Protein phosphatase 2A cooperates with the autophagy-related kinase UNC-51 to regulate axon guidance in Caenorhabditis elegans

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SUMMARY
UNC-51 is a serine/threonine protein kinase conserved from yeast to humans. The yeast homolog Atg1 regulates autophagy (catabolic membrane trafficking) required for surviving starvation. In C. elegans, UNC-51 regulates the axon guidance of many neurons by a different mechanism than it and its homologs use for autophagy. UNC-51 regulates the subcellular localization (trafficking) of UNC-5, a receptor for the axon guidance molecule UNC-6/Netrin; however, the molecular details of the role for UNC-51 are largely unknown. Here, we report that UNC-51 physically interacts with LET-92, the catalytic subunit of serine/threonine protein phosphatase 2A (PP2A-A), which plays important roles in many cellular functions. A low allelic dose of LET-92 partially suppresses axon guidance defects of weak, but not severe, unc-51 mutants, and a low allelic dose of PP2A regulatory subunits A (PAA-1/PP2A-A) and B (SUR-6/PP2A-B) partially enhanced the weak unc-51 mutants. We also found that LET-92 can work cell-autonomously on axon guidance in neurons, and that LET-92 colocalized with UNC-51 in neurons. In addition, PP2A dephosphorylated phosphoproteins that had been phosphorylated by UNC-51. These results suggest that, by forming a complex, PP2A cooperates with UNC-51 to regulate axon guidance by regulating phosphorylation. This is the first report of a serine/threonine protein phosphatase functioning in axon guidance in vivo.

KEY WORDS: C. elegans, Axon guidance, PP2A, Serine/threonine protein kinase, Serine/threonine protein phosphatase

INTRODUCTION
In the development of the nervous system, neurons extend their axons to precise targets. In this process, axon guidance molecules, expressed on cell membranes or in the extracellular milieu, provide positional information (Tessier-Lavigne and Goodman, 1996; Yu and Bargmann, 2001; Dickson, 2002; Chilton, 2006; Killean and Sybingleco, 2008). To do this, the axon guidance molecules bind to receptors on the growth cone, a specialized structure at the growing axonal tip, and induce cytoskeletal changes in the growth cone.

A conserved axon guidance molecule, UNC-6/Netrin, is required for dorsoventral axon guidance in C. elegans (Hedgecock et al., 1990; Ishii et al., 1992; McIntire et al., 1992; Hao et al., 2001) and is expressed by ventral cells (Wadsworth et al., 1996; Asakura et al., 2007). Two C. elegans UNC-6 receptors are UNC-5 and UNC-40/DCC, which belong to the immunoglobulin superfamily. Each has a single transmembrane domain (Leung-Hagestein et al., 1992; Chan et al., 1996), and both are required for ventral UNC-6 to repulse axons that are fated to extend dorsally (Wadsworth, 2002). Ventrally extending axons, however, are attracted to UNC-6 and require only the UNC-40 receptor for this response. The dorsoventral guidance of C. elegans axons is also regulated by a conserved axon guidance molecule, SLT-1/Slit (Hao et al., 2001). SLT-1 is expressed by dorsal muscles and some ventrally extending axons are repelled by it. Two of the C. elegans SLT-1 receptors are SAX-3/Robo and EVA-1. Each has a single transmembrane domain (Zallen et al., 1998; Fujisawa et al., 2007), and SAX-3 belongs to the immunoglobulin superfamely. EVA-1 has two lectin-like galactose binding domains in its ectodomain. UNC-6 and SLT-1 act partially redundantly in ventrally directed axon guidance (Hao et al., 2001; Fujisawa et al., 2007).

UNC-51 and UNC-14 are essential for the axon guidance of many neurons in C. elegans (Hedgecock et al., 1985; Desai et al., 1988; McIntire et al., 1992; Mörike et al., 2003; Lai and Garriga, 2004; Siddiqui and Culotti, 2007). UNC-51 is a conserved serine/threonine protein kinase that is homologous to yeast Atg1 and human ULK (Ogura et al., 1994; Matsuura et al., 1997, Straub et al., 1997; Yan et al., 1998). All three homologs are required for autophagy, that is, the catabolic vesicle trafficking that is required to survive starvation (Matsuura et al., 1997; Straub et al., 1997; Meléndez et al., 2003; Hará et al., 2008). The function of these UNC-51 homologs in axon guidance is also conserved from C. elegans to mammals (Ogura et al., 1994; Tomoda et al., 1999; Tomoda et al., 2004; Zhou et al., 2007; Ahantarig et al., 2008; Toda et al., 2008). Because, in C. elegans, knocking-down of other autophagy genes does not cause axon guidance defects, the axon-guidance function of UNC-51 is probably effected through a different mechanism than its autophagy function (Ogura and Goshima, 2006). The binding partner of UNC-51, UNC-14, is a novel protein that contains a RUN domain (Ogura et al., 1997). Although the function of the RUN domain in UNC-14 is not known, RUN domains are predicted to play important roles in Rap and Rab family GTPase signaling pathways in vesicle trafficking (Calabria et al., 2001). UNC-51 and UNC-14 together regulate the subcellular localization (trafficking) of the UNC-6 receptor UNC-5 (Ogura and Goshima, 2006). However, the molecular functions of UNC-51 and UNC-14 are largely unknown.
Serine/threonine protein phosphatase 2A (PP2A) is highly conserved from yeast to mammals (Millward et al., 1999; Lechward et al., 2001; Sontag, 2001; Janssens et al., 2008). A 36 kD catalytic subunit (PP2A-C) and a 65 kD regulatory A subunit (PP2A-A) comprise the core structure of PP2A, to which one of a variety of regulatory B subunits can bind, to confer distinct properties on the heterotrimeric holoenzyme. Extensive analysis using PP2A mutants or PP2A inhibitors in cultured cells has revealed the involvement of PP2A in many cellular functions, including cell division, development and apoptosis, and in pathological conditions, such as cancer. For example, in C. elegans, PP2A is involved in vulval differentiation (Kao et al., 2004) and mitotic spindle assembly (Schlaitz et al., 2007). However, the early lethality of the PP2A mutation makes it difficult to analyze the in vivo function of PP2A in neural cells.

Here, we report that LET-92, the catalytic subunit of the C. elegans protein phosphatase 2A (PP2A-C), physically interacts with UNC-51 and that the genes encoding the catalytic and regulatory subunits of PP2A interact genetically with unc-51 to influence axon guidance phenotypes. We also found that LET-92 can work cell-autonomously on axon guidance in neurons and colocalized with UNC-51 in neurons. In addition, PP2A dephosphorylated phosphoproteins that had been phosphorylated by UNC-51. These results suggest that PP2A functions in cooperation with UNC-51 to regulate axon guidance by regulating phosphorylation. This is the first report of a serine/threonine protein phosphatase having an in vivo function in axon guidance.

MATERIALS AND METHODS

Worms

Bristol strain N2 was used as the standard wild-type strain. The worms were handled as described by Brenner (Brenner, 1974). The analyzed strains were made by the crossing or transformation of the original strains shown as follows: I, unc-14(e57), sur-6(sv30), zdfs5(mye-4::gfp), hT2; II, unc-115(tm655), unc-22(s7), let-92(s504), let-92(s677), unc-25(5094), nT1; III, unc-51(e369), unc-51(e80), t(f1), nT1; V, unc-51(e369), unc-51(e80), let-92(sv30), ceh-12(sv312), unc-47::gfp, lin-15(n765ts), sas-3(ky213).

Two-hybrid screening

The C. elegans two-hybrid cDNA library was kindly provided by Robert Barstard (Oklahoma Medical Research Foundation, OK, USA). AH109 (TaKaRa, 630444) was used as the host strain. pGBK-T7 (TaKaRa, 630443) was used to drive the expression of the UNC-51 (276-856) and full-length UNC-14 baits. Library screening was performed as described by the manufacturer (TaKaRa, 630303). let-92 cDNAs were isolated in both screenings.

Isolation of a deletion mutant

The paa-1(tm655) mutant was isolated as described by Gengyo-Ando et al. (Gengyo-Ando et al., 2000). tm655 lacked 1428 base pairs (bp) that included 70.2% of the coding region of the paa-1 gene (http://www.wormbase.org/db/gene?gene=names=WBGene00003901;class=Gene), resulting in a putative null allele.

Genetic analysis

The DD and VC neurons were labeled with oxs12 (unc-47::gfp) (McIntire et al., 1997). The AVM neuron was labeled with by zdfs5 (mye-4::gfp) (Clark and Chiu, 2003). Each animal was mounted on a 5% agarose pad in M9 buffer containing 5% sodium azide and observed using a fluorescence microscope (Axioplan 2, Carl Zeiss). The number of DD and VC axons that reached more than 80% of the way to the dorsal nerve cord in L4 larvae or young adults was counted. Because we enumerated the DD and VC axons exhibiting defective pathfinding, we used the Bonferroni correction for the statistical analysis. For AVM axon guidance, we used a binary, YES/NO, evaluation mode in which an axon was counted as having a defect if it did not reach the ventral nerve cord, or if the axon extended anteriorly or posteriorly before reaching the ventral nerve cord. Therefore, we used a chi-square test for the statistical analysis of these results.

Imaging

A confocal microscope (LSM510, Carl Zeiss) was used to obtain images.

GST pull-down analysis

Glutathione Sepharose 4B beads (GE Healthcare, 17-0756-01), extracts of E. coli that expressed GST::LET-92 (Ogura et al., 2003), and reticulocytes (Promega, L1170) that expressed each of the MYC-tagged proteins were mixed in cold buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 1 mM MgCl2, 0.2% NP-40]. For the expression of MYC-tagged proteins in reticulocytes, we used a pGBK-T7 vector (TaKaRa, 630443). EST clones yk6686c5 (unc-51), yk459c7 (unc-14) and yk678c6 (unc-115) (Lundquist et al., 1998) were used for the constructs. The beads were washed several times with the same cold buffer. Each sample was fractionated by 8% SDS-PAGE and analyzed by western blotting using ECL Advance (GE Healthcare, RN2135). We used an anti-MYC antibody (Santa Cruz, sc-789) for the primary antibody and an HRP-conjugated anti-rabbit IgG (GE Healthcare, NA 934) for the secondary antibody.

Molecular analysis

A 4.3-kb HindIII fragment (bp 20862-25420 of the cosmid F38H4) containing only F38H4.9(unc-92) was sub-cloned into pgEL92P. A 3070 bp paa-1 promoter region was amplified from a cosmid clone F38H4. The DNA fragment was inserted into pPD95.77, resulting in a let-29p::GFPl construct (pgEL92P). A 3070 bp paa-1 promoter region was PCR-amplified from a cosmid clone F48E8. The DNA fragment was inserted into a Venus (Nagai et al., 2002) expression vector p77-CV, resulting in a paa-1p::Venus construct (pp77-CV). A 4123 bp sur-6 promoter region was PCR-amplified from N2 genomic DNA. The DNA fragment was inserted into p77-CV, resulting in a sur-6p::Venus construct (pp77-CV).

A let-92 open reading frame (ORF) was amplified from pBP345, which was previously isolated in our two-hybrid screening as a POS-1 bait (Ogura et al., 2003). The let-92 ORF was inserted into the unc-25 promoter (Jin et al., 1999) ::Venus expression vector p25P-NV, resulting in an unc-25p::Venus:let-92 construct (p25PV-l92). The unc-51 ORF was amplified from yk668c8 and was inserted into an unc-25 promoter::mCherry (Shaner et al., 2004; McNally et al., 2006) expression vector p25P-CmCh, resulting in an unc-25p::unc-51::mCherry construct (p25P-51mCh). An src-2 myristoylation signal ORF corresponding to MGSCIGK (Adler et al., 2005) was inserted into p25P-CmCh, resulting in an unc-25p::src-2 myristoylation signal::mCherry construct (p25P-srcmCh).

PCR-amplified tissue-specific promoters [a mec-7 promoter (Hamelin et al., 1996), a H20 promoter (Shioi et al., 2001), a myo-3 promoter (Okkema et al., 1993), an unc-4 promoter (Miller and Niemeyer, 1995), a ceh-12 promoter (Von Stetina et al., 2007) and an ins-18 promoter (Bílou et al., 2004)] were inserted into C. elegans expression vector p77-T2. The let-92 ORF was inserted into the tissue-specific expression vectors, resulting in mec-7p::let-92(pm7P-L92), H20p::let-92(pH20P-L92), myo-3p::let-92(pmyo3P-L92), unc-4p::let-92(punc4P-L92), ceh-12p::let-92(pceh12P-L92) and ins-18p::let-92(pins18P-L92) constructs.

Transformation of C. elegans

Transformation was performed as described by Mello et al. (Mello et al., 1991). For the let-92 rescue experiments, pRF4, a rol-6 marker (Kramer et al., 1990) was used; 10 ng/µl of the let-92 rescue clone (pL92H3) and 90 ng/µl of pRF4 were injected into the adult [unc-22(sv77) let-92(sv504)/nT7] gonad. For the expression of let-92p::GFPl construct (pgEL92P). paa-1p::Venus::paa1P-CV or sur-6p::Venus::paa6P-CV, MT8189 [lin150765ts X] was used as the transformation strain, and pJM23 was used as the transformation marker (Huang et al., 1994). Each sample of 10 ng/µl DNA and 90 ng/µl pJM23 was injected into the adult gonad.
For other transformation analyses, myo-2::mRFP (pmy2P-mR) (Campbell et al., 2002) was used as the marker (10 ng/µl). pBluescript SK+ (80 ng/µl) was injected into the adult N2 gonad. To detect Venus::LET-92 in VD growth cones, the constructs [pBluescript SK+ (70 ng/µl), pmy2P-mR (10 ng/µl), and pH20P-myO2 (50 ng/µl)] were injected into the adult N2 gonad. For the colocalization analysis, the constructs [pBluescript SK+ (70 ng/µl), pmy2P-mR (10 ng/µl), and pH20P-myO2 (50 ng/µl)] were injected into the adult N2 gonad. To detect Venus::LET-92 in VD growth cones, the constructs [pBluescript SK+ (70 ng/µl), pmy2P-mR (10 ng/µl), and pH20P-myO2 (50 ng/µl)] were injected into the adult N2 gonad.

Biochemical analyses
CMVp::unc-51::FLAG, CMVp::unc-51 (ΔAIKAI, a kinase dead form): FLAG and CMVp::unc-14::HA constructs were kindly provided by Gian Garriga (Lai and Garriga, 2004). pEGFP-N3 (Clontech, 632313) was used for GFP expression. CMVp::let-92::HA, CMVp::unc-51::HA, and CMVp::unc-14::HA constructs were made by inserting an ORF or a C. elegans is shown aligned with the PP2A-C of human (PP2AC-beta isoform) (Khew-Goodall et al., 1991). D. melanogaster (Orgad et al., 1990) and S. pombe (Paal) (Kinoshita et al., 1990). Asterisks indicate conserved amino acids. Amino acids that are altered by the let-92 mutations are indicated by arrowheads. The number of amino acid residues and overall identity between LET-92 and the other PP2A-Cs are shown at the ends of each amino acid sequence. The CDNA sequence of let-92 has been deposited in the DNA Data Bank of Japan (DDBJ) under accession number AB108533. (B) LET-92 interacted directly with both UNC-51 and UNC-14 in a GST pull-down assay. Abbreviations: U51, MYC::UNC-51; U14, MYC::UNC-14; U115, MYC::UNC-115. Arrowheads indicate the physically interacting proteins.

RESULTS
The catalytic subunit of protein phosphatase 2A (PP2A-C) interacts physically with UNC-51 and UNC-14
To analyze the function of UNC-51 and UNC-14 of C. elegans, we screened for their interacting proteins using the yeast two-hybrid system. From the screenings, we identified the F38H4.9 cDNA, which encodes the catalytic subunit of protein phosphatase 2A. An HRP-conjugated anti-mouse IgG (GE Healthcare, NA931), anti-rat IgG (GE Healthcare, NA935) or anti-rabbit IgG (GE Healthcare, NA934) was used as the secondary antibody. λ-PPase (NEB, P0753S) was used for the non-specific dephosphorylation control. PP2A-AC (Millipore, 14-111), which contains the catalytic and the regulatory A subunits of human PP2A, was used as the secondary antibody.

Axon guidance by PP2A and UNC-51
For other transformation analyses, myo-2::mRFP (pmy2P-mR) (Campbell et al., 2002) was used as the marker (10 ng/µl). pBluescript SK+ was used to equalize the amount of DNA in the transformations. For experiments to evaluate cell autonomy, constructs [pBluescript SK+ (80 ng/µl), pmy2P-mR (10 ng/µl) and pL29H13 (10 ng/µl)], [pBluescript SK+ (70 ng/µl), pmy2P-mR (10 ng/µl), pm7p-dc2 (unc-7::dsRed2, 5 ng/µl) and pm7P-L92 (10 ng/µl)], and [pBluescript SK+ (60 ng/µl), pmy2P-mR (10 ng/µl), pm7P-L92 (10 ng/µl), pm25PV-L92 (10 ng/µl)] were injected into the adult N2 gonad. For the V AB-8 analysis, we added phosphatase inhibitor (PhosSTOP, 1 mM NaCl, 0.5% Triton X-100, protease inhibitor (complete, Roche, 1 836 443). Cells were harvested in cold buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 100 mM DTT, 100 mM EDTA, 100 mM MgCl2, 100 mM MnCl2, 0.02 units) in reaction buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EGTA, 2 mM DTT, 0.01% Brij 35, 2 mM MnCl2, 0.02 units] in reaction buffer [20 mM HEPES (pH 7.0), 1 mM DTT, 1 mM MnCl2, 100 µg/ml BSA] (Ito et al., 2000) or in the PP2A reaction buffer only.

DEVELOPMENT
conserved amino acids. The third, s504, had a mutation at the splice acceptor site of the first intron. We found that let-92 was expressed in almost all cells, including many neurons, at all stages (Fig. 2A).

A low allelic dose of LET-92 suppresses the DD and VD axon guidance defects of unc-51 mutants

To analyze the functional relationship among UNC-51, UNC-14 and LET-92, we examined their genetic interactions in dorsally directed DD and VD axon guidance (Fig. 3A), as unc-51 and unc-14 mutants show axon-guidance defects in these neurons (McIntire et al., 1992; Ogura and Goshima, 2006). All of the alleles (s504, s677 and s2021) of let-92 are completely lethal in early larvae (Rogalski and Baillie, 1985). We found that the let-92(s504) homozygous mutants had minor DD and VD axon guidance defects (data not shown); however, the dead let-92 worms showed the ‘clear’ phenotype, which was probably caused by abnormal osmotic regulation. Therefore, we could not determine whether the axon guidance defects were primary defects or if they were secondary to the larval lethality. In addition, although let-92(s504) homozygotes die as early larvae, let-92(RNAi) resulted in early embryonic lethality (data not shown), indicating that the maternal product functions during embryogenesis. As DD neurons are born in the embryo (Sulston et al., 1983), their axon guidance could be affected by the maternal product. To avoid these issues, we examined the effect of a low allelic dose of the let-92 gene. Heterozygotic let-92 mutants were healthy and active, appeared identical to wild-type worms and had no axon guidance defects (Fig. 4). The low allelic dose of LET-92 produced in let-92(s504/+ ) and let-92(s677+/+) heterozygotes partially suppressed the axon guidance defects of a weak unc-51 allele, unc-51(ks38::Tc1); Fig. 3C; Fig. 4). However, let-92(s504+) did not suppress the defects of the severe unc-51(e369) allele (Fig. 4). These results suggest that LET-92 negatively regulates the functions of UNC-51 in dorsally directed DD and VD axon guidance, and that some level of UNC-51 function is required for the LET-92 function. We also analyzed the effect of low allelic dose let-92 on animals carrying the null allele unc-14(e57) (Ogura et al., 1997). However, we did not detect a genetic interaction (Fig. 4). Some level of UNC-14 function also may be required for the LET-92 function.

In the course of the study, we noticed that heterozygotic mutants for unc-22 [unc-22(s7/+)], which encodes muscle protein Twichin (Waterston et al., 1980; Benian et al., 1989), also suppressed the DD and VD axon guidance defects in unc-51(ks38::Tc1) and unc-
Low allelic dose of PP2A regulatory subunits (PAA-1/PP2A-A and SUR-6/PP2A-B) enhances the unc-51 defects in DD and VD axon guidance

The heterotrimeric PP2A holoenzyme contains one catalytic (PP2A-C) and two regulatory (PP2A-A and PP2A-B) subunits (Millward et al., 1999; Lechward et al., 2001; Sontag, 2001; Janssens et al., 2008). To analyze the effect of these genes on UNC-51 function, we examined the genetic interactions among paa-1, sur-6 and unc-51.

No mutants of the paa-1 gene were available; therefore, we made a deletion-null allele, paa-1(tm655), which caused complete early larval lethality, just as in homozygous let-92 mutants. Like the dead let-92 worms, the dead paa-1(tm655) larvae showed the ‘clear’ phenotype and paa-1(tm655)(RNAi) caused early embryonic death (data not shown). Therefore, we examined the effect of a low allelic dose of the paa-1 gene. The paa-1(tm655+/+) heterozygotes were healthy and active, appeared identical to wild-type worms and had no axon guidance defects (Fig. 4). The only reported null allele of the sur-6 gene, sur-6(sv30) (Kao et al., 2004), shows maternal effect lethality. That is, sur-6(sv30) homozygotes of sur-6(sv30+/+) heterozygous mothers grow to adulthood. However, the homozygote eggs never hatch, showing that the maternal product functions during embryogenesis. As with let-92, because the DD neurons are born during embryogenesis (Sulston et al., 1983), DD axon guidance could be affected by the maternal product. To avoid this issue, we used the same strategy of examining animals expressing a low allelic dose of the sur-6 gene. The sur-6(sv30+/+) heterozygotes were identical to wild-type animals and had no axon guidance defects (Fig. 4).

We found that the low allelic dose of paa-1 and sur-6 partially enhanced the axon guidance defects of DD and VD motor neurons in the unc-51(ks38::Tc1) mutants (Fig. 4), suggesting that these regulatory subunits positively regulate UNC-51. PAA-1 and SUR-6 probably inhibit LET-92 activity, indirectly causing the upregulation of UNC-51 activity in the DD and VD axons. Both paa-1 and sur-6 were expressed almost ubiquitously, including in many neurons at all stages, similar to the expression of let-92 (Fig. 2B,C).

A low allelic dose of LET-92 partially suppresses the AVM axon guidance defect of the unc-51 mutant

UNC-51 and UNC-14 are required for the axon guidance of many neurons (Hedgecock et al., 1985; Desai et al., 1988; McIntire et al., 1992; Mörcck et al., 2003; Lai and Garriga, 2004; Siddiqui and Culotti, 2007). Furthermore, UNC-51, UNC-14 and LET-92 are expressed in many neurons (Fig. 2A) (Ogura et al., 1994; Ogura et al., 1997). Therefore, we hypothesized that PP2A might regulate UNC-51 and UNC-14 in other neurons in addition to the dorsally directed DD and VD neurons. We next looked for genetic interactions among let-92, unc-51 and unc-14 by assessing the pathfinding phenotype of the AVM axon. The AVM is a mechanosensory neuron that resides in the lateral region of C. elegans (White et al., 1986). The axon of the AVM neuron first extends ventrally and then, after reaching the ventral nerve cord, extends anteriorly (Fig. 5A).

We first found that unc-51 and unc-14 mutants had defects in the ventral guidance of the AVM axon (Fig. 5B,C). As we expected, let-92(s504+/+) heterozygotes partially suppressed the AVM axon guidance defect in the unc-51(ks38::Tc1) mutant (Fig. 5E). However, let-92(s504+) did not affect the AVM axon guidance defect in the unc-14(e57)-null mutant (Fig. 5E). Therefore, some UNC-14 function may be required for LET-92 to act in axon guidance.

Mutations in both unc-51 and unc-14 enhance the unc-6 mutant phenotype, but suppress the sla-1 mutant phenotype, in AVM axon guidance

The molecules involved in the ventral guidance of the AVM axon in C. elegans have been extensively studied (Walthall and Chalfie, 1988; Hao et al., 2001; Yu et al., 2002; Gitai et al., 2003; Chang et al., 2004; Chang et al., 2006; Quinn et al., 2006; Fujisawa et al., 2007).
Ventrally expressed UNC-6 attracts the A VM axon ventrally, and the dorsally expressed SLT-1 repels it. These pathways are partially redundant; approximately 50% of the animals carrying the null mutations unc-6(ev400) or slt-1(eh15) showed abnormal A VM axon pathfinding (Fig. 5D; Fig. 6) and nearly 100% of unc-6(ev400); slt-1(eh15) double mutants showed such defects (Fig. 6).

To determine whether LET-92, UNC-51 and UNC-14 function in the UNC-6 or SLT-1 pathways, we examined the genetic interactions among unc-51, unc-14, unc-6 and slt-1. We found that the AVM axon guidance phenotypes observed in the unc-51(ks38::Tc1) and unc-14(e57) mutants resulted from the additive effects of inhibiting the SLT-1 pathway and enhancing the UNC-6 pathway, and that the abnormal A VM guidance phenotypes in the unc-51 and unc-14 mutants (Fig. 5B,C) result from defects in the SLT-1 pathway. As let-92 partially suppressed the phenotypes of unc-51(ks38::Tc1), LET-92 may negatively regulate the function of UNC-51 in the SLT-1 pathway (Fig. 7A) or positively regulate its function in the UNC-6 pathway (Fig. 7B).

We also found that the unc-14(e57) mutation partially suppressed the AVM axon guidance phenotype caused by unc-51(ks38::Tc1), a severe mutant allele of unc-51 (Fig. 6), suggesting that UNC-14 can negatively regulate the UNC-6 pathway without UNC-51.

LET-92 can function cell-non-autonomously in AVM axon guidance
As UNC-51 functions cell-autonomously in neuronal axon outgrowth (Lai and Garriga, 2004), we next examined the cell autonomy of let-92 effects on AVM axon guidance. We found that the expression of let-92 in the AVM neurons did not rescue the phenotype (Fig. 8), suggesting that LET-92 can function cell-non-autonomously in the axon guidance. Recently, we found that unc-51 regulated localization of UNC-6 in ventral neurons (VA, VB and AVG) (T. Asakura, unpublished data). Therefore, we examined the let-92 cell autonomy in the ventral neurons. We found that
expression of *let-92* in the ventral neurons (VA, VB and AVG) did not rescue the phenotype (Fig. 8). We examined the cell autonomy in body wall muscles in which SLT-1 and UNC-6 are expressed (Hao et al., 2001; Asakura et al., 2007). We found that the expression of *let-92* in the body wall muscles did not rescue the phenotype (Fig. 8). Finally, we found that pan-neuronal expression of *let-92* rescued the phenotype (Fig. 8). These results suggest that *unc-51* and *let-92* can cell-non-autonomously work in some neurons in the AVM axon guidance.

**PP2A dephosphorylates phosphoproteins phosphorylated by UNC-51**

How do UNC-51 and LET-92 cooperate to regulate the axon guidance in neurons? We tested two hypotheses. First, as UNC-51 is a serine/threonine kinase and LET-92 is a serine/threonine protein phosphatase, we hypothesized that LET-92 dephosphorylates phosphoproteins that are phosphorylated by UNC-51 and that the balance in phosphorylation created by these enzymes is important for axon guidance. Second, we hypothesized that the activity of LET-92 is regulated by phosphorylation by UNC-51. Given that UNC-51 phosphorylates UNC-14, VAB-8 and UNC-51 itself in cultured cells and in vitro (Lai and Garriga, 2004), we first examined whether PP2A could dephosphorylate the phosphoproteins that were phosphorylated by UNC-51 in vitro. We found that the phosphoproteins UNC-14, VAB-8 and UNC-51, which were phosphorylated by UNC-51, were dephosphorylated by human PP2A-AC, which included the catalytic C and regulatory A subunits (Fig. 9A-D), suggesting that LET-92 regulates their activity by its dephosphorylation activity. By contrast, we did not detect phosphorylation of LET-92 or GFP by UNC-51 in vitro (Fig. 9E,F), suggesting that UNC-51 does not regulate LET-92 activity, and that UNC-51 does not randomly phosphorylate proteins. These results supported the hypothesis that LET-92 dephosphorylates phosphoproteins that are phosphorylated by UNC-51, and that the phosphorylation balance generated by these enzymes may be important for axon guidance.

**LET-92 colocalizes with UNC-51 in the DD and VD neurons**

To examine their subcellular localizations, we expressed Venus::LET-92 and UNC-51::mCherry in DD and VD neurons. We found that Venus::LET-92 and UNC-51::mCherry were largely colocalized in the cell bodies and axons in the DD and VD neurons (Fig. 10A-C). Their punctate appearance in the axons may reflect their localization to small transport vesicles. In addition, we found that Venus::LET-92 localized to the margins of the VD growth cones (Fig. 10D-F), where receptors of axon guidance molecules should be located. As UNC-51 regulates the localization of UNC-5, a receptor of UNC-6 (Ogura and Goshima, 2006), LET-92 may cooperate with UNC-51 to regulate the localization of such receptors at the margins of the VD growth cones.

**DISCUSSION**

**PP2A regulates the function of UNC-51, probably by forming a complex with it**

Protein kinases and phosphatases play important roles in signal transduction pathways (Hunter, 2000). UNC-51 is a serine/threonine protein kinase and its kinase activity is essential for axon guidance.
PP2A is a serine/threonine protein phosphatase (Millward et al., 1999; Lechward et al., 2001; Sontag, 2001; Janssens et al., 2008). Thus, UNC-51 and PP2A have antagonistic enzymatic activities. We found that UNC-51 physically and genetically interacted with PP2A, and that PP2A dephosphorylated phosphoproteins that were phosphorylated by UNC-51, including UNC-51. In addition, we found that LET-92 colocalized with UNC-51 in axons, and that LET-92 could act in neurons.

PP2A interacts with several serine/threonine protein kinases and regulates their kinase activity by dephosphorylation (Millward et al., 1999). For example, PP2A forms a stable complex with Ca2+-calmodulin-dependent protein kinase IV (CaMKIV) and negatively regulates its activity by dephosphorylation (Westphal et al., 1998). In this case, PP2A elicits a rapid downregulation of CaMKIV after its activation. Thus, the physical interaction between these proteins probably speeds up the enzymatic reaction of PP2A. By contrast, PP2A also forms a stable complex with the serine/threonine protein kinase Raf-1 and positively regulates its activity by dephosphorylation (Abraham et al., 2000). In this case, PP2A removes an inhibitory phosphate from Ser 259 of Raf-1. Thus, PP2A can positively regulate kinase activity as well. Therefore, the simplest hypothesis for the UNC-51 and LET-92 interaction is that the kinase activity of UNC-51 is regulated by the dephosphorylation activity of LET-92 in a complex that includes these two proteins (Fig. 11A). Similar to the case of CaMKIV, rapid dephosphorylation by PP2A may be important for downregulating UNC-51 function.

As UNC-51 has autophosphorylation activity, a kinase that phosphorylates UNC-51 may be UNC-51 itself. Alternatively, by forming a complex, UNC-51 and LET-92 may rapidly regulate the phosphorylation level (and thus the activity) of an unidentified in vivo substrate required for axon guidance (Fig. 9).
UNC-51 is expressed on ventral cells, where it acts as a repellent, and UNC-5 is expressed on the axons, where it mediates the transduction of positional information. Recently, we reported that UNC-51 and UNC-14 regulate the subcellular localization of UNC-5, probably by regulating its trafficking (Ogura and Goshima, 2006). We proposed that the subcellular localization of UNC-5 is correlated with its functional activity.

Our genetic results showed that, in DD and VD axon guidance, LET-92 negatively regulated the function of UNC-51 and that its regulatory subunits, PAA-1 and SUR-6, positively regulated UNC-51. As PP2A dephosphorylated the auto-phosphorylated UNC-51, a simple and attractive hypothesis is that PP2A regulates the effect of UNC-51 on the subcellular localization of UNC-5 by dephosphorylating UNC-51. In *D. melanogaster*, UNC-51 binds and phosphorylates UNC-76, which is a kinesin heavy chain adaptor protein necessary for kinesin-1-mediated axonal transport, to control the motor-cargo assembly for synaptic vesicle transport (Toda et al., 2008). The phosphorylation of UNC-76 by UNC-51 is important for the binding of UNC-76 to Synaptotagmin-1, a synaptic vesicle protein. In *C. elegans*, the binding partner of UNC-51, UNC-14, acts as an adaptor protein for kinesin-1-dependent synaptic vesicle transport (Sakamoto et al., 2005). As PP2A dephosphorylated phospho-UNC-14 that was phosphorylated by UNC-51, UNC-51 and PP2A may regulate UNC14 in its role as an adaptor protein for the unidentified motor protein that transports UNC-5. As Venus::LET-92 localized at the margins of the VD growth cones, LET-92 may cooperate with UNC-51 to regulate the UNC-5 localization at the margins of the VD growth cones.

**Possible functions of PP2A and UNC-51 in AVM axon guidance**

Ventrally expressed UNC-6 attracts the AVM axon ventrally, and the dorsally expressed SLT-1 repels it (Hao et al., 2001). The UNC-6 and SLT-1 pathways have partially redundant functions in AVM axon guidance. In the UNC-6 pathway, an UNC-6 receptor, UNC-40, receives the UNC-6 information, which is transduced via CED-10/Rac, UNC-115/abLIM and UNC-34/Enabled (Gitai et al., 2003). CED-10 acts upstream of UNC-115, and UNC-34 functions in another pathway. The UNC-40–UNC-34 pathway is inhibited by CLR-1, a receptor protein phosphatase (RPTP) (Chang et al., 2004). MIG-10/lamellipodin and AGE-1/Pi3K probably contribute to the neurally asymmetric axon formation through UNC-40 and CED-10 (Chang et al., 2006; Quinn et al., 2006; Quinn et al., 2008).

In the SLT-1 pathway, SLT-1 binds to the co-receptors SAX-3 and EVA-1 (Zallen et al., 1998; Fujisawa et al., 2007). The information is then partly transmitted by UNC-34 (Yu et al., 2002). Conversely, RPM-1 and CLEC-38 negatively regulate the expression levels of the receptors SAX-3 and UNC-40, respectively (Li et al., 2008; Kulkarni et al., 2008).

Our genetic results suggest that UNC-51 and UNC-14 negatively regulate the UNC-6 pathway, but positively regulate the SLT-1 pathway. Similar to DD and VD axon guidance, in which UNC-51 and UNC-14 regulate the localization of UNC-5 (Ogura and Goshima, 2006), UNC-51 and UNC-14 may regulate the localization (or trafficking) of molecules important for the signal transduction pathways of UNC-6 and SLT-1. Recently, we found that UNC-51 and UNC-14 regulated localization (secretion) of UNC-6 in ventral neurons (T. Asakura, unpublished data), suggesting that UNC-51 and UNC-14 can cell-non-autonomously act on the AVM axon guidance. Our results also suggest that UNC-51 with LET-92 can cell-non-autonomously act on the AVM axon guidance in some neurons that are different from the UNC-6 secretory cells. UNC-51 and UNC-14 probably participate in multiple functions on the AVM axon guidance.

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**Fig. 11. Models for the regulation of UNC-51 function by LET-92.**

(A) The activity of UNC-51 may be regulated by phosphorylation via the dephosphorylation activity of LET-92 in the complex. X is an unknown kinase that phosphorylates UNC-51. **(B)** The balance between the kinase activity of UNC-51 and the phosphatase activity of LET-92 in the complex may be important for the function of an unknown substrate (Y) required for axon guidance.
Our genetic results suggest that, in AVM axon guidance, UNC-14 can negatively regulate the UNC-6 pathway without UNC-51. This is different from the DD and VD neurons, in which UNC-14 cannot work without UNC-51 (Ogura and Goshima, 2006). Although UNC-51 and UNC-14 physically interact and regulate the axon guidance of many neurons in C. elegans, their molecular mechanisms for axon guidance may differ from neuron to neuron.

Because the low allelic dose of LET-92 partially suppressed the abnormal pathfinding of the AVM axon in the weak unc-51 mutant, we propose two possible functions for LET-92 in AVM axon guidance: (1) LET-92 negatively regulates the UNC-51 function for the SLT-1 pathway (Fig. 7A), and (2) LET-92 positively regulates the UNC-51 function for the UNC-6 pathway (Fig. 7B). These two models are not necessarily mutually exclusive. That is, LET-92 may participate in both pathways. We found that let-92 cell-non-autonomously worked in some neurons in the AVM axon guidance.

In the neurons, unc-51 and let-92 may regulate secretion of an unknown factor that regulates activity of UNC-6, SLT-1 or their receptors. As we only analyzed the effects of the low allelic dose of LET-92, we cannot exclude cell-autonomous function of LET-92 in AVM axon guidance.

Future work
In this paper, we report that PP2A cooperates with UNC-51 to regulate axon guidance in C. elegans. However, some of the molecular details of these axon guidance mechanisms are still unclear. Among the remaining questions are, how do PP2A and UNC-51 regulate axon guidance in DD and VD neurons? Does PP2A participate in localization of UNC-5, which is regulated by LET-92? How do PP2A, UNC-51 and UNC-14 regulate UNC-6 and SLT-1 signaling in the AVM neuron? Our future research will seek the answers to these questions. Finally, analysis of the mammalian homologs of UNC-51 and PP2A will reveal whether PP2A has evolutionarily conserved roles in axon guidance.

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