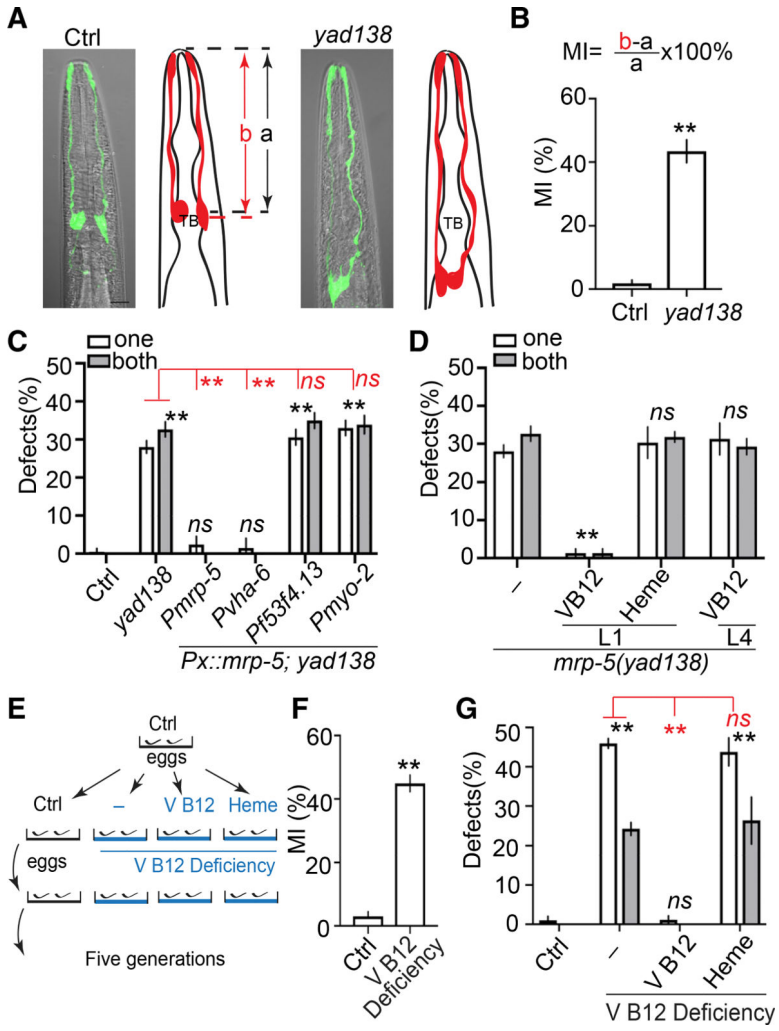

Figure 1. Vitamin B12 Is Required for Terminating Glial Migration

(A) *yad138* causes over-migration of AMsh glia. Confocal images and schematic representation of AMsh glia in control and *mrp-5(yad138)* animals expressing *Pf53f4.13::GFP (yadIs48)* are shown. TB, pharyngeal terminal bulb. Scale bar, 10 μm.

(B) Quantification of Migration Index (MI) in control and *mrp-5(yad138)* animals. MI is calculated as (b − a)/a × 100%. As illustrated in (A), “a” represents the distance between the tip of nose and the center of the pharyngeal terminal bulb. “b” shows the distance between the tip of nose and the center of the AMsh cell bodies.

(C) *mrp-5* functions in the intestine to regulate Amsh migration. White and gray bars show the percentage of animals with over-migration defects in one AMsh or both AMsh glia, respectively. *Pvha-6* is an intestine-specific promoter, *Pf53f4.13* drives expression only in AMsh glia, and *Pmyo-2* is a pharyngeal-muscle-specific promoter.

(D) Injection of vitamin B12, but not heme, rescues *mrp-5(lf)* phenotypes.

(E) A schematic showing how vitamin B12 deficiency was induced.

(F) Quantification of MI of AMsh glia in animals cultured in control and vitamin-B12-deficient conditions.

(G) Dietary supplementation of vitamin B12, but not heme, rescued AMsh migration defects in vitamin-B12-deficient conditions. Data show the percentage of animals with AMsh migration defects.

In (B) and (F), data are represented as mean ± SEM. One-way ANOVA test; **$p < 0.01$; each point represents at least 30 worms. In (C), (D), and (G), data are represented as mean ± SEM. Two-way ANOVA test; **$p < 0.01$; ns, no significant difference. Each point represents three experiments of at least 50 worms each.

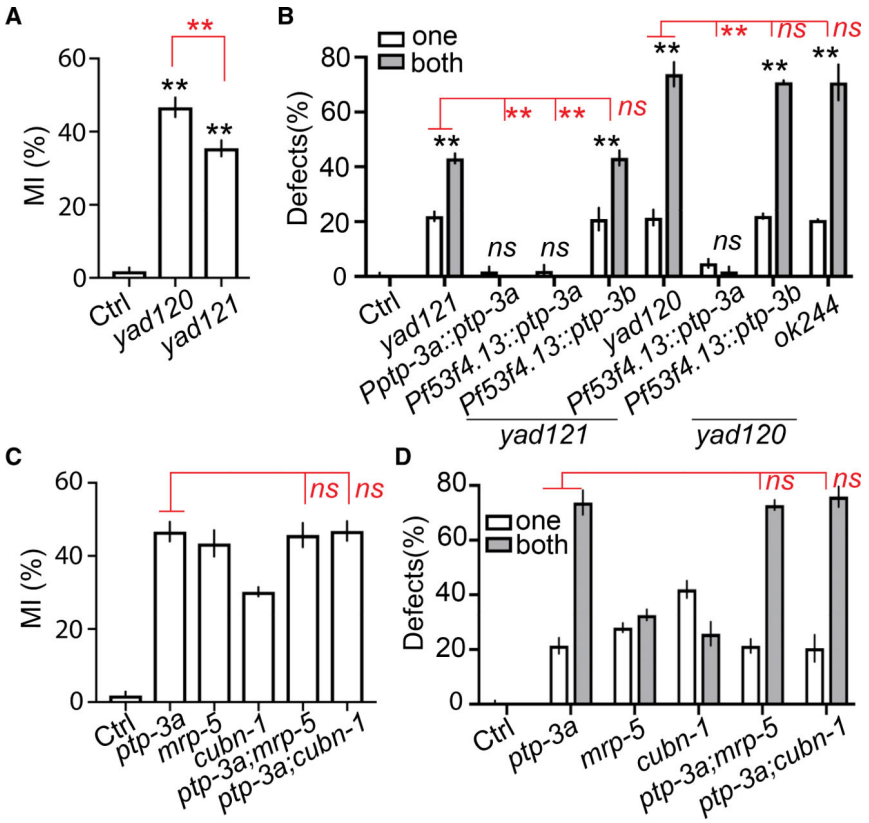

Figure 2. *ptp-3a* Functions in the Same Genetic Pathway as *mrp-5* and Vitamin B12 in Regulating Glial Migration

(A) Quantification of MI in control, *ptp-3a(yad120),* and *ptp-3a,b(yad121)* animals.

(B) *ptp-3a,* but not *ptp-3b,* regulates AMsh migration. White and gray bars show the percentage of animals with over-migration defects in one AMsh or both AMsh glia, respectively. *Pf53f4.13* drives expression only in AMsh glia.

(C and D) Double mutants of *ptp-3a;mrp-5* and *ptp-3a;cubn-1* display similar defects as in *ptp-3a* single mutants. Quantification of MI of AMsh glia

(C) and the percentage of animals with over-migration defects (D) are shown.

In (A) and (C), data are represented as mean ± SEM. One-way ANOVA test; **p < 0.01; each point represents at least 30 worms. In (B) and (D), data are represented as mean ± SEM. Two-way ANOVA test; **p < 0.01; each point represents three experiments of at least 50 worms each.

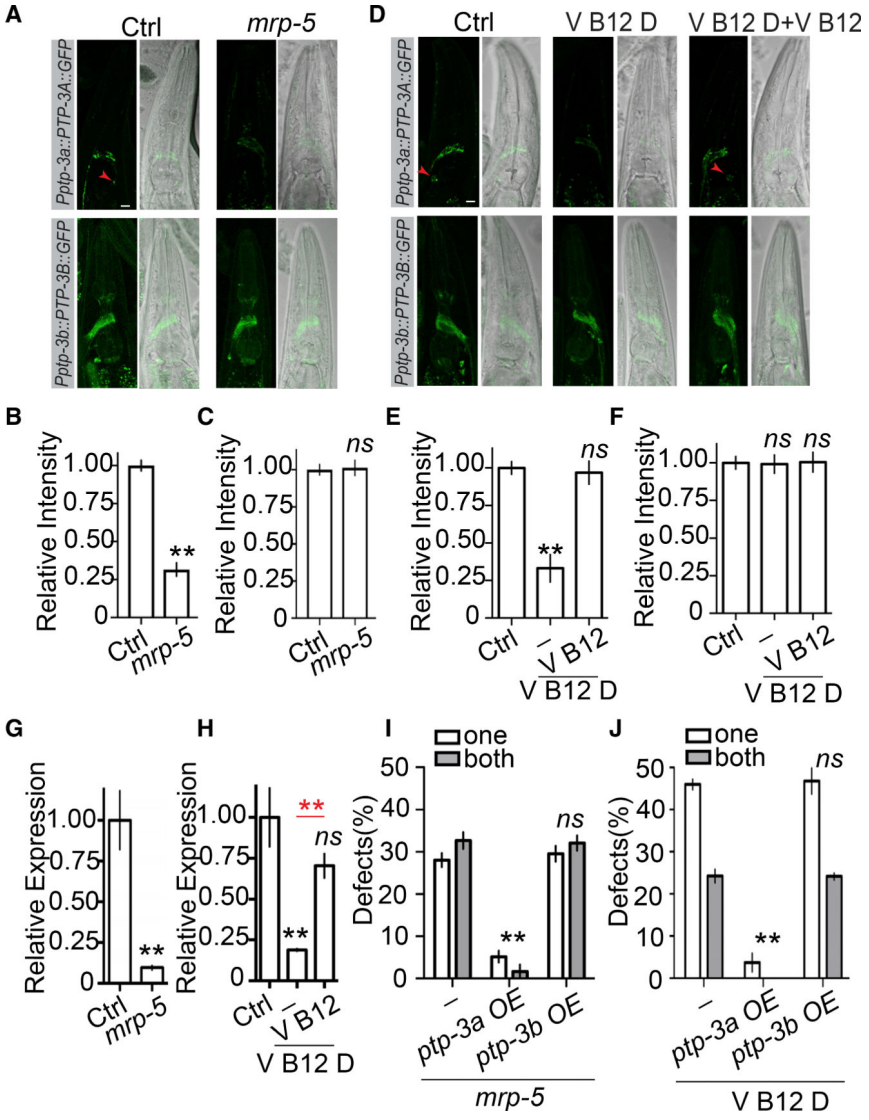

Figure 3. *mrp-5* and Vitamin B12 Regulates AMsh Migration through Isoform-Specific Control of *ptp-3a,* but Not *ptp-3b,* Expression

(A–C) Loss offunction in *mrp-5* decreases PTP-3A, but not PTP-3B, protein levels. Confocal images (A, left panel, GFP; right panel, differential interference contrast [DIC] merged with GFP; scale bar, 10 μm) and quantification (B and C) show the expression level of PTP-3A and PTP-3B in control and *mrp-5(lf)* animals. The red arrowheads point to AMsh cell bodies.

(D–F) Vitamin B12 deficiency suppresses PTP-3A, but not PTP-3B, expression. Confocal images (D, left panel, GFP; right panel, DIC merged with GFP; scale bar, 10 μm) and quantification (E and F) show the expression level of PTP-3A and PTP-3B in control vitamin-B12-deficient and vitamin-B12- supplemented conditions.

(G and H) Real-time PCR results show that loss of function in *mrp-5* (G) or culturing animals in vitamin-B12-deficient conditions (H) decrease *ptp-3a* mRNA levels. A housekeeping gene, *cdc-42,* was used as an internal control.

(I) Overexpression of *ptp-3a,* but not *ptp-3b,* in AMsh cells suppresses migration defects in *mrp-5(lf)* mutants.

(J) Overexpression of *ptp-3a,* but not *ptp-3b,* in AMsh cells suppresses migration defects caused by vitamin B12 deficiency.

In (B), (C), (E), and (F), data are represented as mean ± SEM. One-way ANOVA test; **p < 0.01; each point represents at least 20 worms. In (G)–(H), data are represented as mean ± SEM. One-way ANOVA test; **p < 0.01; each point represents three experimental replicates. In (I)–(J), data are represented as mean ± SEM. Two-way ANOVA test; **p < 0.01; each point represents three experiments of at least 50 worms each.

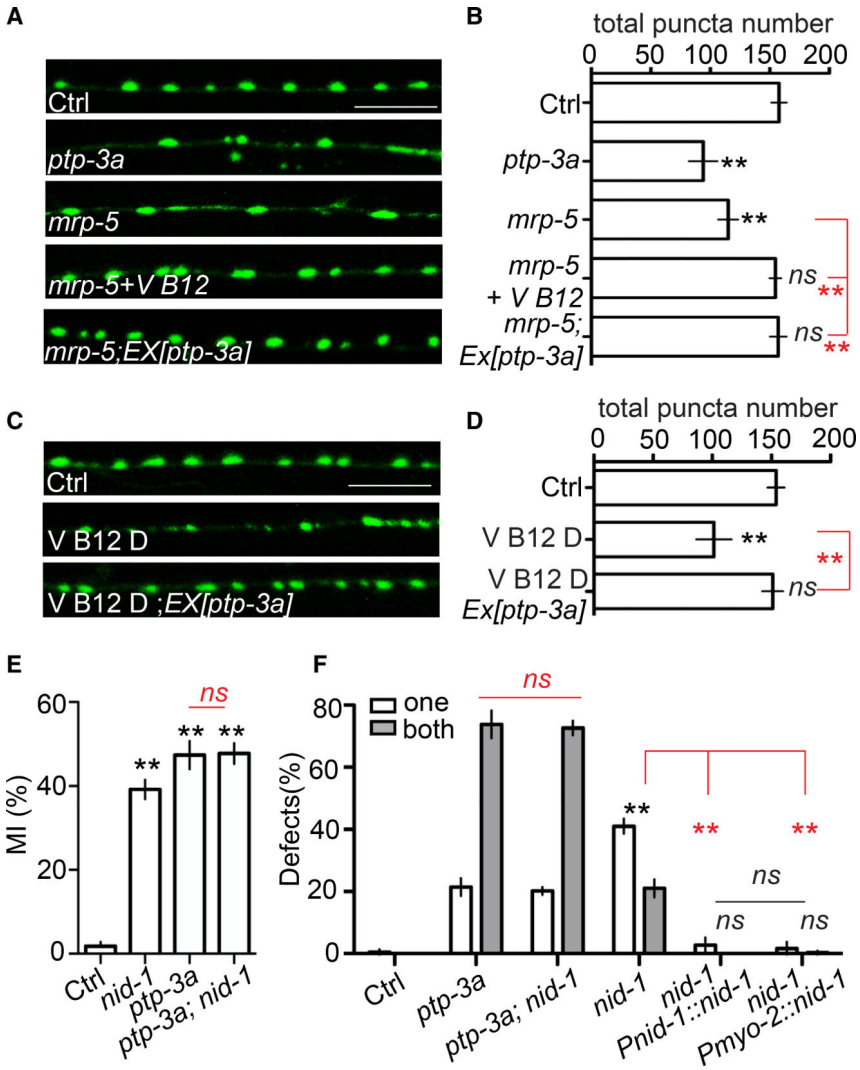

Figure 4. Vitamin B12 and *mrp-5* Regulate Synapse Formation through *ptp-3a,* and the Interactions of *nid-1* with *ptp-3a* Play an Important Role in Both Synapse Formation and Glial Migration

(A and B) *mrp-5* regulates synapse formation through vitamin B12 and *ptp-3a.* Confocal images (A) show GABA neuron synapses visualized by SNB-1::GFP (*juIs1)* at the dorsal cord (scale bar, 10 μm), and quantification data (B) show the total SNB-1::GFP puncta number in the dorsal cord.

(C and D) Vitamin B12 regulates synapse formation through *ptp-3a.* Confocal images (C) show GABA neuron synapses visualized by SNB-1::GFP *(juIs1*) at the dorsal cord (scale bar, 10 μm), and quantification data (D) show the total SNB-1::GFP puncta number in the dorsal cord.

(E and F) *nid-1* functions together with *ptp-3a* to regulate AMsh migration.

(E) Quantification of MI in control, *nid-1, ptp-3a(yad120),* and *nid-1;ptp-3a(yad120)* animals.

(F) The percentage of animals with over-migration defects. *Pmyo-2* is a pharyngeal-muscle-specific promoter.

In (B), (D), and (E), data are represented as mean ± SEM. One-way ANOVA test; **p < 0.01; each point represents at least 20 worms. In (F), data are represented as mean ± SEM. Two-way ANOVA test; **p < 0.01; each point represents three experiments of at least 50 worms each.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
Escherichia coli OP50	http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html	N/A
Escherichia coli DA1977	Caenorhabditis Genetics Center (CGC)	N/A
Chemicals, Peptides, and Recombinant Proteins		
1-phenoxy-2-propanol	TCI America, Portland, OR	Cat#P0118, CAS RN:770-35-4
Vitamin B12 (Cyanocobalamin)	Sigma	Cat#V6629, CAS RN:68-19-9
Hemin	Sigma	Cat#H9039, CAS RN:16009-13-5
Dil Stain	Molecular Probes, Eugene, OR	Cat#:D282, CAS RN:41085-99-8
Critical Commercial Assays		
iScript Reverse Transcription Supermix for RT-qPCR	Bio-Rad Laboratories	Cat#1708840
iTaq Universal Probes Supermix	Bio-Rad Laboratories	Cat#1725131
TaqMan Gene Expression Assays – *cdc-42*	Thermo Scientific	Cat#:4331182 Assay ID:Ce02435136_g1
TaqMan Gene Expression Assays – *ptp-3*	Thermo Scientific	Cat#:4448892 Assay ID: Ce02406216_g1
Experimental Models: Organisms/Strains		
C. elegans strains, see Table S1	This paper	N/A
Recombinant DNA		
Pmyo-2::mrp-5	This paper	PNYL1218
Pf53f4.13::mrp-5	This paper	PNYL1219
Pf53f4.13::ptp-3a	This paper	PNYL1215
Pf53f4.13::ptp-3b	This paper	PNYL1216
Pptp-3A::ptp-3a::HA	This paper	PBH21
Punc-25::ptp-3a	This paper	PNYL1217
Pf16f9.3::mCherry::H2B	This paper	PNYL1142
Fosmid WRM0627BF04	Yishi Jin	WRM0627BF04
lrp-1 RNAi 1	This paper	PNYL1280
lrp-1 RNAi 1	This paper	PNYL1281
Pf53f4.13::sid-1(cDNA)	This paper	PNYL1279
Pf53f4.13::mCherry	This paper	PNYL696
Pnid-1::nid-1::GFP	Brian Ackley/ Jim Kramer Lab	pEVL54
Software and Algorithms		
Graphpad Prism 6	Graphpad Software	N/A
Zeiss Zen Black	Zeiss	N/A
StepOnePlus Software v2.1	Applied Biosystems	N/A