A Ubiquitin E2 Variant Protein Acts in Axon Termination and Synaptogenesis in *C. elegans*

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ABSTRACT

In the developing nervous system, cohorts of events regulate the precise patterning of axons and formation of synapses between presynaptic neurons and their targets. The conserved PHR proteins play important roles in many aspects of axon and synapse development from *C. elegans* to mammals. The PHR proteins act as E3 ubiquitin ligases for the dual-leucine-zipper-bearing MAP kinase kinase kinase (DLK MAPKKK) to regulate the signal transduction cascade. In *C. elegans*, loss of function of the PHR protein RPM-1 (Regulator of Presynaptic Morphology-1) results in fewer synapses, disorganized presynaptic architecture, and axon overextension. Inactivation of the DLK-1 pathway suppresses these defects. By characterizing additional genetic suppressors of *rpm-1*, we present here a new member of the DLK-1 pathway, UEV-3, an E2 ubiquitin conjugating enzyme variant. We show that *uev-3* acts cell autonomously in neurons, despite its ubiquitous expression. Our genetic epistasis analysis supports a conclusion that *uev-3* acts downstream of the MAPKK *mkk-4*, and upstream of the MAPKAPK *mak-2*. UEV-3 can interact with the p38 MAPK PMK-3. We postulate that UEV-3 may provide additional specificity in the DLK-1 pathway by contributing to activation of PMK-3 or limiting the substrates accessible to PMK-3.
INTRODUCTION

Chemical synapses are specialized cellular junctions that enable neurons to communicate with their targets. An electrical impulse causes calcium channel opening and consequently stimulates synaptic vesicles in the presynaptic terminals to fuse at the plasma membrane. Neurotransmitter activates receptors on the postsynaptic membrane and triggers signal transduction in the target cell. In order for this communication to occur efficiently, the organization of the proteins within these juxtaposed pre- and post- synaptic terminals must be tightly regulated (JIN and GARNER 2008). Previous studies in C. elegans have identified RPM-1, a member of the conserved PHR (for Pam/Highwire/RPM-1) family of proteins, as an important regulator for the synapse (SCHAEFER et al. 2000; ZHEN et al. 2000). Recent functional studies of other PHR proteins have shown that they are also required for a number of steps during nervous system development including axon guidance, growth, and termination (BLOOM et al. 2007; D'SOUZA et al. 2005; GRILL et al. 2007; LEWCOCK et al. 2007; LI et al. 2008; WAN et al. 2000).

The signaling cascades regulated by the PHR proteins have been identified using genetic modifier screens (COLLINS et al. 2006; DIANTONIO et al. 2001; LIAO et al. 2004; NAKATA et al. 2005) and biochemical approaches (GRILL et al. 2007; WU et al. 2007). These studies reveal that a major function of PHR proteins is to act as ubiquitin E3 ligases (JIN and GARNER 2008). In C. elegans, RPM-1 regulates the abundance of its substrate, the MAPKKK DLK-1, and controls the activity of the MAP kinase cascade composed of three additional kinases, MAPKK MKK-4, p38 MAPK PMK-3, and MAPKAPK MAK-2 (NAKATA et al. 2005; YAN et al. 2009). This signaling cascade further
regulates the activity of the CCAAT/enhancer binding protein (C/EBP), CEBP-1, via a mechanism involving 3'UTR mediated mRNA decay.

Signal transduction involving MAP kinases can be fine-tuned using multiple mechanisms to ensure optimal signaling outputs (RAMAN et al. 2007). For example, scaffold proteins for MAP kinases can provide spatial regulation of kinase activation in response to different stimuli (REMY and MICHNICK 2004; WHITMARSH 2006). Small protein tags such as ubiquitin have also been shown to control the activation of kinases. Specifically, in the IKK pathway ubiquitination via Lys63 chain formation catalyzed by the Ubc13/Uev1a E2 complex and TRAF6 E3 ligase is required for TAK1 kinase activation (SKAUG et al. 2009).

To further the understanding of the DLK-1 pathway in the development of the nervous system, we characterized a new complementation group of rpm-1(lf) suppressors. These mutations affect the gene uev-3, a ubiquitin E2 conjugating (UBC) enzyme variant (UEV). UEV proteins belong to the UBC family, but lack the catalytic active cysteine necessary for conjugating ubiquitin (SANCHO et al. 1998). The best characterized UEV proteins are yeast Mms2 and mammalian Uev1A, both of which act as the obligatory partner for the active E2 Ubc13 and function in DNA repair and IKB pathways, respectively (DENG et al. 2000; HURLEY et al. 2006). In addition, UEV proteins, such as Tsg101 can also regulate endosomal trafficking (BABST et al. 2000). We find that similar to other members of the DLK-1 pathway, uev-3 functions cell autonomously in neurons. uev-3 genetically acts downstream of mkk-4 and upstream of mak-2. UEV-3 can bind PMK-3 in heterologous protein interaction assays. We
hypothesize that UEV-3 may be acting to add specificity to the DLK-1 pathway by binding to PMK-3 for its activation or for selecting specific downstream targets.

**MATERIALS AND METHODS**

*C. elegans* genetics: *C. elegans* strains were maintained as described (BRENNER 1974). The suppressors were isolated from rpm-1(ju44); syd-2(ju37); juls1 or rpm-1(ju44); syd-1(ju82); juls1 animals mutagenized with 50 mM EMS (NAKATA et al. 2005). Suppressor mutations were outcrossed multiple times against wild type (N2) or juls1[Punc-25 SNB-1::GFP] strains. Specificity of suppression of rpm-1(lf) was tested by crossing sup; rpm-1; syd-2; juls1 to rpm-1; juls1 males. Double mutants were constructed following standard procedures, and the genotypes were confirmed by allele-specific nucleotide alterations determined by DNA sequencing or restriction enzyme digest.

Cloning of *uev-3*: We mapped the suppression activity of *uev-3(ju587)* in the *rpm-1; syd-2* double mutant strain to Chromosome I near +4 using the single-nucleotide polymorphism mapping strategy (DAVIS et al. 2005; NAKATA et al. 2005). For fine mapping of *uev-3 (ju587)*, we constructed dpy-5 *uev-3 (ju587)* unc-75; *rpm-1; juls1* strain. Following crossing to the Hawaiian strain CB4856, recombinant animals of phenotypic Dpy non-Unc or Unc non-Dpy that were mutant for *rpm-1* were selected, and the presence of *uev-3 (ju587)* in each recombinant was determined by observing *juls1* marker expression. *uev-3 (ju587)* was mapped between *snp_F14B4[1]* and *snp_M04C9[1]*, within a 90 kb interval including about 19 predicted genes. We performed RNAi against the predicted genes using a sensitized strain *eri-1(mg366)*;
rpm-1(ju23); syd-2(ju37), and found that uev-3 RNAi caused suppression of rpm-1; syd-2 phenotypes. ju593, ju638, and ju639 were determined to be alleles of uev-3 based on linkage to chromosome I and non-complementation test. DNA sequence analyses of these suppressor mutations were carried out following standard procedures, and the nucleotide alterations were confirmed in independent PCR reactions.

Molecular biology and expression constructs: We determined the gene structure and the full-length transcripts of uev-3 by RT-PCR and 5′ RACE analyses. Total RNAs were prepared using TRIzol reagents (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions, and cDNAs were generated using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). 5′ RACE kit (Roche Applied Science, Indianapolis, IN) was used with the following pair of primers to amplify the 5′ region of uev-3: SP1 and YJ3861 ccattgacacgttgagattc; and SP2 and YJ3846 acgtttagacactcctccc. DNA sequence analysis of eight cloned 5′ RACE products revealed a SL2 splice leader in all, indicating that uev-3 is transcribed as the downstream gene in the operon with rab-5. We obtained full-length uev-3 cDNA by RT-PCR using YJ3852 ggggacaagtttgtacaaaaaagcaggctccaaaatgtccgatcaacctgg and YJ3853 ggggaccactttgtacaaaaagctgggttatgaaattccaatgacatc. The DNA sequences of the cDNA clone (pCZ729) verified and corrected the predicted uev-3 exon and intron boundaries. The uev-3 expression constructs in C. elegans were generated following standard procedures or using Gateway Cloning Technology (Invitrogen, Carlsbad, CA), and the details of the clones are in Table S2. For yeast two-hybrid studies, full length cDNA or fragments of cDNA for dlk-1, mkk-4, pmk-3, mak-2, or uev-3 were cloned to either pBTM116 vector to be expressed as GAL4 activation domain fusion protein, or
into pACT2 vector to be expressed as LexA DNA binding domain fusion protein, as described in Table S2.

**Neuronal morphology and synapse analyses:** We observed GFP or SNB-1::GFP using *mils32[Pmec-7-GFP]* or *juls1[Punc-25-SNB-1::GFP]* in one-day old adult animals either live or anesthetized in 1% 1-phenoxy-2-propanol (TCI America, Portland, OR) in M9 buffer. Images were captured either on a Zeiss Axioplan 2 microscope with Chroma HQ filters or a Zeiss LSM510 confocal microscope.

**Germline transformation and transgenic analyses:** Transgenic animals were usually generated by injecting DNA at a dilution series (1 ng–50 ng/µl) following standard procedures (Mello *et al.* 1991), using either pRF4 *rol-6(dm)* or *Pttx-3-RFP* as coinjection markers. For each construct, two to thirteen independent transgenic lines were analyzed.

**Yeast two hybrid:** Yeast two-hybrid assays were performed using pACT2 and pBTM116 vector backbones (Clontech, Mountain View, CA). The yeast strain L40 [MATa his3D200 trp1-901 leu2-3, 112 ade2 LYS2::(lex-Aop)4-HIS3 URA3::(lexAop)8-lacZ GAL4 gal80] was used. The yeast transformation was performed by the lithium acetate method and selected on Trp-, Leu-, and His-selection plates. Pairs of plasmids were co-transformed into yeast strain L40, and selected on –Leu –Trp plates. For interaction assay, single clones were picked from each transformation and cultured to OD₆₀₀ = 1. Yeast cells were pelleted by centrifugation, washed 3 times and resuspended, and plated in a dilution series of 10 to 10000 times by pipeting 5 µl per spot onto Histidine selection plates containing 3mM 3-AT. β-galactosidase assays were
performed following the Clontech Yeast Protocols Handbook (FIELDS and STERNGLANZ 1994).

**cebp-1 mRNA analysis by qRT-PCR:** RNA isolation and cDNA preparation were performed as above. Power SYBR® Green PCR Master Mix kits (Applied Biosystems, Foster City, CA) were used for the PCR reactions and the ABI Prism® 7000 Sequence Detection System was used for real time PCR. cDNAs were amplified using following primers: *cebp-1* pair (gcacgacaagatgaagagg and gcatgcgttgctctttca) amplifies 183 bp; *ama-1* pair (actcagatgacactcaacac and gaatacagtcaacgacggag) amplifies 128 bp.

**Protein interaction studies in 293T mammalian cells:** Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cells were transfected with a total 5 ug of DNA containing various expression vectors. After 24 h, cells were collected and washed once with phosphate-buffered saline (PBS), and lysed in 0.4 ml of 0.1% NP-40 lysis buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 2 mM dithiothreitol (DTT), Protease inhibitor (Roche Applied Science, Indianapolis, IN) and Phosphatase inhibitor (Nacalai, San Diego, CA). Cellular debris was removed by centrifugation at 10,000 g for 5 min. FLAG epitope-tagged proteins were immunoprecipitated with anti-FLAG monoclonal antibody M2 (Sigma, St. Louis, MO). For immunoblotting, aliquots of immunoprecipitates and whole cell lysates were resolved on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to Amersham Hybond-P membranes (GE Healthcare, Piscataway, NJ). The membranes were immunoblotted with anti-HA rabbit polyclonal antibody Y-11 (Santa Cruz Biotechnology Inc, Santa Cruz, CA). The bound antibody was visualized with
horseradish peroxidase-conjugated antibody to rabbit IgG using the Amersham ECL Advance Western blotting detection kit (GE Healthcare, Piscataway, NJ).
RESULTS

**UEV-3 is a Ubc/E2 variant protein:** Previous analyses of the suppressors of rpm-1 loss of function (lf) mutants revealed five loci, defining *dlk-1, mkk-4, pmk-3, mak-2* and *cebp-1* (NAKATA et al. 2005; YAN et al. 2009). We devised a non-complementation test scheme and identified four alleles belonging to a new complementation group: *ju593, ju587, ju638*, and *ju639*. We mapped this suppressor locus to an interval of ~90kb on the right arm of Chromosome I (Figure 1A). We used a combination of RNAi and transgenic expression of cosmid DNAs from the region to locate the gene affected. We found that the 6kb Eco105I - SpeI fragment of the cosmid F26H9 contained the rescuing activity for the suppression of *rpm-1(ju44)* by *ju587*. The genomic DNA fragment contains two predicted genes: *rab-5* and *uev-3*. By RT-PCR and 5’ RACE analyses, we determined that *uev-3* transcripts contained an SL2 splice leader, confirming that *rab-5* and *uev-3* form an operon, with *uev-3* as the downstream gene (Materials & Methods). DNA sequence analysis from *ju587, ju593*, and *ju638* identified single nucleotide alteration at various splice acceptor sites, while *ju639* is a 26 base pair deletion from amino acid 277 in the sixth exon (Figure 1B, Table S1). Moreover, we performed RNAi of *uev-3* in a sensitized genetic background and observed partial suppression of *rpm-1(lf)* (Table S1). These analyses are consistent with the suppressor mutations causing loss of function in *uev-3*.

*uev-3* is one of the three ubiquitin-conjugating (UBC) E2 enzyme variant (UEV) proteins in *C. elegans* (JONES et al. 2002; KIPREOS 2005). It is composed of 356 amino acids, with the UEV domain from residues 168 to 324 (Figure 1B). UEV proteins are similar to UBC E2 enzymes but lack the critical cysteine residue that is required for a
transient interaction between E2 and ubiquitin (Pickart and Eddins 2004; Sancho et al. 1998) (Figure S1). Alignment of the UEV domain of UEV-3 with other UBC and UEV proteins reveals motifs with high similarity; for example, the HxN tripeptide motif, which is important for proper folding of the active site region in UBC proteins, is conserved in UEV-3 (Figure S1) (Gudgen et al. 2004). The overall sequence of UEV-3 is most similar to C. elegans UEV-2, followed by UBC-3 and UBC-7 in C. elegans (Figure 1C), and divergent from canonical UEVs (see later).

**uev-3(lf) suppresses the defects in motor and mechanosensory neurons of rpm-1(lf):** rpm-1(lf) mutants display irregularly shaped and sized presynaptic terminals in the motor neurons (Zhen et al. 2000). The mutants also show axon termination defects in the mechanosensory neurons (Schaefer et al. 2000). Despite these defects, rpm-1(lf) animals appear superficially wild type in the overall nervous system architecture and locomotory behavior. The uev-3 mutants alone also develop normally, and exhibit no discernable abnormalities in the motor and mechanosensory neurons (Figure 2). However, in an rpm-1(lf) background, mutations in uev-3 can ameliorate the defects in motor neuron synapses and touch axon patterning.

We determined the extent of rpm-1(lf) suppression by uev-3(lf). We first analyzed the presynaptic puncta patterns and numbers using juls1 [Punc25-SNB-1::GFP], a marker that visualizes presynaptic terminals in GABA motor neurons (Hallam and Jin 1998). In wild-type animals, this marker shows a pattern of uniformly sized and spaced fluorescent puncta along the dorsal and ventral cords, and on average, 158.9 puncta are visible in the dorsal cord (Figure 2A). rpm-1(lf) mutants have fewer puncta, averaging 87.1 puncta in the dorsal cord. The remaining GFP puncta in rpm-1 mutants
are often enlarged and disorganized in distribution. Single mutants of \textit{uev-3(ju587)} or \textit{uev-3(ju639)} have an average 165.9 or 160.4 puncta per dorsal cord, respectively, and SNB-1::GFP puncta patterns are similar to wild-type. Both \textit{uev-3(ju587); rpm-1(If)} and \textit{uev-3(ju639); rpm-1(If)} double mutants show significant suppression of the \textit{rpm-1} phenotype, increasing SNB-1::GFP puncta number to an average of 116.9 and 111.6 in the dorsal cord, respectively.

We also examined the touch neuron morphology using the \textsl{mul532 [Pmec-7-GFP]} marker (CH'NG \textit{et al.} 2003). In wild-type animals, the ALM cell body lies laterally in the mid-body region and sends a longitudinal axonal projection anterior into the pharyngeal region of the animal where a process branches into the nerve ring and forms synapses (Figure 2B). The PLM cell body resides in the tail and sends a projection anterior into the mid-body of the animal, terminating posterior to the ALM cell body. PLM cells also extend a synaptic branch to the ventral cord to form synapses onto its partners. In \textit{rpm-1(If)} mutants, both ALM and PLM axons frequently overextend beyond their normal termination sites and loop posteriorly or into the ventral cord, described as “ALM hook” or “PLM hook” defects, respectively (Figure 2B). Additionally, the PLM synaptic branch is frequently missing. Although low levels of ALM and PLM defects are detected in \textit{uev-3(ju587)} and \textit{uev-3(ju639)} mutants, both mutations significantly suppressed \textit{rpm-1(If)} (Figure 2B). The degree of suppression of \textit{rpm-1(If)} by both alleles of \textit{uev-3} is comparable to those observed for the mutants in the DLK-1 MAPK cascade (NAKATA \textit{et al.} 2005). \textit{uev-3(ju639)} has a slightly stronger suppression effect on the mechanosensory neuron phenotypes, so we have designated \textit{ju639} as the cannonical mutation of the gene.
**uev-3 acts cell autonomously in presynaptic neurons:** We determined the transcriptional expression pattern of the *rab-5* and *uev-3* operon using 1.8 kb of the 5’ upstream sequences of the operon to drive GFP expression. GFP is observed in all tissues, and noticeably present in ventral cord neurons (Figure 3A). We next addressed whether UEV-3 acts cell autonomously in neurons by expressing *uev-3* cDNA driven by tissue-specific promoters in *uev-3; rpm-1* mutants (Tables S2, 3). We examined functional rescue of the motor neuron synaptic phenotypes by quantitating SNB-1::GFP puncta numbers (Figure 3B). Expression of *uev-3* driven by a pan-neuronal promoter, or a motor neuron specific promoter rescues the suppression of *rpm-1(lf)* to similar degree comparable to that of transgenic expressing *uev-3* genomic DNA. The muscle promoter driven transgene did not show any effects on the suppression in *uev-3; rpm-1* mutants. Similarly, we expressed *uev-3* cDNA in touch neurons, and observed significant rescue of the suppression on the mechanosensory neuron phenotypes (Figure 3C). As in motor neurons, the muscle-driven promoter did not show any rescue activity in *uev-3; rpm-1* mutants. The transgenes alone or in a *uev-3* background did not cause any significant defects (Figure 3C, data not shown for *[Pmyo-3-uev-3]* and *[Prgef-1-uev-3] transgenes alone). These results thus demonstrate that *uev-3* is required cell autonomously in presynaptic neurons.

**uev-3 acts downstream of mkk-4, upstream of mak-2 in the DLK-1 MAPK cascade:** To learn how *uev-3* functions in the DLK-1 MAPK cascade, we performed four lines of experiments. First, we made pair-wise loss of function mutant combinations between *uev-3* and the MAPK genes, and measured the suppression of motor neuron puncta numbers in *rpm-1(lf)* (Figure 4A). For example, *uev-3; rpm-1* have
116.9 SNB-1::GFP puncta per dorsal cord, and *pmk-3; rpm-1* have 130.7 puncta per dorsal cord. The triple mutants *uev-3; pmk-3; rpm-1* show a mean 129.9 puncta number in the dorsal cord, and do not suppress *rpm-1* any stronger than either double mutants. This analysis is consistent with the interpretation that *uev-3* acts in the same pathway with MAPK genes *dlk-1, mkk-4*, and *pmk-3*.

Second, to place *uev-3* within the DLK-1 MAPK pathway, we took advantage of the observations that transgenic expression of either wild type *mkk-4(++)* or a phospho-mimetic *mkk-4(DD)* in a wild type background causes gain of function phenotypes (NAKATA et al. 2005). The animals carrying these extra-chromosomal arrays display an uncoordinated movement, with disorganized synaptic puncta resembling those of *rpm-1(lf)* (Figure 4B). Loss of function in *pmk-3*, a gene downstream of *mkk-4*, can suppress both the synaptic and behavior defects associated with either *mkk-4(++)* or *mkk-4(DD)* transgene, whereas loss of function in *dlk-1*, which acts upstream of *mkk-4*, only suppresses the effects of *mkk-4(++)* (NAKATA et al. 2005). We found that when either the *mkk-4(++)* or *mkk-4(DD)* transgene is in the *uev-3(lf)* background, the body size and movement phenotypes of the transgenic animals are abolished, and the average total synaptic GFP puncta are increased significantly (Figure 4B). Thus, *uev-3* behaves genetically similar to *pmk-3* and likely acts downstream of *mkk-4*.

Third, we have recently identified that the MAP kinase activated protein kinase MAK-2 and the transcription factor CEBP-1 function downstream of PMK-3 (YAN et al. 2009). Transgenic overexpression of a phospho-mimetic MAK-2, *mak-2(EE)* causes a gain-of-function defect resembling *mkk-4(gf)* transgenes, which are suppressed by loss of function in *cebp-1* but not *pmk-3* (YAN et al. 2009)(Figure 4C). We found that *uev-3(lf)*
does not suppress the *mak-2(EE)* gain of function defects (Figure 4C), consistent with a conclusion that *uev-3* likely acts upstream of *mak-2*.

Lastly, we examined the levels of *cebp-1* mRNA transcripts in *uev-3* mutants. The DLK-1 MAP kinase cascade regulates *cebp-1* by controlling the levels of *cebp-1* mRNA (Yan *et al.* 2009). We performed quantitative RT-PCR on RNAs isolated from mixed-stage animals, and compared the levels of *cebp-1* transcripts to those of *ama-1*, the large subunit of RNA polymerase II (Sanford *et al.* 1983). In *rpm-1* mutants, *cebp-1* transcript levels are elevated compared to wild type because DLK-1 is not degraded, allowing high-level of MAP kinase signaling to promote the stability of *cebp-1* mRNA (Figure 4D). In both *rpm-1; dlk-1* and *rpm-1; uev-3* mutant strains, the transcript levels of *cebp-1* are comparable to wild type levels. All together, these four lines of evidence show that *uev-3* functions in the DLK-1 MAPK pathway, at the step between *mkk-4* and *mak-2*.

**The canonical UEV-1 and Ubc-13 do not suppress *rpm-1*:** Biochemical studies of canonical UEV proteins in yeast and mammalian cells, such as Mms2 and Uev1A, respectively, have established that the UEV domain functions as an obligatory subunit for an active Ubc, Ubc13 (Broomfield *et al.* 1998; Deng *et al.* 2000; Xiao *et al.* 1998). The Uev1A/Ubc13 E2 complex catalyzes Lys63 poly-ubiquitin chain formation (Hofmann and Pickart 1999; Vandemark *et al.* 2001). The ortholog of Mms2 or Uev1A in *C. elegans* is UEV-1, which can interact with UBC-13 (Gudgen *et al.* 2004). The UEV domain of the UEV-3 is very divergent from the canonical UEV (Figure 1C, Figure S1), and also shows limited degree of similarities to that of UEV-1 (14.7% identity and 30.6% similarity) (Figure 5A). Residues known to be important for Uev1 binding to either its
cognate Ubc13 or ubiquitin do not seem to be conserved in UEV-3 (MORAES et al. 2001; VANDEMARK et al. 2001) (Figure 5A). In addition, UEV-3 has extended N-terminal sequences and short C-terminal tail (Figure 5B). The sequence comparisons raise the question whether UEV-3 may retain functions similar to those of UEV proteins in other organisms.

We tested whether uev-1 and ubc-13 interacted with rpm-1. The uev-1 gene is a small gene, with its coding sequences less than 1kb, and resides in an operon as the upstream gene (Figure 5B). A deletion allele, ok2610, removes 496bp starting 142 bp in the promoter of the operon and ending 69bp in the third exon of uev-1, and is likely a null mutation. The homozygous ok2610 animals are viable, develop normal touch neurons, and do not suppress rpm-1(lf) (Figure 5D). We also examined a deletion allele of ubc-13, tm3546, which breaks in the first exon and would lead to a premature stop at amino acid 88 (Figure 5B). We observed no genetic suppression of rpm-1(lf) by ubc-13(tm3546) (Figure 5D). Moreover, overexpression of uev-1 did not bypass the requirement of uev-3 (Figure 5D). We also made a chimeric gene in which we replaced the UEV domain and C-terminus of UEV-3 with the UEV domain from UEV-1 (Figure 5C). Intriguingly, we found that transgenic expression of this UEV chimeric protein in neurons rescued the suppression of rpm-1(lf) in the uev-3; rpm-1 background to similar levels as does the expression of the full-length uev-3 (Figure 5D). With the caveat of overexpression, this result suggests that despite its divergency, the UEV domain of UEV-3 could have a function similar to that of the canonical UEV domain of UEV-1. We therefore wanted to test whether UEV-3 might require any other UBC. C. elegans has 22 annotated UBC genes. We analyzed available deletion or mutant alleles for several
UBC genes, but observed no genetic interactions with *rpm-1(lf)* (Table S1). We also performed dsRNAi for 19 *ubc* genes in *eri-1; rpm-1; muls32* strain and did not observe detectable suppression of *rpm-1(lf)* (data not shown). We also tested protein interactions between UEV-3 and UBC genes using yeast two hybrid assays, and were not able to detect a positive interaction with UEV-3, out of eleven UBC genes and two UEV genes tested (data not shown). In summary, these studies suggest a scenario in which the UEV domain of UEV-3 might have a canonical function like UEV-1, but UEV-3 might likely have distinct partners for its function.

**UEV-3 can bind PMK-3:** To better characterize the relationship between UEV-3 and the DLK-1 MAPK cascade, we asked whether UEV-3 could interact with the kinases by performing yeast two-hybrid assays between UEV-3 and all four kinases and CEBP-1. We detected interactions between UEV-3 and PMK-3, but not between UEV-3 and the other three kinases or CEBP-1 (Figure 6A, data not shown for *dlk-1, mkk-4, cebp-1*). We attempted to narrow down the interacting domains using bait expressing only the UEV domain or N-terminal domain of UEV-3, but were hindered by self-activation of the N-terminal expression construct, and were not able to observe strong interactions between either domain alone and PMK-3 (Figure 6A). We further tested the binding interactions between UEV-3 and PMK-3 by co-immunoprecipitation studies in heterologous 293T cells. Although wild type PMK-3 was not detectable in the immunocomplex with UEV-3, we observed co-immunoprecipitation between UEV-3 and a mutant PMK-3 in which the catalytic active site was mutated (Figure 6B). Such catalytic active site mutants of MAP kinases are often used to detect transient interactions between MAP kinases and their substrates or interacting partners (Han et
al. 1997). Lastly, we asked if this protein interaction might play roles in the localization or abundance of each protein. We generated transgenic animals expressing functional PMK-3::GFP or UEV-3::GFP in neurons. Both tagged proteins show ubiquitous expression in cytosol and nucleus (Figure 6C,D). When each transgene was crossed into pmk-3 or uev-3 mutants, we did not observe major differences in the localization pattern or expression level of either transgene. Moreover, overexpression of pmk-3(+) in uev-3; rpm-1 mutants does not cause any detectable effects, nor does the overexpression of uev-3(+) in pmk-3; rpm-1 mutants (data not shown). Together with the genetic epistasis analyses, these data suggest that UEV-3 and PMK-3 are likely functional partners.

**DISCUSSION**

The conserved DLK kinases have recently emerged as key regulators of axon and synapse development in the nervous systems of both vertebrates and invertebrates (Po et al. 2010). The mechanistic dissection of the DLK signal transduction cascade has only just begun. In this study, we identified and characterized a new member of the DLK-1 kinase pathway, UEV-3, a previously uncharacterized ubiquitin conjugating enzyme/E2 variant. Like other MAPKs known to function in the DLK-1 pathway, loss of function of uev-3 on its own is grossly wild type, but shows specific suppression of rpm-1 in axon termination and synapse formation phenotypes. Our genetic epistasis studies reveal that uev-3 acts in the DLK-1 MAPK pathway, downstream of mkk-4 and upstream of mak-2. Based on our studies of UEV-3 protein and its tentative binding interactions with PMK-3, we propose that UEV-3 may act as a co-factor for PMK-3, for
example, to modulate PMK-3 activation or to recognize substrates, resulting in fine-
tuning the DLK-1 signal transduction cascade.

A ubiquitin conjugating enzyme variant could provide an additional means for pathway regulation and specificity by delineating the targets of the pathway. The UEV protein, Fts1, has been shown to act as a scaffold between protein kinase B (PKB/Akt) and 3-phosphoinositide-dependent kinase 1 (PDK1) (REMY and MICHNICK 2004). *C. elegans* has three closely-related p38 MAP kinases that appear to be ubiquitously expressed (BERMAN *et al.* 2001). The function of UEV-3 may be to provide specificity for PMK-3 in the DLK-1 MAPK pathway during synaptogenesis and axon termination in neurons. Through the UEV-3 and PMK-3 interaction, substrates important for these processes may be selectively activated. Despite substantial efforts, we have not yet been able to directly test the contribution of UEV-3 in the activation of MAK-2, because of the lack of reagents to detect phosphorylated MAK-2 *in vivo*. However, the idea that UEV-3 could help PMK-3 to affect kinase activation, such as that of MAK-2, would be similar to those revealed by the role of Uev1/Ubc13 in TAK1 kinase activation in the IkK pathway (DENG *et al.* 2000; WANG *et al.* 2001).

Defining the roles of proteins linked to ubiquitination in the nervous system has been a major advance in the past decade (TAI and SCHUMAN 2008). Comparing to what we have learned about the E3 ubiquitin ligases, relatively little is understood about the functional specificity and regulation of E2 enzymes. A classic example is the *Drosophila* Bendless (MURALIDHAR and THOMAS 1993; THOMAS and WYMAN 1984), which is implicated in synapse function but acts distinctly different from the E3 ligase Highwire (UTHAMAN *et al.* 2008). Emerging expression studies suggest that UEV proteins are
widely expressed in the developing nervous system (Watanabe et al. 2007). However, their functions are largely unknown. Our transgenic studies suggest that despite the sequence divergency of the UEV domain, UEV-3 might act in a manner similar to canonical UEV proteins. Nonetheless, with the limitations of available reagents, we were not able to identify a cognate E2 ubiquitin conjugating enzyme for UEV-3.

An intriguing possibility remains that UEV-3 may be functioning on its own to help add additional regulation in the RPM-1 pathway. It is well established that the UEV protein Tsg101/Vps23 acts in the endocytic pathway to bind ubiquitinated proteins and remove them from the membrane (Katzmann et al. 2002). The endosomal sorting complex required for transport (ESCRT-1) contains Vps23 and two other Vps proteins necessary for recognizing and sorting cargo endocytosed at the plasma membrane. Vps23 binds ubiquitin conjugated to proteins at the plasma membrane and together with the ESCRT-II and -III complexes, target proteins are sorted through multivesicular bodies in the endosomal pathway. A recent study has shown that a splice variant of human Uev1 has an extended N-terminal domain, which can target the protein to endosomal-like organelles and confer regulation to EGF receptor signaling, possibly through protein degradation (Duez et al. 2010). UEV-3 is unusual in that it has a long N-terminal extension, which could have a regulatory role in UEV-3 function. This idea would be consistent with the observation that overexpression of the chimeric UEV-3-UEV-1 mimics the activity of full-length UEV-3, whereas overexpression of UEV-1 does not. The RPM-1 pathway has previously been connected to vesicular regulation through the biochemical interaction between RPM-1 and the RabGEF GLO-4 (Grill et al. 2007). A tempting possibility is that UEV-3 may provide crosstalk between the GLO-4 and
DLK-1 pathways by binding ubiquitinated proteins through its UEV domain and acting in the endosomal pathway. Potential future directions would be to identify UEV-3 interacting proteins to aid in further elucidating UEV-3’s mechanism in axon termination and synapse formation.

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**FIGURE LEGENDS**

**Figure 1.** *uve*-3 is a ubiquitin E2 variant. **A.** *uve*-3 locus on Chromosome I. The Eco1051-SpeI genomic fragment from cosmid F26H9 fully rescues the suppression of *rpm-1*(*lf*) by *uve*-3 mutations. Black box, coding sequences; white box, 3’ UTR; and lines, promoter or intronic sequences. **B.** Illustration of *uve*-3 gene structure and positions of the mutations. Boxes, exons; and black shading, UEV domain. **C.** Dendrogram of UEV-3 with close homologs (ClustalW) *Ce, C. elegans; Dm, D. melanogaster, and Hs, H. sapiens.*

**Figure 2.** *uve*-3 suppresses *rpm-1* defects in motor neuron synapse formation and mechanosensory neuron axon termination. **A.** Top left schematic illustration of an animal expressing *Punc-25-SNB-1::GFP (juls1)*. Cell bodies (large gray dots) reside in the ventral cord, and synaptic SNB-1::GFP puncta (small green dots), along the ventral and dorsal cords. The epi-fluorescent images below show SNB-1::GFP in the dorsal cords in one-day old adult animals with genotypes as indicated. Scale bar, 10µm. The graph on the right shows the quantification of SNB-1::GFP in the dorsal cord (mean ± SEM). n indicates number of animals scored. Statistics, ANOVA with Bonferroni correction: * P<0.05, ** P<0.01, *** P<0.001, ns = not significant. **B.** Schematic of animal expressing *Pmec-7::GFP (muls32)*, marking one each of the bilaterally symmetric ALM and PLM neurons. Images below show portion of the ALM and PLM axons, corresponding to the dash-boxed regions. The tip of the animal’s nose is indicated with aqua arrows, and ALM and PLM axon termination site is indicated with purple arrows. Red asterisks mark ALM cell body. ALM axon termination defects occur
when the axon tip extends into the tip of the nose and loops back (or hook). PLM axon termination defects include absence of synapse branch or “hook” such that the PLM axon overextends beyond normal termination site and turns ventrally or turns ventrally before normal termination site. The graph shows the suppression of rpm-1 by uev-3 in three categories. n indicates animals scored. Statistics, Fischer Exact Test, comparing the PLM “hook” defects: * P<0.05, ** P<0.01, *** P<0.001, ns = not significant.

**Figure 3.** UEV-3 functions cell autonomously in presynaptic neurons. **A.** The 1.8 kb promoter of the operon driven GFP (ixEx2 [Puev-3-GFP]) expression in many tissues (left), including the motor neurons of the ventral cord (white arrows, right). **B.** Presynaptic expression of UEV-3 rescues the suppression of rpm-1 by uev-3 in the GABAergic motor neurons. Prgef-1, 3.5kb pan-neural promoter; Punc-25, 1.2kb GABAergic motor neuron promoter, and Pmyo-3, 1kb body muscle promoter. Quantification of SNB-1::GFP puncta in the dorsal nerve cord of young adults is shown as mean ± SEM; n as indicated. Statistics, ANOVA with Bonferroni correction: * P<0.05, ** P<0.01, *** P<0.001, ns = not significant. **C.** Cell-autonomous rescue of the suppression of rpm-1 by uev-3 in touch neurons driven by the Pmec-4 promoter. n indicates animals scored. Statistics, Fischer Exact Test, comparing the PLM “hook” defects: * P<0.05, ** P<0.01, *** P<0.001, ns = not significant.

**Figure 4.** uev-3 acts in the DLK-1 MAPK cascade, downstream of mkk-4, and upstream of mak-2. **A.** uev-3(lf) does not further enhance the suppression of rpm-1 in the motor neuron synapses by pmk-3 or mkk-4 or dlk-1. Numbers are mean ± SEM, n
as indicated. Statistics, ANOVA with Bonferroni correction compared with rpm-1 single mutant: * P<0.05, ** P<0.01, *** P<0.001, ns = not significant. B. uev-3 functions downstream of mkk-4 MAPKK. Transgenic animals overexpressing wild-type MKK-4 [mkk-4(++)], juEx490 or expressing the constitutively active version of MKK-4 [mkk-4(DD), juEx669] display similarly abnormal synaptic patterns (juls1), uncoordinated locomotion, and small body size (left). The phenotypes of both types of transgenes are suppressed by uev-3(ju587), and quantitation is shown on the right (mean ± SEM); ANOVA with Bonferroni correction: * P<0.05, ** P<0.01, *** P<0.001, ns = not significant, n as indicated. C. uev-3 acts upstream of mak-2. Expression of a phosphomimetic MAK-2(EE) causes gain-of-function effects, which is suppressed by cebp-1, but not by uev-3. n as indicated. Statistics, Fischer Exact Test * P<0.05, ** P<0.01, *** P<0.001, ns = not significant. D. uev-3 acts in the dlk-1 pathway to regulate levels of cebp-1 transcripts, qRT-PCR levels of cebp-1 mRNAs normalized against ama-1. Statistics, Student’s t test: * P<0.05, ** P<0.01, *** P<0.001, ns = not significant, n=3.

**Figure 5.** Functional comparison of UEV-1/UBC-13 and UEV-3. A. Alignment of UEV-1 and UEV-3 UEV domains. Black boxes, identical residues and grey boxes, similar residues. Asterisk, conserved proline and tryptophan residues; caret, aspartic acid residue at the position where both UEV proteins lack the active cysteine. Solid line above residues in UEV-1 31-39 indicates the conserved region that would be important for interacting with Ubc13. Circled Ser, Thr and Ile residues have been shown to be on the interface of S. cerevisiae Mms2 with ubiquitin, which are not conserved in UEV-3.
**B.** Illustration of the *uev-1* and *ubc-13* genes and mutations. **C.** Schematics of DNA constructs used in transgenic lines in panel D. **D.** Quantification of genetic interactions between *uev-1*, *ubc-13* and *rpm-1* and transgenic UEV expression. Statistics, Fischer Exact Test * P<0.05, ** P<0.01, *** P<0.001, ns = not significant.

**Figure 6.** UEV-3 likely binds PMK-3. **A.** UEV-3 interacts with PMK-3 but not with MAK-2 in yeast two-hybrid interaction assay, Trp-Leu- plates (left panel), lacZ assay (middle panel), and Trp-Leu-His- plates containing 3mM 3-AT (right panel). UEV-3(FL), full-length UEV-3; UEV-3(UEV), UEV domain only; UEV-3(NTD), N-terminal domain, BD is binding domain and AD is activating domain. **B.** HA-UEV-3 coimmunoprecipitated with FLAG-PMK-3(AQY) mutant but not with FLAG-PMK-3 wild type when coexpressed in 293T cells. **C.** Functional PMK-3::GFP (*juEx675 [Prgef-1-PMK-3::GFP]*) in neurons is seen in cytoplasmic and nuclear compartments in wild type, and is unaltered in *uev-3(ju587)* background. Arrows indicate neurons in the ventral cord. **D.** Functional UEV-3::GFP in mechanosensory neurons (*juEx2118 Pmec-4-GFP::UEV-3*) localizes to cytoplasm and nucleus, and is unaltered in *pmk-3* mutant background.
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A

B

FLAG-PMK-3 (wt)  
FLAG-PMK-3 (AOF)  
HA-UEV-3

IP: α-FLAG  
IB: α-HA  
HA-UEV-3

IP: α-FLAG  
IB: α-FLAG  
FLAG-PMK-3 (wt or AOF)

Whole cell lysate  
IB: α-HA  
HA-UEV-3

C

Prgef-1  
PMK-3  
GFP

wild type  
uev-3

D

PmeC-4  
GFP  
UEV

wild type  
pmk-3

10 µm  
10 µm