Molecular characterization of Pegarn: a *Drosophila* homolog of UNC-51 kinase

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Abstract We have isolated and characterized the gene encoding a *Drosophila melanogaster* homolog of *Caenorhabditis elegans* UNC-51 (uncoordinated movement-51): Pegarn. Developmental Northern blot shows the Pegarn gene is expressed at all stages of development. The protein is detected throughout the *Drosophila* third instar larval central nervous system (CNS) in axons projecting out from the ventral ganglion and in the optic anlagen of the optic lobe. Heterozygous Pegarn mutant embryos show defects in larval axonal neuronal patterning, but survive to adulthood. Homozygous mutants have an even more deformed pattern of neuronal development and do not survive through the larval stages. The data from this research suggest the critical roles of Pegarn in CNS and PNS axonal formation in *Drosophila melanogaster* and indicates its similar role in other multicellular species.

Keywords Uncoordinated movement-51 · Ventral ganglion · Central nervous system · Commissures · Connectives · Midlines

Introduction

Protein kinases play key roles in eukaryotic signal transduction pathways [1] and have been shown to influence the

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dynamics of cytoskeletal organization during neuronal migration and neurite formation [2–4]. There are numerous protein serine/threonine kinases that function in cytoskeletal modifications that affect the motility of neurons and their growth cones in the developing brain [5]. Axonal elongation is required for the formation of complex neuronal networks. In the nematode *C. elegans*, more than 10 genes are needed for normal axon elongation and/or guidance, including UNC-51 [5]. UNC-51 was first described as an uncoordinated (UNC) mutant in the nematode *C. elegans* [6]. This gene affects the growth of amphid and phasmid axons in *C. elegans* [7]. Mutation in the UNC-51 gene results in various abnormalities in axonal elongation and axonal structures in that most of the neurons exhibit premature termination or dysregulated growth. Toth et al. [8] found that inactivation of UNC-51 influences neuronal degeneration in *C. elegans*. Neuronal abnormalities have been observed in all species where UNC-51 mutants have been examined to date; hence, this gene product is likely to play an important role in construction of the neural networks [9]. A human homolog of UNC-51, designated ULK1 has been cloned and characterized [10]. Homology search analysis showed that ULK1 has 41% overall amino acid similarity to UNC-51 [10]. ULK-1 interacts with proteins involved in vesical transport and axonal elongation in mammalian neurons [11]. A murine homolog of UNC-51, designated UNC 51.1, has also been identified [12]. UNC 51.1 encodes a serine/threonine kinase which is expressed in granule cells in the cerebellar cortex and participates in the program of gene expression leading to the formation of granule cell axons [12]. Mouse ULK2 was identified by Yan et al. [13] and is involved in an uncharacterized signaling pathway in mammalian cells [13]. UNC-51 and ULK1 regulate neural differentiation [14]. Zhou et al. [15] suggested that ULK1/2 proteins

regulate filopodia extension and neurite branching during sensory axon outgrowth.

We have isolated the *Drosophila* homolog of UNC-51. This protein is expressed in neuronal cells of the *Drosophila melanogaster* central nervous system throughout development. Mutant flies lacking UNC-51 show lethality during the late larval stages. Our results show that the UNC-51 kinases have homologous function in multicellular organisms. We have named this protein Pegarn which is a Thai word, meaning "unable to function normally since some part is missing."

Materials and methods

PCR reactions to amplify cDNA that encodes kinases

PCR with degenerate oligonucleotides was performed to amplify putative kinase gene sequences from a cDNA pool made from heads of *Drosophila* polyA+ RNA. The first oligonucleotide encodes the sequence H/Y R D L K P E/D N which is within the conserved catalytic domain VI of ser/thr kinases (ATP binding sites) [16–18]. The reverse complement of the second oligonucleotide encodes the sequence Y I/L/M A P E I/V I/L, which is conserved domain VIII in ser/thr kinases, and is generally found between 135 and 180 nt downstream from the first oligonucleotide [19].

Cloning of Pegarn cDNA

A λgt10 *Drosophila* cDNA library from Stratagene was screened with a 150 bp fragment of Pegarn probe labeled with (^{32}P) dCTP, using the NEBlot labeling kit from New England Biolabs following the protocol from the manufacturer [20]. The sequence of the cDNA was compared to the genomic sequence from the *Drosophila* genomic database Berkeley *Drosophila* Genome Project (BDGP).

Plasmid rescue

The cytological location of Pegarn was used to identify possible mutant fly lines from Flybase (http://flybase.bio.indiana.edu). Two potential P element insertion lines, EP(3)3348, and EP(3)3193 were identified. The genomic sequences flanking the P elements were rescued using the protocol supplied by the BDGP. The sequence of the genomic DNA flanking the P element insertion confirmed that the insertions were located in the Pegarn gene.

Lethality test

Three crosses were set up in the fly houses in the 25°C incubator. The first cross was Canton S crossed with w;

Cyo/Bl; TM2/TM6b, the second was EP(3)3348 which has the genotype of (P[w$^+$]/TM6b), and the third was EP(3)3193 which has the genotype (P[w$^+$]/TM6b). Both EP(3)3348 and EP(3)3193 lines contain the balancer TM6B but differ in the positions of P element insertion. The offspring (400 embryos of each type of cross) were transferred into four vials (100 embryos/vial) for three different sets of the experiments. The number of pupa appeared and adult flies hatched from the pupal cases were determined.

Developmental northern blots

Total RNA was isolated from embryos at different time stages: 4 h, 12 h, 1 day, 2 day, 4 day, 6 day, 8 day and adult, using an RNA isolation kit (Ambion) according to manufacturer's protocol. Total RNA of adult wild type head and body and of the whole animal from heterozygote EP(3)3348 (adult homozygous lethal) and homozygous EP(3)3193 were also isolated. All RNA were run on formaldehyde gels and transferred to nitrocellulose membranes. The blots were probed with Pegarn cDNA labeled with (^{32}P) dCTP using NEBlot Kit from New England Biolabs. In order to control for loading, RNA blots were also hybridized with the RP49 ribosomal protein gene.

Plasmid construction and making GST-fusion protein of Pegarn

Approximately 810 bp of Pegarn encoding the least conserved region (amino acid 350–620) was generated by PCR and subcloned into BamHI-EcoRI sites of pGEX-3X (Pharmacia Biotech). The oligonucleotides used were: forward primer flanked with BamHI 5′-CGGGATCCGTG TCCTGTGCGA-3′ and reverse primer flanked with EcoRI 5′-CGGAATTCTGCTCGCGATCCAT-3′. The construct was transformed into *Escherichia coli* strain BL21 (protease negative strain) and GST fusion protein was produced and purified using Glutathione-Sepharose 4B beads (Pharmacia Biotech) according to manufacturer's protocol.

Pegarn-GST fusion protein was run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 10% acrylamide resolving gel and transferred to Nitrocellulose membrane. The membrane was probed with anti-GST antibody to confirm the production of Pegarn-GST fusion protein of the correct relative size. Rat polyclonal antiserum was generated against Pegarn fusion protein by Pocono Rabbit Farm, and affinity purified using GST fusion protein bound to CNBr beads (Pharmacia-biotech). Proteins were extracted from the head and body of Canton S (CS) flies, heads of EP(3)3348 and EP(3)3193 flies and separated on a 10% SDS-PAGE, transferred to the membrane and probed with purified Pegarn antibody (1:500). For loading

control, the membranes were also probed with anti-E7 tubulin antibody. The signals were detected using chemiluminescence reagents from Sigma and assayed according to the manufacturer's protocol.

Immunofluorescence

Ten micron thick sections of adult wild type fly heads and bodies were fixed in 4% paraformaldehyde in $1\times$ PBS, permeabilized with cytoskeletal buffer (10 mM Hepes pH 7.4, 200 mM sucrose, 3 mM $MgCl_2$, 50 mM NaCl, 0.5% Triton-X-100, and 0.02% NaN_3), washed and incubated with purified rat anti-Pegarn polyclonal antobody (1:25) at 4°C for 24 h. The slides were washed with 0.1% saponin in $1\times$ PBS and secondary antibody (FITC anti-rat) 1:500 was added, incubated at room temperature for 1 h, washed and mounted in VECTASHIELD (Vector Laboratories, Inc.).

EP(3)3348/GFP fly line was generated by the cross between EP(3)3348 which has the genotype of $(P[w^+]/$ TM6B) with the fly line that contain GFP [w;Sb/TM3 $P\{w^+$ GFP actin}ser]. Their offspring that contain both P element and GFP were selected and maintained. Embryos of EP(3)3348/GFP were collected and sorted under a fluorescent dissecting microscope using a GFP filter from Zeiss. Twelve hour embryos of each type were collected, dechorionated, fixed with 4% paraformalehyde and heptane for 1 h with gentle shaking. The vitellin membrane was removed with a needle and washed with PBT ($1\times$ PBS with 0.3% Triton-X-100) before applying the primary antibody, rat anti-Pegarn (1:100). CNS of third instar larvae were dissected and fixed for 1 h with 4% paraformaldehyde, washed with PBT, and incubated with rat-anti Pegarn primary antibody (1:50). All secondary antibodies used: FITC, Texas Red, and Cy5 are from Jackson Laboratory and used at 1:200, 1:400, and 1:100 respectively. For co-labeling, monoclonal antibody mouse 22C10 and mouse BP102 from Hybridoma Bank (http://www.uiowa.edu/∼dshbwww/index.html) were used at 1:2 or less. All images were collected by a Biorad 1024 scanning confocal microscope.

Results

cDNA sequence of Pegarn kinase

We employed PCR using degenerate oligonucleotides on cDNA which was made by random-primed reverse transcription of mRNA from the wild type fly heads to amplify approximately 150 bp from putative kinases [16, 19]. The sequences of isolated fragments were compared by BLAST analysis against NCBI databases. One fragment was identified as a potential homolog of UNC-51. This 150 bp fragment was used to screen a *Drosophila* cDNA library (λgt10 library).

Approximately 1,260 bp of Pegarn cDNA was identified. The sequence was aligned with the genomic sequence available from the Berkeley *Drosophila* Genome Project (BDGP) with the Accession number **AAF49879** [21]. The 5′ terminus of this gene was confirmed by primer extension.

Amino acid sequences of Pegarn and alignment with potential homologs

The Pegarn cDNA has an open reading frame that encodes a protein of 835 amino acids with a calculated MW of 90.6 kDa. It is smaller than the human ULK-1 (1050 amino acids; Accession **AAC32326**), mouse ULK-1 (1051 amino acids; Accession **AAC40118**), and about the same size as UNC-51 (856 amino acids; **Accession CAA86114**) (Fig. 1a). The basic structure of the UNC-51 kinases of these organisms contains three major parts: the kinase domain, prolineserine (PS) rich domain, and carboxyterminal domain. The kinase domain of these kinases is the most highly conserved region. Compared to human ULK-1, Pegarn has an overall 30% amino acid identity (67% identity in the kinase domain) and 41% similarity. Compared to mouse ULK-1, Pegarn has 30% identity (68% identity in the kinase domain) and 42% similarity. And compared to *C. elegans* UNC-51, Pegarn has 33% identity (64% identity in the kinase domain) and 38% similarity. All percent identities and similarities were calculated with the GeneWorks program. These proteins were aligned using ClustalW. The remarkable high level of amino acid conservation between these species which are evolutionarily separated by over 650 million years indicates a great likelihood of conservation of function.

Genomic organization and mutant lines

The genomic organization of Pegarn is shown in Fig. 1b. The Pegarn gene consists of 10 exons (pink boxes) and 9 introns (green boxes). The sizes of the introns range from approximately 60–6,500 bp. Exon sizes range from 72 to 1,080 bp (Fig. 1b). The Pegarn gene mapped to the 69E1–69E2 region of chromosome 3L of *D. melanogaster*. Using the cytological location of this gene, we searched the available stocks of mutant flies (http://flybase.bio.indiana.edu) and found two Enhancer-Promoter (EP) lines [EP(3)3348 and EP(3)3193] generated by Rorth [22]. The EP(3)3348 line has a P element inserted at the first exon, 5 bp downstream from the first ATG of Pegarn gene. The P element in EP(3)3193 is inserted in the second intron of the genomic structure of this gene (Fig. 1b).

Lethality test

The EP(3)3348/TM6B (adult homozygous lethal) line has a P element inserted at the 5′ end of Pegarn gene. Another line,
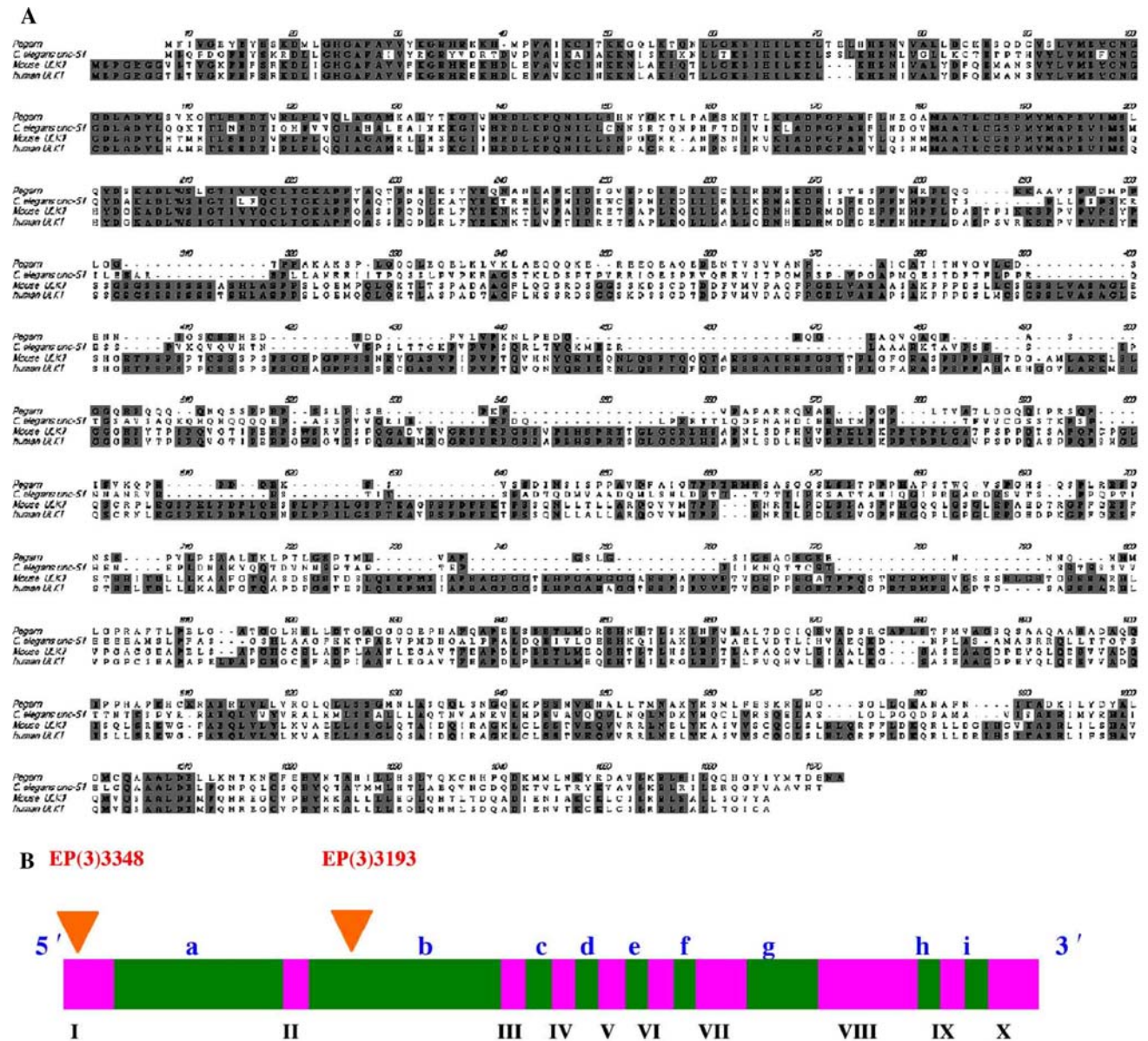
A

B **EP(3)3348**   **EP(3)3193**

Fig. 1 (**a**) Amino acid alignment of *C. elegans* UNC-51, human ULK1, mouse ULK1, and Pegarn by the ClustalW program. Identical residues are shaded. (**b**) Genomic organization and relative positions of the mutant EP lines. Pegarn kinase contains 9 introns (green boxes). The sizes of introns a to i are approximately, 6,500, 9,000, 160, 160, 60, 50, 347, 60, and 60 bp respectively. Exons are represented by pink boxes. The exons sizes from I to X are 788, 174, 87, 126, 72, 162, 282, 1080 141, and 236 bp respectively. Also shown in this figure are the relative location of P element insertions of the EP lines, EP(3)3348 and EP(3)3193

EP(3)3193/TM6B (adult homozygous viable), has the P element inserted at the second intron of the genomic structure of this gene (Fig. 1b). Both EP(3)3348 and EP(3)3193 lines were reported to have reduced viability. To confirm this information, we performed three types of crosses. In the first, wild type flies were crossed with flies that have the genotype of w; CyO/Bl; TM2/TM6B as a control experiment. The second and the third crosses involved EP(3)3348/TM6B and EP(3)3193/TM6B.

The percent of viability in early development of these two EP lines and the control experiment were determined. From

the results of the lethality test (Table 1), the offspring of wild type flies crossed with double balancer, pupal cases appeared 73% and the adult flies hatched 71%. For EP(3)3193 cross, pupa cases appeared 60% and adult flies hatched 58%. Viable adult flies of EP(3)3193 offspring should be 75% due to the balancer TM6B containing in the fly (fly homozygous for the balancer will die). Some embryos also died during the transfer into the vials. Considering pupal cases appeared and adult flies hatched, the two numbers are almost equal for the control experiment and of EP(3)3193 (adult homozygous viable). In contrast, EP(3)3348 which has the P element inserted at the

Table 1 Lethality tests

Type of fly	P element position in Pegarn structure	% Pupa appeared	% Adult fly hatched
EP(3)3348/TM6B	5′ of gene	52	28
EP(3)3193/TM6B	2nd intron	60	58
CS/TM6B	–	73	71

Three crosses were performed in 25°C incubator. The eggs of wild type flies crossed with w; CyO/Bl; TM2/TM6b, and crosses of two EP lines, EP(3)3348/TM6B and EP(3)3193/TM6B were transferred into vials. The percentage of pupae appearing and adult flies eclosing was determined

5′end of Pegarn gene, pupal cases appeared 52% and adult flies hatched 28%. This is a clear reduction of % of adult flies hatched when compared to % pupal cases appeared. Furthermore, the stage of lethality of EP(3)3348 also was determined. Homozygous larva of EP(3)3348 can develop into pupa but they could not metamorphose to the adult. We distinguished the homozygote and heterozygote larva from their shapes, long and tubby for homozygote and heterozygote larva respectively. Therefore, the most likely stage of the death of EP(3)3348 is in the third instar larval stage.
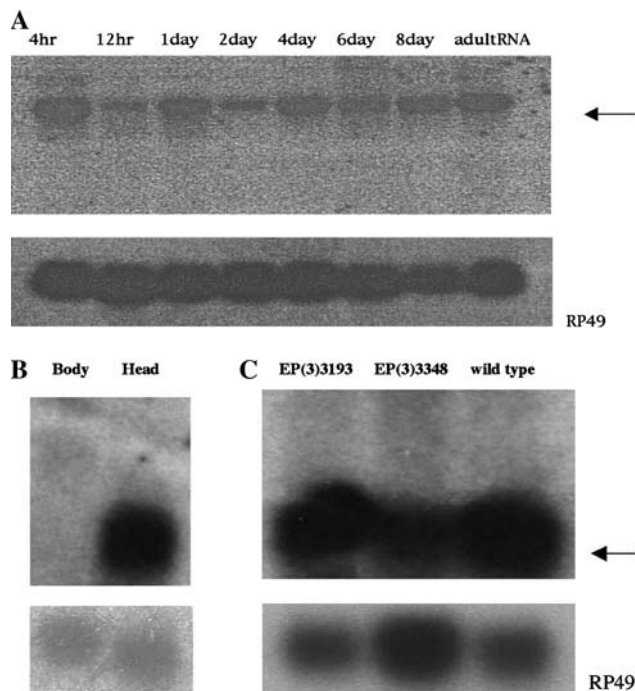

Fig. 2 Developmental Northern Blot of Pegarn. (**a**) Total RNA from 4 h, 12 h, 1 day, 2 day, 4 day, 6 day, 8 day and adult: embryos and flies were transferred to the Nitrocellulose membrane and probed with Pegarn cDNA. (**b**) Total RNA of adult wild type fly head and body. (**c**) Total RNA from mutants EP(3)3348, EP(3)3193 and wild type flies were used. EP(3)3348 heterozygote flies were over a balancer (TM6B). The arrow indicates 3.5 kb transcripts. The lower panels of each blot are the controls for loading of RP49

The P element inserted at the second intron of EP(3)3193 shows no reduction of adult flies hatched compared to the total numbers of pupal cases appeared, same as in the control experiment. This result implied that the insertion at the second intron of the genomic DNA does not affect on expression of the Pegarn gene.

Expression of Pegarn mRNA and protein

To examine the expression of Pegarn mRNA, we extracted total RNA from embryos at different time stages and Northern blot analysis was performed using a Pegarn cDNA probe. The results of this developmental analysis showed that the Pegarn mRNA is expressed at all stages of development with lower expression at the 12 h and 2 day time points (Fig. 2a). The 3.5 kb transcript (arrow) detected corresponds to the size of the entire cDNA sequence. A Northern blot was done using mRNA from the head or the body of wild type flies and the two EP lines with insertions in the gene. Pegarn mRNA was detected only in the head, not in the body of the adult fly (Fig. 2b). This is similar to the expression of UNC-51 in *C. elegans* which is mostly in the head region of the worm [9]. Heterozygote viable EP(3)3348 adult flies (homozygous lethal adults) show reduced mRNA expression compared to wild type (Fig. 2c). EP(3)3348 heterozygote flies were over a balancer (TM6B). This result indicated that the insertion at 5′ end of Pegarn affects the
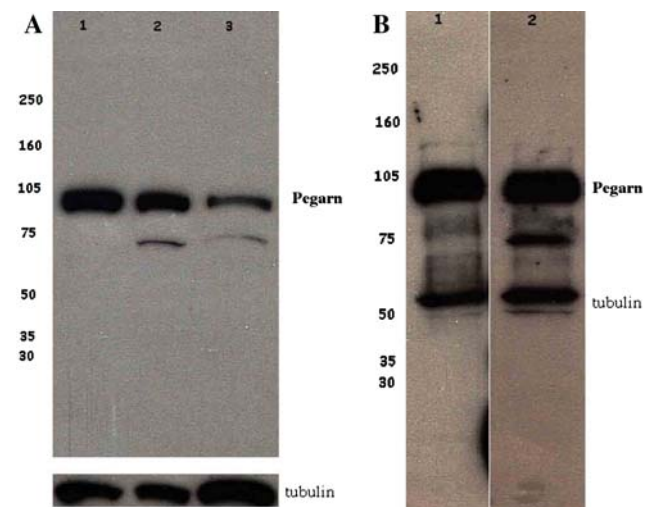

Fig. 3 Western blotting of head and body of wild type fly, heads of EP(3)3348 and EP(3)3193. Protein was extracted from the head and body of wild type flies, heads of EP(3)3348 and EP(3)3193 and Western blot analysis was performed with anti-Pegarn purified antibody. The lists of the numbers on the side of each figure represent molecular weight marker. (**a**) Lane 1, 2 and 3 are wild type heads, wild type bodies, and heterozygous EP(3)3348 heads respectively. Antibody to tubulin for the loading control is shown at the bottom of the Fig. 3. (**b**) Lane 1 and 2 are wild type heads and EP(3)3193 homozygous heads respectively. Antibody to tubulin was used for the detection of protein loading and shown in the same blot

Fig. 4 Expression pattern of Pegarn protein in wild type animals by immunofluorescence staining. (**a**) Adult wild type fly heads with purified Pegarn antibody, (**b**) pre-immune serum, (**c**) larval CNS axon (in the brain hemisphere): purified Pegarn antibody, (**d**) pre-immune on same tissue, (**e**–**j**) expression pattern in the axons projecting out from the ventral ganglion co-staining with monoclonal antibody 22C10 (red), Pegarn antibody (green) and merge (yellow) vs. (**k**) pre-immune. The images of Fig. 4e–g and h–j are taken from the same ventral ganglion but from different focal planes. The thorax and (**l**) the abdomen (**n**) of adult fly bodies with purified Pegarn antibody vs. (**m**, **o**) pre-immune sera around the same area
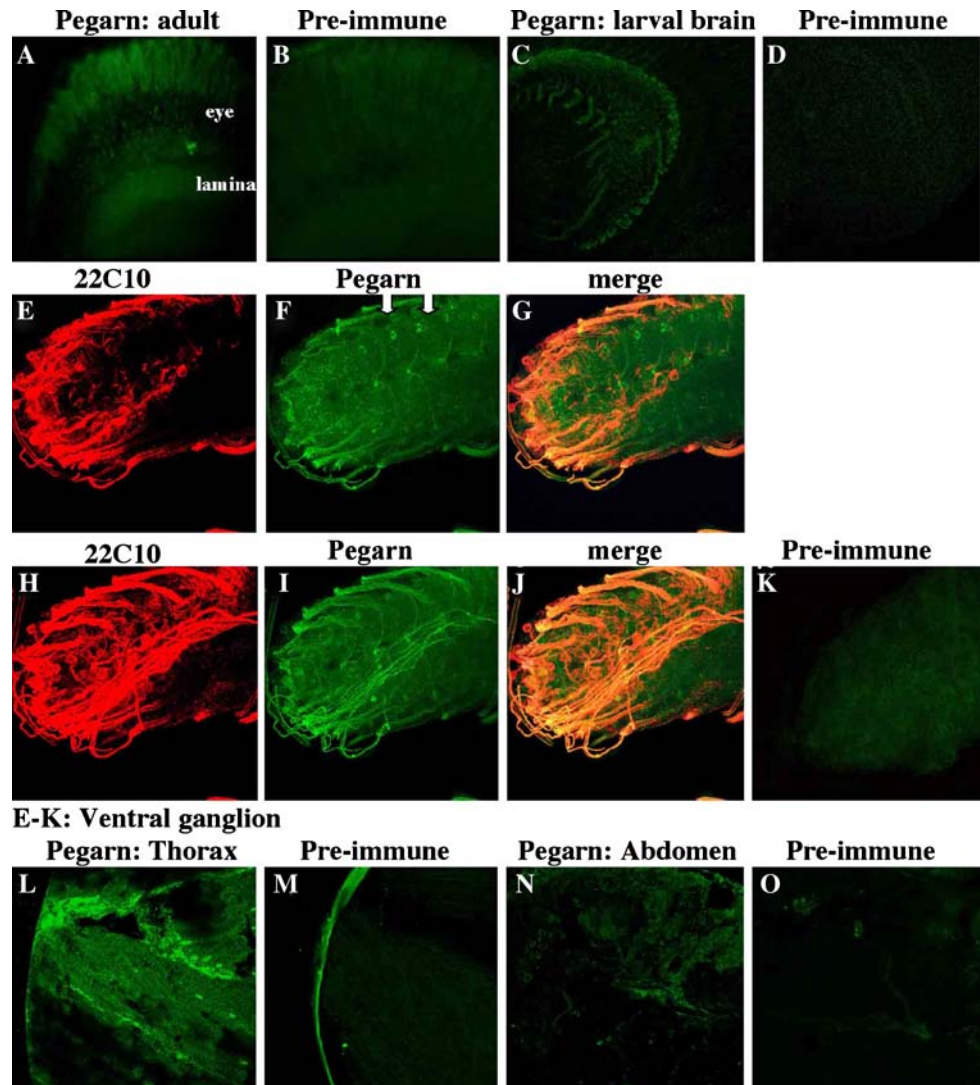
expression of this gene. There was no difference in the level of mRNA expressed in EP(3)3193 compared to wild type indicating that this second intron insertion does not affect the expression of Pegarn.

To determine the tissue-specific expression of this kinase, we generated polyclonal antibodies against the least conserved sequences (amino acid 350–620) to lower cross-reactivity of the antibody. Proteins were extracted from the head and body of adult wild type flies, heads of EP(3)3348 (heterozygous), and also heads of EP(3)3193 (homozygous) and used in Western blot analysis. The Pegarn protein is detected in all head, body and EP lines (Fig. 3a, b). The size of the detected protein matches well to the predicted MW of 91 kDa. Although this result seems to contradict the Northern blot analysis which showed no Pegarn mRNA expression in the body, the detection of Pegarn protein in the body is probably due to the transport of the protein down motor axons and into the body (see below). Reduced expression of Pegarn protein was seen in heterozygous

EP(3)3348 heads (Fig. 3a; lane 3). In contrast, homozygous EP(3)3193 flies seem to have the same level of protein expression as wild type (Fig. 3b). This is further evidence indicating that the insertion of the P element in the second intron has no effect on the expression of this gene. Pre-immune serum of the same animal did not react with Pegarn protein (data not shown). Preabsorption with Pegarn GST-fusion protein blocked reactivity with the 91 kDa band. The protein detected at approximately 70 kDa was not related to Pegarn protein because it was still detected after the Pegarn antibody was pre-absorbed with Pegarn-GST fusion protein, whereas the 91 kDa bands disappeared (data not shown).

Specific localization of Pegarn protein

The Northern blot of Pegarn with head and body of the wild type fly indicated that this mRNA is head specific. Therefore, immunofluorescence experiments with adult fly

heads were carried out. The experiments were done by incubation of 10 μm thick frozen sections of adult wild type fly heads with purified Pegarn antibody using FITC anti-rat secondary antibody. The Pegarn protein was detected in neurons of the eyes and optic lamina (Fig. 4a), while no signal was seen with preimmune serum (Fig. 4b). Immunofluorescence staining in the adult fly bodies was done as well. Even though Pegarn protein is expressed in the adult body, most of the expression was seen in the thorax and very little in the abdomen (Fig. 4l, n). This result corresponded with staining patterns seen in the ventral ganglion of third instar larvae (thorax of the adult) (Fig. 4e–j). No Pegarn protein was detected in the same area when staining with pre-immune serum (Fig. 4m, o). Since lethality occurred during pupation, the expression pattern of Pegarn in third instar larval CNS was determined. Pegarn protein is expressed in the neuropils of the optic lobe anlagen (Fig. 4c) and also in the motor axons that project from of the ventral ganglion of the CNS (Fig. 4e–j). Double labeling with 22C10 (specific marker for neurons) confirmed the expression of Pegarn in the

neurons as parts of the Pegarn signal co-labeled with 22C10. Pre-immune serum did not interact with any tissues (Fig. 4d, k). Pegarn protein is also present within specific cells of each hemisegment in the ventral ganglion (Fig. 4f; arrows) but the identity of these cells is as yet unknown.

Wild type vs. heterozygote and homozygote mutant axonal and neuronal patterns

Since most Pegarn EP(3)3348 mutants die in the pupal cases, immunofluorescence of the third instar larvae and 12-h embryos were performed in wild type, heterozygote and homozygote mutants (Figs. 5–7) to determine if visible abnormal neuronal patterns which might cause the lethality could be detected. Within the larval brain lobes, Pegarn was expressed in the optic lobe neuropils of two crescent-shaped clusters of neuroblasts in the lateral part of both globular brain hemispheres which are called optic anlagen (Fig. 5), In the optic lobe of third instar larvae, expression was seem in the area of inner and outer optic anlage, Bolwig nerve, and

Fig. 5 Immunofluorescence of third instar larvae brain hemisphere. (**a**–**c**) Wild type, (**d**–**f**) heterozygous mutant, (**g**–**i**) homozygous mutant. Each row represents Pegarn staining (green), 22C10 staining (red) and merge (yellow). The expression patterns were seen in the neuropils of the optic lobe anlagen with reduced expression level in homozygote mutants. EP(3)3348 heterozygote flies were over a balancer (TM6B)
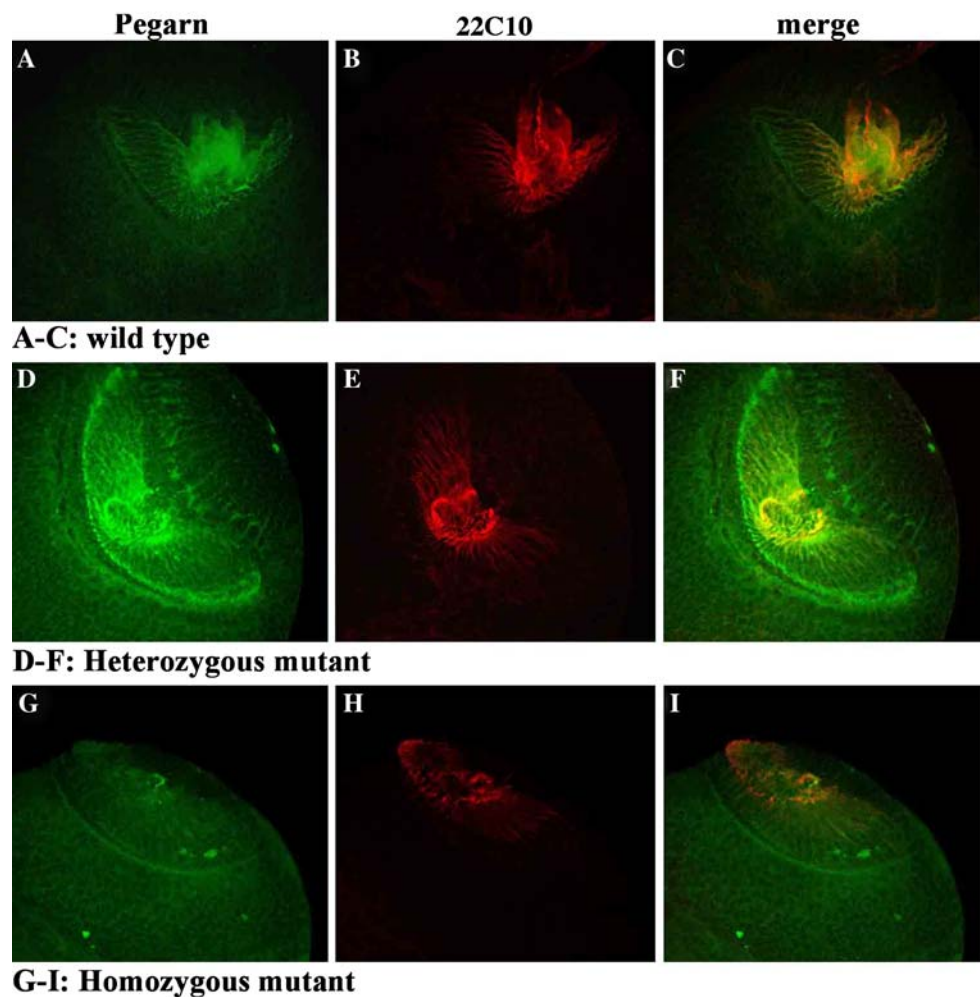
central body fiber (Figs. 4c, 5a–c) of the optic lobe. Heterozygote 3348 and wild type fly staining patterns were similar: the same localization and the same level of expression. In the homozygous 3348 mutant, immunoreactivity to Pegarn was significantly reduced (Fig. 5g) relative to the 22C10 signal (Fig. 5h). Figure 6a–c shows the double staining of Pegarn and 22C10 in the wild type embryo. Pegarn protein localized in CNS axons (Fig. 6b; arrows a and b) and motor axons projecting out from the midline (Fig. 6b; arrow heads). The co-localization with 22C10 was seen in CNS axons (Fig. 6c). This result would be consistent with a role for Pegarn in CNS formation and motor axon outgrowth.

Since Pegarn staining was seen in the commissures (Fig. 6b; b arrows) and connectives (Fig. 6b; a arrows), we also carried out co-staining of wild type embryos with Pegarn and monoclonal antibody BP102 (Fig. 6d–f). BP102 stains the CNS axons and also is a marker for the regular pattern of commissures and connectives (the tracts of nerve fibers passing from one side to the other of the CNS) (http://www.uiowa.edu/~dshbwww/info.html). Pegarn and BP102 co-localized in the CNS axons as shown in Fig. 6f. These

staining results are supportive of a potential role for Pegarn protein in CNS axonal formation or the formation of commissures and connectives. Preimmune serum showed no reactivity with the embryo (Fig. 6g).

Since the EP(3)3348 insertions is an adult homozygous lethal, a line was generated that contains a balancer chromosome marked with Green Fluorescence Protein (GFP) across from the insertion (EP(3)3348/TM6B GFP). This allowed the identification of an embryonic homozygote by virtue of their lack of GFP fluorescence [23]. The flies were mated and 12-h embryos were sorted under a fluorescent dissecting microscope to separate homozygotes and heterozygotes. In heterozygote mutant embryos, Pegarn protein is expressed in the connectives (Fig. 7a, d). The connectives were distorted and broken into pieces as seen in Fig. 7d (arrows) compared to wild type in Fig. 7j. The expression of motor axons projecting out from the midline (a special embryological region around which the future nervous system develops) was also reduced. The neuronal development pattern was dramatically disorganized (Fig. 7b, e) when staining with 22C10. These results, combined with Pegarn's localization pattern, indicates a role for Pegarn in

Fig. 6 Immunofluorescence of the wild type 12-h embryos. (**a-c**) Double staining of 22C10 and Pegarn. The 22C10 (red) staining in (**a**), Pegarn (green) in (**b**) and merge (yellow) in (**c**). (**d–f**) Double staining of 12-h embryos with monoclonal antibody BP102 (red) and Pegarn (green) and merge (yellow). (**g**) Pre-immune staining
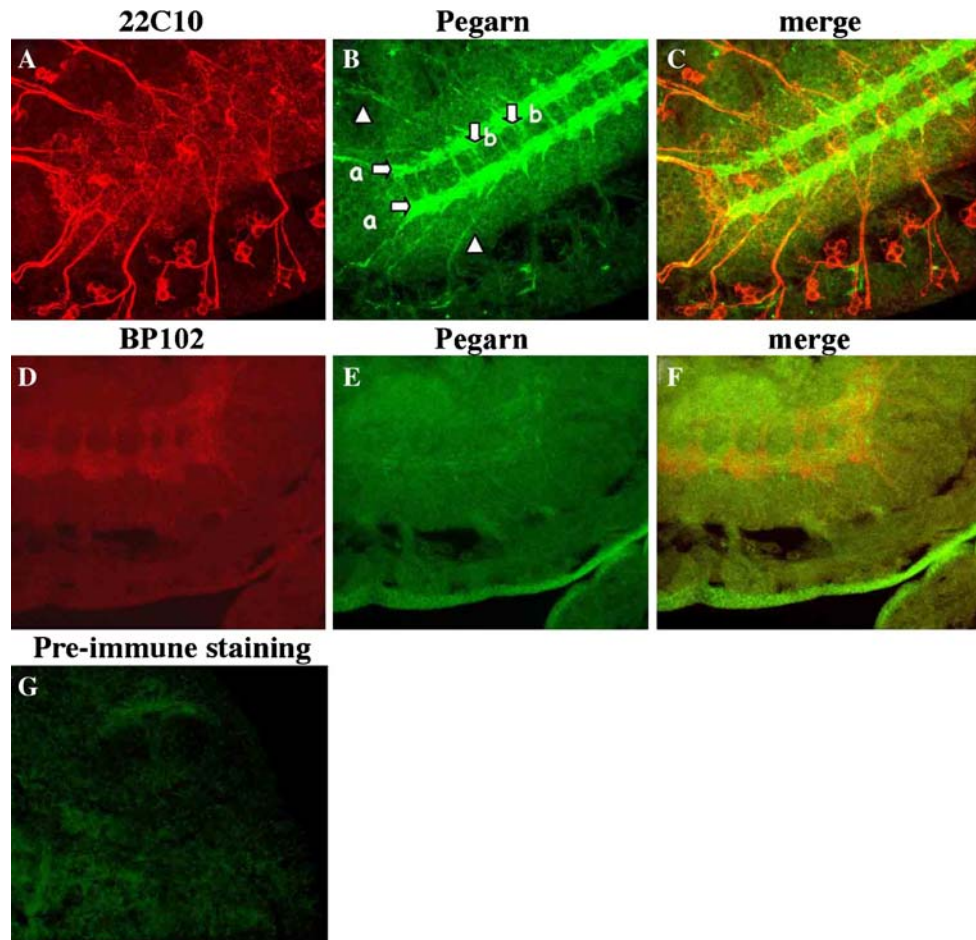
Fig. 7 Immunofluorescence of 12-h mutant embryos (**a–i** and **k–l**) and **j** (wild type embryo). (**a–f**) Heterozygote mutant embryos co-staining of 22C10 (red) with Pegarn (green), and merge (yellow). All EP(3)3348 flies were heterozygote over a balancer (TM6B). (**g–i**) Homozygote mutant embryos co-staining of 22C10 (red) and Pegarn (green) and merge (yellow). The secondary antibody used for Pegarn were Cy5 but further processed by Adobe photoshop and changed the color from blue to green channel. (**j–l**) The images of BP102 staining in wild type, heterozygote and homozygote Pegarn mutant embryos respectively. EP(3)3348 heterozygote flies were over a balancer (TM6B)
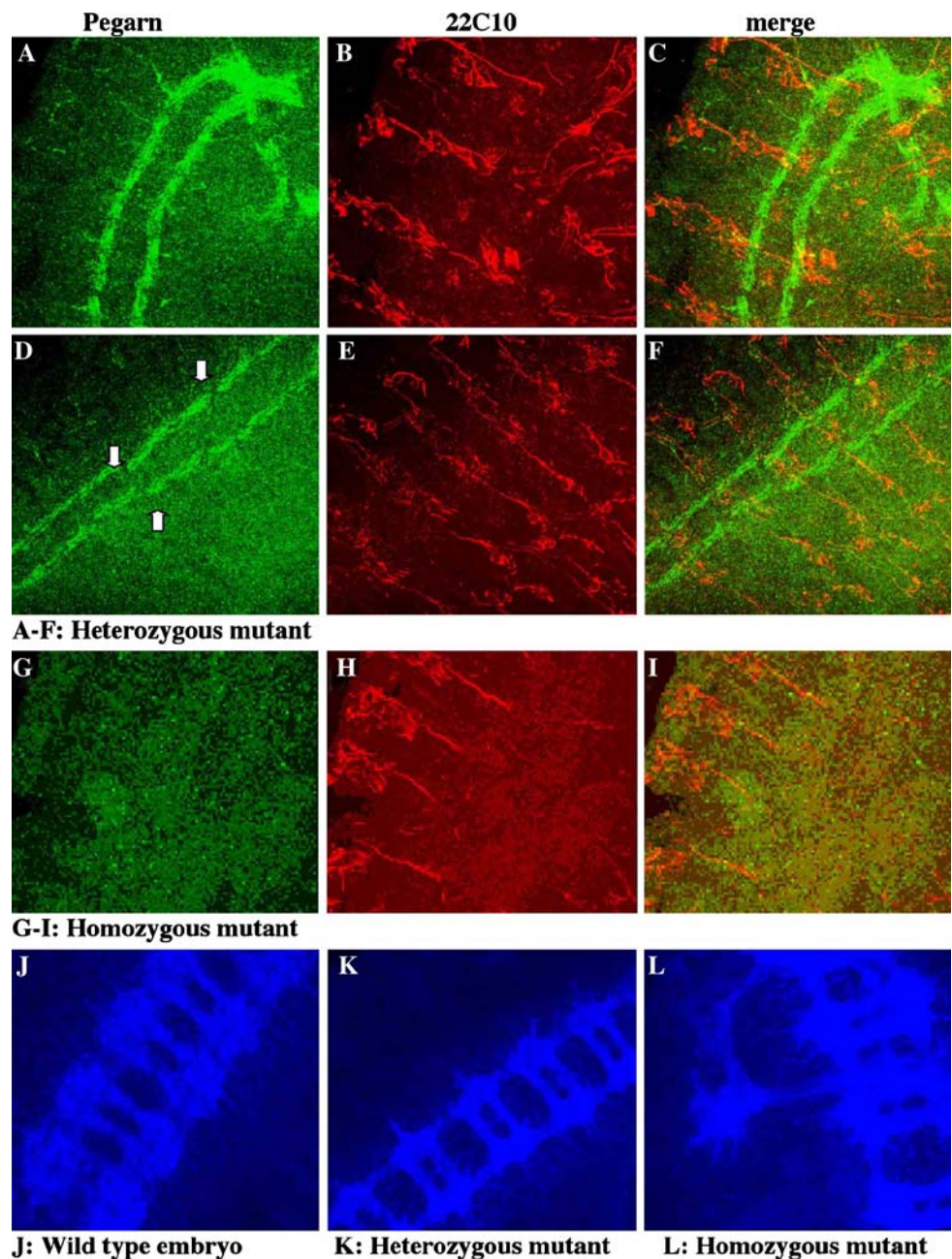
neuronal development. Immunofluorescence staining with BP102 shows increased distortion of the commissures in the homozygote mutant (Fig. 7l) but not in heterozygote mutant (Fig. 7k). Staining with only BP102 does not show a reduction in the staining strength, indicating that there is not a dramatic loss of the CNS axons, just reduced connectivity in the mutants.

Figure 7g–i shows co-staining with antibody against Pegarn protein and 22C10 in a homozygote mutant embryo. Pegarn protein is not detected in the homozygote mutant embryos (Fig. 7g) indicating that this insertion may cause a null phenotype. Neurons in the mutant embryos

show reduced projection (Fig. 7h) indicating that Pegarn is important in neuronal pathfinding. In addition, the neuronal patterns are also deformed. When staining with BP102 alone, homozygote Pegarn mutant embryos show a more disorganized pattern of CNS axons (commissure and connective structures) (Fig. 7l) when compared to wild type (Fig. 7j). There were additional neurons between the two connectives (Fig. 7l). CNS axons also extended in abnormal directions (Fig. 7l). These results unveil a significant function of the Pegarn protein in motor axon outgrowth and in the correct formation of commissures and connectives.

Discussion

In *Drosophila*, one of the main tasks of the midline cells is to provide cues for incoming axons from neural cells of the neuroectoderm. These cues help guide axons along and across the midline. Longitudinal axon fibers form the inter-segmental connectives. Transverse fibers form two commissures in each segment, a posterior and an anterior commissure [24]. Our results suggested that Pegarn might be involved in the formation of longitudinal axon fibers and transverse fibers. There are several genes in *Drosophila* that are involved in the formation of commissures, such as commissureless (comm), roundabout (robo), and netrins (Net). Comm and robo encode components of attractive and repulsive signaling system at the midline [25]. Netrin functions as an instructive guidance cues. Deletion of Netrin genes gives rise to thinner and sometimes absent commissures and occasional breaks in the longitudinal tracts [26, 27]. In addition, there are phosphorylation events of protein kinase A and protein kinase G involved in the function of Netrin. Ogura and Goshima [28] suggested that UNC-51 and UNC-14 in *C. elegans* regulate the subcellular localization of the Netrin receptor UNC-5. Pegarn might be one of candidate protein kinases that might work together with the many other genes involved in the complex functions needed to form complete commissures and connectives.

The function of UNC-51 appears to be highly conserved among species which are evolutionary distant. We now add our *Drosophila* homolog of UNC-51 which might be involved in CNS axon formation and in neuronal development. Organisms as diverse from one another as *Drosophila* and human share a nervous system structure based on the midline [29–31]. Studies by Okazaki et al. [11] also suggested the role of ULK-1 human homolog of UNC-51 kinase in axonal elongation in mammalian neurons. Furthermore, in 2007, the study of Zhou et al. found that RNAi knockdown of mouse ULK1/2 resulted in impaired endocytosis of nerve growth factor (NGF), excessive axon arborization, and severely stunted axon elongation. The data from our research suggested a conserved function for UNC-51 kinases among the multicellular organisms. Whether the mechanisms of axonal developmental regulation by Pegarn will be conserved with other species need more biochemical studies in the future which might reveal important processes of neuronal development in human or mammalian nervous systems and related neuronal diseases.

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