DMob4/Phocein Regulates Synapse Formation, Axonal Transport, and Microtubule Organization

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DMob4/Phocein Regulates Synapse Formation, Axonal Transport, and Microtubule Organization

For Joost Schulte, Katharine J. Sepp, Ramon A. Jorquera, Chaohong Wu, Yun Song, Pengyu Hong, and J. Troy Littleton

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The monopolar spindle-one-binder (Mob) family of kinase-interacting proteins regulate cell cycle and cell morphology, and their dysfunction has been linked to cancer. Models for Mob function are primarily based on studies of Mob1 and Mob2 family members in yeast. In contrast, the function of the highly conserved metazoan Phocein/Mob3 subfamily is unknown. We identified the Drosophila Phocein homolog (DMob4) as a regulator of neurite branching in a genome-wide RNA interference screen for neuronal morphology mutants. To further characterize DMob4, we generated null and hypomorphic alleles and performed in vivo cell biological and physiological analysis. We find that DMob4 plays a prominent role in neural function, regulating axonal transport, membrane excitability, and organization of microtubule networks. D Mob4 mutant neuromuscular synapses also show a profound overgrowth of synaptic boutons, similar to known Drosophila endocytotic mutants. DMob4 and human Phocein are >80% identical, and the lethality of D Mob4 mutants can be rescued by a human phocein transgene, indicating a conservation of function across evolution. These findings suggest a novel role for Phocein proteins in the regulation of axonal transport, neurite elongation, synapse formation, and microtubule organization.

Introduction

The monopolar spindle-one-binder (Mob) family of zinc binding proteins is conserved from yeast to humans (Stavridi et al., 2003; Ponchon et al., 2004; Li et al., 2006; Mrkobrada et al., 2006). Mobs are found in complexes with kinases and may function as activating subunits, similar to cyclins (Weiss et al., 2002; Devroe et al., 2004; Hergovich et al., 2005; Wei et al., 2007). The founding Mob members, Saccharomyces cerevisiae Mob1p and Mob2p, activate Dbf-2 kinases and facilitate exit from mitosis (Luca et al., 2001; Weiss et al., 2002; Stoepel et al., 2005; Mrkobrada et al., 2006). Mob isoforms have also been localized to neuronal dendrites in mammals, in which NDR (nuclear Dbf-2) kinases regulate dendritic branching (Zallen et al., 2000; Baillat et al., 2001; He et al., 2005; Emoto et al., 2006). These findings suggest that Mob proteins may have important functions within postmitotic neurons, in addition to regulating cell proliferation.

Whereas S. cerevisiae has two Mobs, Drosophila melanogaster, Caenorhabditis elegans, and Danio rerio have four, and Homo sapiens has seven (Li et al., 2006; Mrkobrada et al., 2006). Although roles in regulation of cell proliferation by Mob1 and cell polarity by Mob2 have been characterized, little is known about the third subfamily, Phocein/Mob3 (Colman-Lerner et al., 2001; Hou et al., 2003; Lai et al., 2005; Wei et al., 2007; Praskova et al., 2008). Phocein is highly expressed in Purkinje cell dendrites and spines (Baillat et al., 2001; Haeberlé et al., 2006). Biochemical studies indicate that Phocein interacts with various vesicle trafficking proteins, including Striatin, Eps-15, Dynamin-1, and Nucleoside-Diphosphate Kinase (NDPK) (Baillat et al., 2001, 2002). However, no loss-of-function analysis of Phocein has been conducted. In vivo knockdown of Striatin blocks dendrite formation, suggesting that the Phocein/Striatin complex may regulate neuronal morphology (Bartoli et al., 1999).

Phocein is also a component of the STRIPAK complex (for Striatin interacting phosphatase and kinase), which contains protein phosphatase 2A (PP2A), Ste-kinases, Dynein, and several other interactors (Goudreault et al., 2008). The presence of serine-threonine kinases and phosphatases in the STRIPAK complex suggests that it may act as an on/off switch that governs target phosphorylation states (Pi and Lisman, 2008). In neurons, PP2A associates with microtubules and regulates its dynamics by alternating microtubule-associated protein (MAP) phosphorylation (Sontag et al., 1996; Tournebize et al., 1997; Aranda-Orgilés et al., 2008). These interactions suggest that the STRIPAK/PP2A complex may regulate neuronal microtubules through interactions with a variety of effector proteins, including Phocein. As such, Phocein may regulate a host of neuronal functions in vivo.

Here we describe the first in vivo knock-out analysis of phocein/DMob4 (hereafter referred to as D Mob4) in metazoans. Within neurons, we find that D Mob4 is required for axonal transport, synaptic development, and normal organization of microtubule networks. Genetic rescue of D Mob4 mutant phenotypes with human phocein suggests a conserved role of this protein...
family in neurons. These findings highlight a new function for the metazoan-specific Phoecin/Mob3 subfamily in the regulation of neuronal structure and function.

Materials and Methods

DMob4 mutant strains. To isolate DMob4 (CG3403) mutant alleles, a P-element excision screen was conducted using y,w $^{67C}$ P $^{[E] P e g 2}$ EY23407 and the transposase source y,w $^{1118}$, avo $^5$ CyO, BPal $^{[52 \cdot 3, E v o l]}$. EY23407 is a P-element insertion in the 59 untranslated region (UTR) of DMob4 in exon 1, and it is 33 bp upstream of the DMob4 start codon, as confirmed by inverse PCR and sequencing of the P-element ends. Putative w/Ey $^\Delta$ imprecise excision alleles arising from the screen were balanced over CyO and scored for homozygous lethality. A subsequent round of PCR screening of the homozygous lethal lines with oligonucleotides spanning the EY23407 insertion site (Pr3cdna forward, 59 TCAGCTTCAGGATACACCTATGCGGGAT; Pr3cdna reverse, 59 TCGAGAATCATCCCTTTGATGACCG) identified strains containing small deletions of the D Mob4 locus. Using these oligonucleotide pairs, a 729 bp band is obtained in wild types. Lethal stocks that yielded PCR products of $<729$ bp were identified as being excision mutants. These bands were analyzed by DNA sequencing to map the excision breakpoints. D Mob4 $^{E Y 2 L 5 0 7}$ contains a 116 bp deletion starting at 33 and is missing part of exon 1 and the downstream intron; at the 39 breakpoint also at 33. This deletion removes all of exon 1, the intervening intron, and part of exon 2. The Ey $^\Delta$ H1123 strain is a homozygous viable precise excision allele of y,w $^{67C}$ P $^{[E] P e g 2}$ EY23407.

DMob4 rescue strains and analysis. The Drosophila D Mob4 cDNA LD21194 (Drosophila Genomics Resource Center, Indiana University, Bloomington, IN) was PCR amplified with BgII/SpeI-tailed oligonucleotides (59 GGAGATCTCATCGGGCCGTCTGAG; 59 CACTCTGATCCGAGATCTCTACTACG) flanking the open reading frame of DMob4 and then directionally cloned into a BgII/SpeI fragment into the pValum vector system (for vermillion attB LoxP UAS MCS), for the generation of transgenic lines (Markstein et al., 2008; Ni et al., 2008). For the human phoecin rescue construct, we repeated the same strategy as for DMob4. The human phoecin variant-1 (GenBank accession number NM_015387.2) cDNA clone (OriGene Technologies) was used to make rescue constructs. We repeated the same strategy as for DMob4. For double-stranded RNA (dsRNA) production, the DMob4 antibody was affinity purified on HiTrap NHS-activated HP columns (1.0 ml) according to the specifications of the manufacturer (Amersham).

Immunohistochemistry. Staining of embryos and larvae was performed according to standard protocols (Rothwell and Sullivan, 1999). In brief, embryos were fixed in PEM (0.1 M pipes, 2 mM EGTA, 1 mM MgSO4, pH 6.95) buffer and 4% formaldehyde for 20 min and washed/permobilized in PBS plus 0.05% Tween 20. Larvae were reared at 21°C, and wandering third instars were dissected in calcium-free HL3.1 saline (Feng et al., 2004), and the larvae were fixed for 30 min in HL3.1 and 4% formaldehyde and incubated for 5 d before Western blot analysis.

Full-length DMob4 antibody. Full-length DMob4 was PCR amplified from the expressed sequence tag (EST) LD21194 (Drosophila Genomics Resource Center) using XhoI and KpnI tailed oligonucleotides (59 primer, CCTCTGAGATGAAGTGCAGGC; 39 primer, GTGTTCCATACGGCCTCTGCAAA) and subcloned into the pGem-T Easy vector system (Promega). DMob4 clones were sequenced and subcloned into pTrcHis A (Invitrogen) as an XhoI/KpnI fragment for the production of N-terminal 6x-His-tagged protein. The tagged DMob4 was purified from BL-21 (DE3) Escherichia coli with 1.0 ml HisTrap HP nickel column (GE Healthcare) and was injected into guinea pigs (Invitrogen) for antibody production. For in situ applications, the DMob4 antibody was affinity purified on HiTrap NHS-activated HP columns (1.0 ml) according to the specifications of the manufacturer.

RNA in situ hybridizations. In situ hybridization studies of DMob4 in embryos were performed as described previously (Kearney et al., 2004). To produce a linearized template for digoxigenin (DIG)-labeled RNA probe synthesis, the full-length DMob4 EST LD21194 (Drosophila Genomics Resource Center) was PCR amplified, with oligonucleotides specific to the pOT2a backbone vector (OT2a forward, 59 GCCGATTCATTAATGCA-GCGG; OT2a reverse, 59 GCCGATTCATTAATGCA-GCGG) and subcloned into the pGem-T Easy vector system (Promega). DMob4 clones were sequenced and subcloned into pTrcHis A (Invitrogen) as an XhoI/KpnI fragment for the production of N-terminal 6x-His-tagged protein. The tagged DMob4 was purified from BL-21 (DE3) Escherichia coli with 1.0 ml HisTrap HP nickel column (GE Healthcare) and was injected into guinea pigs (Invitrogen) for antibody production. For in situ applications, the DMob4 antibody was affinity purified on HiTrap NHS-activated HP columns (1.0 ml) according to the specifications of the manufacturer.

DNA in situ hybridizations. In situ hybridization studies of DMob4 in embryos were performed as described previously (Kearney et al., 2004).

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treated mashed banana to 50% and penicillin–streptomycin to a final concentration of 50 IU/ml and 50 μg/ml, respectively (Mediatech). Mutant larvae were transferred to fresh yeast/banana/penicillin–streptomycin media daily until they attained the third-instar stage. For neuromuscular junction (NMJ) morphology analysis, supernumerary boutons were defined as strings of five or fewer boutons that extend from the central NMJ axis. The following antibodies and concentrations were used: 22C10 mouse monoclonal antibody (mAb) at 1:5 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), mouse anti-acetylated α-Tubulin clone 6-11B-1 at 1:250 (T7451; Sigma-Aldrich), mouse anti-tyrosinated α-Tubulin TUB-1A2 at 1:1000 (T9028; Sigma-Aldrich), guinea pig anti-DMob4 at 1:600, rabbit anti-HRP rhodamine at 1:150 (Jackson ImmunoResearch), nc82 at 1:200 (Developmental Studies Hybridoma Bank), and rabbit anti-synaptotagmin-1 (Syt-1) at 1:500 (Littleton et al., 1993). Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies were used at 1:400 (Invitrogen). Tissue preparations were imaged on a Leica TCS-SP2 confocal LSM microscope.

**Western blot analysis and biochemical experiments.** For standard SDS-PAGE/Western blot analysis, 1.5 larvae per lane were loaded onto gels. For quantitative Western blot analysis, total protein levels were determined in lysates (10 larvae/100 μl lysis buffer: 20 mM HEPES, pH 7.5, 50 mM KCl, 2 mM EGTA, 1 mM MgCl₂, and 0.2% NP-40 plus protease inhibitors) using a BCA protein assay kit (Pierce), and then 6.5 μg/lane was loaded onto gels. To reduce background bands on Western blots, the viscera of the third-instar larvae were removed before homogenization in

![Figure 1](image-url)

**Figure 1.** RNAi knockdown of DMob4 in primary cultures alters neurite morphology. A, Fluorescent microscopy images of Drosophila primary neural cultures derived from embryos expressing GFP. Cultures were either untreated (control) or subjected to DMob4 RNAi using DMob4-specific dsRNA amplicons (DRSC4993 and DRSC29567). B, Quantification of DMob4 RNAi phenotypes in primary neural cultures. Custom algorithms were used to extract and quantify image features from automated microscopy digital files \((n=192\) per treatment group). For small neurumes, a threshold of \(\log _{10} \text { area } \leq 5.6 \, \mu m^2\) was set. The number of small neurumes per image was tabulated as a percentage, and images were binned into groups as displayed in the histogram (bins: 58–67, 67–70, 71–75, or 75–79%). For short neurites, a threshold was set at \(\geq 6.5\) and \(\leq 16.1 \, \mu m\) (bins: 63–65, 65–67, 67–68, 68–69, and 69–70%). For long neurites, a threshold was set at \(\geq 25.8 \, \mu m\) (bins: 8.9–9.9, 9.9–10.1, 10.1–12, 12–13, and 13–15%). For neurite strength, representing neurite thickness, bins were set at increments of 31.4 pixel intensity units (PIU). Analysis using all image features indicated that the control is statistically different from treatment groups (control vs DRSC4993, \(p = 2.22 \times 10^{-16}\); control vs DRSC29567, \(p = 1.41 \times 10^{-6}\)), and the two treatment groups are also statistically different from each other \((p = 0.002)\). WT, Wild type. C, Western blot analysis of cell cultures treated with DMob4 dsRNA amplicons. DMob4 protein levels are decreased by \(-75\%\) with DRSC4993 or DRSC29567. No apparent increase in DMob4 knockdown is achieved using a combination of the two amplicons \((n=8)\). Dlg was used as a loading control. D, DMob4 mRNA in situ hybridization to embryos with DMob4 antisense transcript (blue). DMob4 expression is abundant in early embryos at stage 5 and widely expressed until stage 13, when it becomes enriched in the ventral nerve cord (arrow, stage 14). DMob4 negative control (sense transcript) fails to label the ventral nerve cord [arrow, stage 14, (−) cntrl]. Anterior is to the right, and ventral is down. Scale bars, 100 μm.
sample buffer (standard SDS-PAGE) or lysis buffer (quantitative SDS-PAGE). For immunoblotting, antibodies were used at the following concentrations: mouse anti-Discs large (Dlg) (4F3; Developmental Studies Hybridoma Bank) at 1:1000, guinea pig anti-DMob4 at 1:2000, rabbit anti-Synaptogyrin at 1:10,000, mouse anti-α-tubulin clone B-5-1-2 at 1:5000 (T5168; Sigma-Aldrich), and IR700- and IR800-conjugated secondary antibodies at 1:3000 (Rockland Immunochemicals). Western blots were imaged and quantified using an Odyssey infrared scanner (Li-Cor).

Adult behavioral analysis. Viability assays were performed at 21°C. Virgin male and female Drosophila were collected in separate vials (15 flies per vial) and flipped onto fresh media three times per week. The number of deceased animals was noted at the time of each flip. Longevity plots were analyzed separately for the males and females of a given strain and then data were pooled, because there was no statistically significant difference between sexes. The numbers of flies used to obtain the long-term difference between sexes. The data were pooled, because there was no statistically significant difference between sexes. The numbers of flies used to obtain the long-term difference between sexes. The data were pooled, because there was no statistically significant difference between sexes.

Electrophysiology. Evoked postsynaptic currents were recorded from ventral longitudinal muscle 6 at abdominal segment A3 in third-instar larvae using two-microelectrode voltage clamp (OC725; Warner Instruments) at ~80 mV holding potential (Acharya et al., 2006). All experiments were performed in modified HL3 solution composed of the following (in mM): 70 NaCl, 5 KCl, 4 MgCl2, 2 CaCl2, 10 NaHCO3, 115 sucrose, and 5 HEPES-Na, pH 7.2. Data acquisition and analysis were performed using pClamp software (Molecular Devices). For stimulation, nerves were cut close to the ventral ganglion and sucked into a pipette filled with working solution. The nerve was stimulated at frequencies indicated in each experiment using a programmable pulse stimulator (Master-8; A.M.P.L.) with a 0.1 ms duration. Nerve threshold for evoked release was estimated in each experiment (minimal stimulation), and the intensity of stimulation was increased twofold (maximal stimulation).

Results

DMob4 regulates neurite outgrowth in Drosophila primary cultures

We identified DMob4 (CG3403) in a genome-wide RNAi screen for genes required for neurite outgrowth or regulation of neuronal morphology (Sepp et al., 2008). We selected DMob4 for additional characterization because of its homology with other Mob
Table 1. Lethal phase analysis of DMob4 allelic combinations at 21°C

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<th>Strain</th>
<th>Genotype</th>
<th>Second-instar larvae collected (n)</th>
<th>Stage achieved</th>
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<tr>
<td>Precise excision control</td>
<td>EYΔHV1223</td>
<td>50</td>
<td>100% (Pupa) 96% (Adult)</td>
</tr>
<tr>
<td>DMob4 null</td>
<td>EYΔHV1223 DmMob4 &amp; DmMob4</td>
<td>30</td>
<td>0% (Pupa) 0% (Adult)</td>
</tr>
<tr>
<td>Ubiquitous rescue (DMob4)</td>
<td>DmMob4 &amp; elav-Gal4</td>
<td>115</td>
<td>97% (Pupa) 66% (Adult)</td>
</tr>
<tr>
<td>Ubiquitous rescue (hPhocein)</td>
<td>DmMob4 &amp; elav-Gal4 UAS-DMob4</td>
<td>100</td>
<td>95% (Pupa) 47% (Adult)</td>
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<tr>
<td>Neuronal rescue (DMob4)</td>
<td>Elav-GAL4; DmMob4 &amp; UAS-DMob4</td>
<td>50</td>
<td>24% (Pupa) 0% (Adult)</td>
</tr>
<tr>
<td>Muscle rescue (DMob4)</td>
<td>DmMob4 &amp; UAS-DMob4</td>
<td>50</td>
<td>0% (Pupa) 0% (Adult)</td>
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Table 2. Rescue of DMob4 lethality using tissue-specific drivers and Drosophila or human transgenes

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Generation of DMob4 mutant alleles

The Drosophila Mob4 locus resides at cytological band 42C5 and spans 1.5 kb. DMob4 is closely flanked by Rab2 and CG3270 (Fig. 2B). To further characterize the role of DMob4, we generated null mutations in the locus using a P-element excision screen with the EY23407 element. We isolated a precise excision allele EY23407. The DMob4\textsuperscript{EY23407} strain is homozygous lethal. Because the insertion does not disrupt the DMob4 open reading frame, we conducted an excision screen with DMob4\textsuperscript{EY23407} to generate definitive null alleles. Because the locus is compact, with Rab2 and CG3270 within a few hundred base pairs flanking DMob4 (Fig. 2B), we generated small deletions that had breakpoints entirely within the DMob4 gene. We screened 1329 white-eyed DMob4\textsuperscript{EY23407} \textsuperscript{L3} and DMob4\textsuperscript{EY23407} \textsuperscript{CyO} excision strains for lethality (lines were designated DMob4\textsuperscript{EY23407} \textsuperscript{L3} or DMob4\textsuperscript{EY23407} \textsuperscript{CyO}) and used PCR to assay for desired deletions. We isolated two novel DMob4 excision mutants: DMob4\textsuperscript{EY\Delta L3} and DMob4\textsuperscript{EY\Delta L307} (Fig. 2B). Both excision lines have 5' breakpoints that begin 33 bp upstream of the DMob4 initiator methionine, at the original insertion site of the EY23407 element. DMob4\textsuperscript{EY\Delta L3} contains a 357 bp deletion, whereas DMob4\textsuperscript{EY\Delta L307} contains a 116 bp deletion. Both excision lines disrupt the initiator methionine of DMob4. We isolated a precise excision allele EY\Delta HV1223, which is homozygous viable, and served as a control for future experiments.

We next performed Western blot analysis to determine whether DMob4\textsuperscript{EY\Delta L3} and DMob4\textsuperscript{EY\Delta L307} deletions alter levels of DMob4 protein expression. For these studies, we generated a guinea pig polyclonal antibody against the full-length DMob4 protein, which is 223 aa (25.7 kDa). The antibody recognizes a ~25 kDa band on Western blots of larval extracts prepared from control animals (precise excision allele DMob4\textsuperscript{EY23407}), consistent with the predicted molecular weight of DMob4. No DMob4 protein was observed on Western blots of larval extracts prepared from homozygous DMob4\textsuperscript{EY\Delta L3} third-instar larvae (Fig. 2C) or from DMob4\textsuperscript{EY23407} (data not shown), indicating that these alleles are protein nulls. DMob4-specific bands were observed on Western blots of extracts prepared from embryo lysates of the same mutants, likely representing DMob4 protein translated from maternally deposited transcripts observed by mRNA \textit{in situ} hybridization (Fig. 1D). Western blot analysis of third-instar larvae prepared from the DMob4\textsuperscript{EY\Delta L307} strain revealed that this mutant produces an N-terminal truncated DMob4 protein. Heterozygous DMob4\textsuperscript{EY\Delta L307}/CyO animals produce two bands on Western blots: one at 25 kDa representing full-length DMob4 protein, and a smaller fragment at 22 kDa representing the truncated protein (Fig. 2C). Homozygous DMob4\textsuperscript{EY\Delta L307} animals only produce the smaller 22 kDa DMob4 protein product (Fig. 2C). Analysis of the DMob4\textsuperscript{EY\Delta L307} DNA sequence revealed an alternative in-frame initiator methionine in exon 2, downstream of the 3' breakpoint of the EY\Delta L307 deletion. The predicted translational product of this truncated transcript is 32 aa shorter than the full-length 223 aa protein, with a molecular weight of 22 kDa, in agreement with the observed molecular weight of DMob4 in homozygous DMob4\textsuperscript{EY\Delta L307}.
Expression levels of the homozygous DMob4Δ1Δ307 mutants appear reduced relative to controls, suggesting that the deletion has less efficient transcription. Thus, DMob4Δ1Δ307 is a hypomorph and produces N-terminal truncated DMob4, whereas DMob4Δ1L3 and DMob4Δ23407 are protein nulls.

Lethal phase analysis and rescue of DMob4 mutants
To determine the lethal phase of DMob4 mutant alleles, we monitored the survival rates of allelic combinations (Table 1). We used the deletion strain Df(2R)42 in cis– to the mutants to control for the possibility of second site mutations on the DMob4Δ1Δ3 chromosomes. Df(2R)42 has breakpoints at 42C2–42D3 and spans the DMob4 locus. Lethal phase analysis revealed that DMob4Δ1Δ3 homozygous mutants die mostly as first- and second-instar larvae, although ~10% can survive to the third-instar stage. Approximately 35% of homozygous DMob4Δ23407 animals survive to the third-instar stage. The least severe allele, the N-terminal truncation mutant DMob4Δ1Δ307, survives to the adult stage and can be maintained as a homozygous stock.

Because Rab2 and DMob4 lie head-to-head on chromosome 2R and their start codons are separated by only a few hundred base pairs, we wanted to ascertain that the lethality observed for DMob4 mutants was not attributable to a loss of Rab2 activity. We therefore crossed DMob4Δ1Δ307 and DMob4Δ1Δ3 to the Rab202699 null allele. Although Rab202699 homozygous lethal, the DMob4Δ1Δ307/Rab202699 and DMob4Δ1Δ3/Rab202699 transheterozygotes are viable to adult stages and have no obvious morphological or behavioral defects, indicating that DMob4Δ23407 and DMob4Δ1Δ3 lesions are specific to DMob4.

The N terminus of Mob proteins is solvent exposed and flexible and does not appear to be an integral part of the conserved globular core (Stavridi et al., 2003; Ponchon et al., 2004; Mrkobrada et al., 2006). The N terminus is conserved in yeast, x-ray crystal structural analysis of Mob1p suggests that the N terminus can interact with the C-terminal core domain and is necessary for homodimer formation. Because the DMob4Δ1Δ307 is adult viable, we extended our longevity analysis into adulthood to more closely examine the physiological consequences of loss of the DMob4 N terminus. We found that DMob4Δ1Δ307 mutants have significantly decreased adult longevity (Fig. 2D). The precise excision allele was used as a control for longevity studies. DMob4Δ1Δ307 homozygous animals had an ~20% decrease in adult longevity, whereas DMob4Δ1Δ3/DMob4Δ1Δ3 mutants had an ~38% decrease in adult longevity. Collectively, these studies suggest that the N-terminal 32 aa are necessary for complete functionality of DMob4.
To confirm that the DMob4 phenotypes we observed were attributable to loss of DMob4, we conducted rescue experiments using a UAS–DMob4 (wild-type) transgene (Table 2; plus sign (+) refers to wild-type chromosome). We also conducted rescue experiments using the human homolog of DMob4: Phocein/Mobkl3 variant-1, to test for evolutionary conservation of function. The human Phocein protein is 80% identical to DMob4 at the amino acid level (Fig. 2A).

For rescue experiments, we used the GAL4/UAS system to drive expression of the wild type constructs in the DMob4EY1463/L3 null mutant background. To control for differences in expression levels of the upstream activating sequence (UAS) transgenes, we used the attP–attB integration system and targeted the UAS–DMob4 and human UAS–phocein attB rescue constructs to the attP2 integration site on chromosome 3 (Markstein et al., 2008; Ni et al., 2008). Using the actin–Gal4 ubiquitous driver, we were able to rescue DMob4EY1463/L3 larval lethality with both DMob4 and human phocein transgenes to a comparative level (Table 2). We conducted PCR analysis on rescued animals to ensure that they were homozygous for the DMob4EY1463/L3 mutant chromosome (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Rescued animals were able to pupate and eclease as adults (Table 2), suggesting that human phocein and Drosophila Mob4 are functionally equivalent orthologs.

RNA in situ analysis revealed that DMob4 is expressed in the nervous system (Fig. 1D). We tested whether the observed DMob4EY1463/L3 larval lethality could be solely attributed to loss of DMob4 function in the nervous system using rescue experiments with the nervous system-specific driver ElavG128, driving expression of DMob4 in the nervous system of DMob4EY1463/L3 null mutants partially rescued lethality. Twenty-four percent of ElavG128/UAS–DMob4 rescued null animals are able to pupate compared with 0% for mutants (Table 2). In contrast, expressing DMob4 in muscles using the MHC–Gal4 driver did not rescue the lethality. ElavG128/UAS–DMob4 rescued pupae failed to eclease, indicating that DMob4 also has essential functions outside the nervous system.

**Tissue distribution and subcellular localization of DMob4**

Anti-DMob4 antiserum was used for immunohistochemical analysis of embryos and larvae to examine the developmental expression of the protein. DMob4 is widely expressed in embryos and larvae. At the syncytial blastoderm stage, DMob4 staining is intense in the cell cortex, below the nuclei (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). Zygotic genes are not yet active at this stage of development, suggesting that DMob4 protein, like the transcript, is maternally loaded into the egg. As cellularization advances, DMob4 localizes to the poles and mitotic spindles of dividing cells, together with a more widespread cytosolic distribution. In late-stage embryos, DMob4 is expressed in all tissues examined, including muscles, trachea, gut, peripheral glia, and neurons, with strong expression in the embryonic ventral nerve cord (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). During larval development, the ubiquitous expression of DMob4 persists. At the NMJ, DMob4 fills synaptic boutons and surrounds nc82-positive active zones (Fig. 3A–C). This periactive zone distribution is common for proteins implicated in endocytosis (Marie et al., 2004). In peripheral nerves, DMob4 is expressed at high levels in axons, but it is also present in the ensheathing glia (Fig. 3H). A fraction of DMob immunostaining in axons is punctate, suggesting that some DMob4 may be associated with vesicles undergoing transport. These staining patterns are abolished in null mutants (Fig. 3D–F), confirming specificity of the antiserum.

**DMob4 mutants display synaptic overgrowth at the NMJ**

Given that RNAi knockdown of DMob4 in primary neural cultures results in excessive branching of neurite processes and that DMob4 is expressed at NMJs, we examined whether loss of the
It has been previously determined that supernumerary bouton formation is driven by endocytic vesicle trafficking abnormalities. To substantiate this theory, we examined the expression levels of endocytic vesicle trafficking proteins in Dap160 mutants and rescued animals. Dap160 mutants displayed an increase in the number of supernumerary boutons when compared to controls (Fig. 4A–D). This increase was consistent with a requirement for Dap160 in synaptic endocytosis. In peripheral nerves because vesicular cargo is transported long distances along elaborated microtubule networks and Dap160 is highly expressed in axons. At the NMJ Dap160, 237 nulls, the presence of multiple abnormal cisternae-like endocytic structures budding from the plasma membrane of presynaptic terminals (Fig. 5B–D). Such cisternae were absent from control animals (Fig. 5A), consistent with a requirement for Dap160 function in synaptic endocytosis. In peripheral nerves of Dap160 mutants, we observed accumulations of microtubule-associated vesicles with larger diameters than in controls (Fig. 5E–H). This may reflect the transport of large abnormal endocytic cisternae or perturbed axonal transport.

Electron microscopy of Dap160 mutants

Because supernumerary boutons are a phenotype observed in several endocytic mutants and rat Phocein interacts with endocytosis/vesicle trafficking proteins (Eps-15, NDPK, and Dynamin-I), we performed electron microscopy on Dap160 nulls to investigate whether there are any anomalies in endocytosis and/or vesicle trafficking in the mutants. We focused on the NMJ because it is a region of active endocytosis of synaptic vesicles and Dap160 is localized in synaptic boutons. Similarly, we examined axons of peripheral nerves because vesicular cargo is transported long distances along elaborated microtubule networks and Dap160 is highly expressed in axons. At the NMJ of Dap160 nulls, we observed the presence of multiple abnormal cisternae-like endocytic structures budding from the plasma membrane of presynaptic terminals (Fig. 5B–D). Such cisternae were absent from control animals (Fig. 5A), consistent with a requirement for Dap160 function in synaptic endocytosis. In peripheral nerves of Dap160 mutants, we observed accumulations of microtubule-associated vesicles with larger diameters than in controls (Fig. 5E–H). This may reflect the transport of large abnormal endocytic cisternae or perturbed axonal transport.

**Dap160 mutants manifest membrane excitability defects**

To quantitatively investigate endocytosis defects, we conducted electrophysiology experiments at third-instar larval NMJs of control and Dap160 mutants using two-electrode voltage clamp (Fig. 6). We first measured EPSCs at low frequency in 2 mM extracellular Ca²⁺. Dap160 mutant larvae displayed a small ~15% reduction in evoked EPSC amplitude (control, 278 ± 52 nA, n = 4; Dap160, 237 ± 36 nA, n = 5), indicating that robust synaptic transmission persisted in the mutants. We next assayed for use-dependent alterations in synaptic function by recording EPSCs during stimulation trains of 10 or 50 Hz in 2 mM extracellular Ca²⁺. Dap160 mutants displayed an increase in the number of supernumerary boutons when compared to controls (Fig. 4A–D). This increase was consistent with a requirement for Dap160 in synaptic endocytosis. In peripheral nerves because vesicular cargo is transported long distances along elaborated microtubule networks and Dap160 is highly expressed in axons. At the NMJ Dap160, 237 nulls, the presence of multiple abnormal cisternae-like endocytic structures budding from the plasma membrane of presynaptic terminals (Fig. 5B–D). Such cisternae were absent from control animals (Fig. 5A), consistent with a requirement for Dap160 function in synaptic endocytosis. In peripheral nerves of Dap160 mutants, we observed accumulations of microtubule-associated vesicles with larger diameters than in controls (Fig. 5E–H). This may reflect the transport of large abnormal endocytic cisternae or perturbed axonal transport.

Figure 7. Axonal transport defects in DMob4 mutants. A, B. DMob4 mutant third-instar larvae have a tail-flip phenotype (B) compared with controls (A). C–F. Peripheral nerves of third-instar larvae communolabeled for Syt-1 (red) and DMob4 (green). Blockade of axonal transport in DMob4EY23407/Df(2R)42 (null) and DMob4EY23407/Df(2R)42 (homozygous mutants) results in accumulation of Syt-1 in large aggregates along the length of peripheral nerves (E, F). Axonal transport defects in DMob4EY23407 mutants can be rescued with the ectopic expression of DMob4 in neurons using the ElavGal4 driver (D). G. Quantification of Syt-1 immunoreactive inclusions present in peripheral nerves of DMob4 mutants, controls, and rescued animals. Scale bar, 20 μm.

protein caused defects in synapse formation in vivo. We stained homozygous DMob4EY23407 and DMob4EY23407 larvae with the neuronal membrane marker anti-HRP to label NMJs. We observed a supernumerary bouton phenotype in the DMob4 mutants, similar to what has been reported previously for several endocytosis mutants, including Dap160 (Fig. 4A–D) (Koh et al., 2004; Dickman et al., 2006). We quantified the supernumerary bouton phenotype at muscle 6/7 and muscle 4 for various combinations and compared the phenotype with Dap160 endocytic mutants (Fig. 4E). At muscle 6/7 and muscle 4, DMob4EY23407 mutants have eightfold more supernumerary boutons than Canton S or the precise excision EY7517233 animals, whereas Dap160 mutants have sixfold more. We also quantified the frequency of supernumerary boutons in Rab20–GFP/DMob4EY23407 transheterozygotes and found no difference from controls, confirming that the observed supernumerary bouton phenotypes are specific to a loss of DMob4. To determine whether DMob4 acts cell autonomously in neurons to regulate synapse formation, we examined DMob4EY23407 homozygous mutants carrying the elavGal4;UAS–DMob4 rescue transgene. Presynaptic expression of DMob4 partially suppresses the supernumerary bouton phenotype observed in nulls. The number of supernumerary boutons in the nulls is reduced by 2.6-fold when DMob4 is driven in the nervous system (Fig. 4B, C, E). This data indicates that DMob4 functions presynaptically to regulate normal synapse formation.

**DMob4 mutants manifest membrane excitability defects**

To quantitatively investigate endocytosis defects, we conducted electrophysiology experiments at third-instar larval NMJs of control and DMob4 mutants using two-electrode voltage clamp (Fig. 6). We first measured EPSCs at low frequency in 2 mM extracellular Ca²⁺. Dap160 mutant larvae displayed a small ~15% reduction in evoked EPSC amplitude (control, 278 ± 52 nA, n = 4; DMob4, 237 ± 36 nA, n = 5), indicating that robust synaptic transmission persisted in the mutants. We next assayed for use-dependent alterations in synaptic function by recording EPSCs during stimulation trains of 10 or 50 Hz in 2 mM extracellular Ca²⁺.
Ca\(^{2+}\) (Fig. 6 A, B). Mutants with defective endocytosis typically display a gradual rundown of EPSCs with tetanizing stimuli as the synaptic vesicle pool depletes (Delgado et al., 2000). DMob4 mutants showed relatively normal synaptic depression during 10 Hz stimulation, but complete failures in EPSCs were observed after \(~4\) s with a faster 50 Hz stimulation train (Fig. 6B). This phenotype was observed in both DMob4\(^{Ey\Delta L3}\) nulls and DMob4\(^{Ey\Delta L3,307}\) hypomorphs and was 100% penetrant in mutants and absent from controls. Mutant larvae that were allowed to recover from the high-frequency stimulation trains displayed normal EPSCs with subsequent 10 Hz stimulations.

Neuronal expression of DMob4 with Elav\(^{F55}\)GAL4 completely suppressed the EPSC phenotype of DMob4\(^{Ey\Delta L3}\) nulls (Fig. 6 A, B), whereas muscle-specific expression did not, indicating that DMob4 is required presynaptically for normal membrane excitability. To further analyze synaptic transmission failures in DMob4 mutants, we tested potential contributing factors by altering stimulation intensities used to trigger action potentials. We observed supernumerary EPSC responses to single nerve stimuli in DMob4 mutants using stronger stimulation (2 \(\times\) nerve threshold) (Fig. 6C), suggesting abnormal membrane excitability in the absence of DMob4. Excitability defects were also found at minimal stimulation intensities required to trigger a response (Fig. 6D). Delays between the onset of EPSC responses were observed at 50 Hz, even early in stimulation trains, consistent with a slower propagation of action potentials in mutant animals. Similar perturbations in membrane excitability properties have been observed in a variety of ion channel mutants, including the \(Na^+/K^+\) ATPase, and the Shaker and Shab potassium channels mutants (Jan et al., 1977; Jan and Jan, 1978; Ueda and Wu, 2006), suggesting that DMob4 is likely to modulate membrane excitability of neurons, in addition to its role in regulating morphology. The all-or-none failures in EPSCs observed in the DMob mutants likely mask any vesicle depletion phenotype characteristic of classical endocytic mutants.

DMob4 mutants have defective axonal transport

While conducting lethal phase analysis on DMob4 mutants, we observed that mutant third-instar larvae have a tail-flip phenotype (Fig. 7 A, B), with the posterior half of the mutant larva paralyzed. This phenotype has been described previously for microtubule motor mutants such as kinesin and dynein that disrupt axonal transport (Martin et al., 1999). We observed the tail-flip phenotype in all DMob4 strains, including DMob4\(^{Ey\Delta L3}\), DMob4\(^{Ey\Delta L3,307}\), and DMob4\(^{Ey\Delta L3,307}\). The tail-flip phenotype was most severe in the DMob4\(^{Ey\Delta L3,307}\) and DMob4\(^{Ey\Delta L3,307}\) null strains.

Because microtubule motor mutants have defects in axonal transport and display a posterior tail-flip phenotype in third-instar larvae, we investigated whether DMob4 mutants also had defects in axonal transport. We immunolabeled homozygous DMob4\(^{Ey\Delta L3}\) and DMob4\(^{Ey\Delta L3,307}\) mutant third-instar larvae for the synaptic vesicle protein Syt-1 and counter-labeled for DMob4 (Fig. 7C–F). Syt-1 is transported in vesicles along microtubules and normally enriches at synapses. Syt-1 has a punctate distribu-
DMob4 mutants have abnormal microtubule organization at synapses, axons, and muscles

Microtubules serve as the main scaffold along which motors such as kinesin and dynein transport vesicular cargo. Microtubules are also necessary to stabilize presynaptic terminals during synaptic development. In mature presynaptic terminals, microtubules adopt a looped structure. Because D Mob4 mutants have abnormal synapse development and defective axonal transport, we investigated whether microtubule networks at synapses and in axons were disrupted. To examine microtubules in control and D Mob4 mutant third-instar larvae, we immunolabeled the animals for the neuronal microtubule-associated protein Futsch (mAb 22C10). We observed abnormal microtubule organization at the synapse and along peripheral nerves of D Mob4 mutants (Fig. 8). In control animals, typical 22C10-positive microtubule loops at terminal boutons were present (Fig. 8A). In D Mob4 homozygous mutant synapses, microtubule loops were either absent or had multiple breaks (Fig. 8B, C). In synapses in which microtubule loops were absent, the 22C10 staining either filled the boutons in a speckled pattern or was weak and diffuse. The microtubule organization along peripheral nerves was also altered in D Mob4 mutant animals. In control animals, microtubule networks ran parallel along the length of the nerves (Fig. 8D). In D Mob4 mutants, microtubule networks had a distorted appearance (Fig. 8E), suggesting that microtubule networks are disorganized at peripheral nerves, and synapses.

To investigate whether the abnormal distribution of the microtubule-associated protein Futsch (mAb 22C10) in D Mob4 mutants was a reflection of overall microtubule disorganization, we examined microtubules directly by immunolabeling for α-Tubulin. Posttranslational modifications of α-Tubulin can be used to monitor different populations of microtubules. Stabilized microtubules are enriched for acetylated α-Tubulin, whereas nascent microtubules are enriched for tyrosinated α-Tubulin (Palazzo et al., 2003; Fukushima et al., 2009). We examined both populations of α-Tubulin in D Mob4 mutants.

In control animals, acetylated α-Tubulin immunolabeling gives a very stereotypic pattern in muscle fibers, with a high concentration around muscle nuclei and fibers radiating away from the nuclei in an elaborate meshwork (Fig. 8F). The muscle nuclei are also aligned along the longitudinal axis of the muscle fibers. In D Mob4 mutants, there is a striking decrease in the extent of acetylated microtubule networks, and the muscle nuclei are more randomly located throughout muscle fibers (Fig. 8G). We quantified the acetylated microtubule signal in muscles as a function of distance from the nuclear membrane (Fig. 8H). In controls, the microtubule signal decreases by 38% at 20 μm from the nucleus, whereas in D Mob4 mutants it is reduced by 70%. These data strongly suggest that stabilized microtubule networks are disorganized in multiple subcellular compartments in D Mob4 mutants. To extend these findings, we stained control and D Mob4

Figure 9. Diminished levels of tyrosinated microtubules in D Mob4 mutants. A–E. Control and D Mob4 mutants double stained for tyrosinated α-Tubulin (Tyr-α-Tub) using monoclonal antibody Tub-1A2 (green) and costained for Syt-1 (red). Individual and merged channels from confocal stacks are presented. A, B. Peripheral nerves. D Mob4 EY/L3 homozygous mutants have decreased levels of Tyr-α-Tub, and individual microtubule bundles are less evident. In mutants, Tyr-α-Tub appears to have a smooth distribution. Syt-1 aggregates are evident in the D Mob4 mutants (B). Stacks spanning half the diameter of a peripheral nerve are presented for increased resolution of microtubule networks. C, D. NMJs. Diminished levels of Tyr-α-Tub is observed in boutons of D Mob4 mutant NMJs (compare arrowheads in green channel in C, D). Stacks spanning half the diameter of a peripheral nerve are shown. E, F. Abdominal muscle. Microtubule network complexity, as well as Tyr-α-Tub levels are also reduced in the muscles of D Mob4 mutants. Single confocal slices are presented. Scale bars, 20 μm.
Discussion

Here we describe the first *in vivo* functional characterization of a Phocein protein in the nervous system using *Drosophila*. Phocein is a member of the Mob family of zinc-binding proteins that are enriched in Purkinje cell dendrites. A function for DMob4 in regulation of neurite branching was suggested from a genome-wide RNAi screen designed to identify genes necessary for neurite outgrowth and morphology (Sepp et al., 2008). Our mutant analysis of DMob4 loss-of-function alleles revealed a host of nervous system defects, including abnormal synaptic development with extensive satellite bouton formation, disrupted axonal transport, disorganized microtubules, and action potential failure during high-frequency stimulation. DMob4 is essential for viability, because null alleles are larval lethal and hyponomorphic alleles have decreased adult longevity. The larval lethality of nulls can be rescued by expression of DMob4 or human phocein, indicating functional conservation and orthology of DMob4 across species. Although other Mob family members have been found to function in mitotic cells to enable cell cycle progression, facilitate apoptosis, or regulate cell morphogenesis (Luca et al., 2001; Weiss et al., 2002; Hou et al., 2003; He et al., 2005; Lai et al., 2005; Praskova et al., 2008), we find that Phocein/DMob4 has a unique role in postmitotic neurons to regulate synapse formation, axonal transport, and microtubule organization in the nervous system.

In yeast, Mobs function as activating subunits of the Dbf-2 family of protein kinases (Komarnitsky et al., 1998; Ho et al., 2002). There are two *Drosophila* Dbf-2 homologs, Tricornered and Warts, which interact with Mob1 (mats) and Mob2 (Justice et al., 1995; He et al., 2005; Lai et al., 2005). The kinase binding partners for *Drosophila* Mob3 and Mob4 are not known. Mutations in *warts* and *mats* cause overproliferation phenotypes, whereas mutations in *tricornered* results in a split denticle belt phenotype (Justice et al., 1995; He et al., 2005). We did not observe these phenotypes in DMob4 mutants, suggesting that it is unlikely to function as an activating subunit for Tricornered or Warts. What might be the target kinase(s) for DMob4? NDPK associates with rat Phocein (Baillat et al., 2002) and is an interesting candidate kinase, given its established roles in endocytosis and microtubule dynamics (Biggs et al., 1990; Krishnan et al., 2001). NDPK is the main enzyme that synthesizes GTP from GDP in many species, and a large fraction of cellular NDPK is associated with microtubules (Postel, 1998). During microtubule polymerization, GTP is bound to tubulin dimers and is necessary for tubulation. During endocytosis, GTP binding by dynamin triggers oligomerization at the necks of clathrin-coated vesicles to drive fission. Based on studies in mammals, Phocein is also part of the STRIPAK protein complex, which contains multiple STE-kinases (Goudreault et al., 2008). As such, several *Drosophila* STRIPAK STE-kinase homologs might also be regulated by DMob4. The mammalian/*Drosophila* STRIPAK complex is likely to be well conserved, because many of the non-kinase components, including Striatin (*Drosophila* Cka), FGF receptor (e.g., *Drosophila* Heartless), Dynein, and PP2A, are present in *Drosophila*.

**DMob4 function in endocytosis and vesicular traffic**

Phocein was found previously to localize to the Golgi apparatus and dendritic spines of Purkinje cells (Baillat et al., 2001; Haebeler et al., 2006). The association of Phocein with proteins that have well-established roles in vesicular traffic and endocytosis (Eps-15, NDPK, and Dynamin-1), in addition to its subcellular localization to sites of active endocytosis, lead to a hypothesis that Phocein may function in membrane budding and vesicle trafficking (Baillat et al., 2001, 2002; Haebeler et al., 2006). Our *in vivo* characterization of DMob4 mutants supports a role for the protein in endocytosis and vesicular traffic. In *Drosophila*, many endocytosis mutants have been identified that show a supernumerary bouton phenotype, including *endophilin*, *synaptojanin*, *dynamin*, *AP180*, and *Dap-160* (Dickman et al., 2006). DMob4 mutants have a supernumerary bouton phenotype comparable to...
with *Dap-160* mutants. The excess synaptic growth in these mutants is predicted to arise from defective endocytic processing of retrogradely released synaptic growth factors such as the TGFβ homolog, Glass Bottom Boat, resulting in excessive signaling and enhanced synapse formation. TEM analysis of *DMob4* mutant NMJs also reveal endocytic cisternae that are characteristic of defective endocytosis (Kosaka and Ikeda, 1983). Similar cisternae have been reported for *eps-15* and *dap-160* mutants (Koh et al., 2007). In mutants with severely impaired endocytosis, such as *shibire*, cisternae occur in large numbers and elongated tubules are evident (Kosaka and Ikeda, 1983). We did not observe such tubules in *DMob4*, implying that the protein is not absolutely required for endocytosis but likely plays a regulatory role. Our observation that N-terminal truncation mutants (*DMob4* 

with phocein suggest that function during mitosis. GFP–*DMob4* transgenes associate with centrioles and kinetochores in dividing *Drosophila* S2 cells (Tammell et al., 2008). Microtubules attach to these structures to generate force to push/pull chromosomes apart and enable their segregation to daughter cells. RNAi knockdown of *DMob4* in S2 cells results in a mono-auster spindle phenotype that arises from a failure of microtubule minus-ends to focus at centrioles. RNAi knockdown of other *Drosophila* Mobs (*DMob1–DMob3*) does not affect spindle focusing, suggesting that *DMob4*/Phocein has a unique role in regulating microtubule dynamics. Although we did not examine mitotic defects in our mutants, we found that microtubule networks are disorganized in multiple cellular compartments, including NMJs, axons, and muscle fibers. How *DMob4* functions to stabilize microtubule networks is currently unclear, but could be mediated through several pathways. The decrease in complexity of acetylated and tyrosinated tubulin networks suggests a role for *DMob4* in organizing microtubule networks.

**DMob4** function in axonal transport and microtubule dynamics

In addition to its role in endocytosis, phocein has been suggested to function during mitosis. GFP–*DMob4* transgenes associate with centrioles and kinetochores in dividing *Drosophila* S2 cells (Tammell et al., 2008). Microtubules attach to these structures to generate force to push/pull chromosomes apart and enable their segregation to daughter cells. RNAi knockdown of *DMob4* in S2 cells results in a mono-auster spindle phenotype that arises from a failure of microtubule minus-ends to focus at centrioles. RNAi knockdown of other *Drosophila* Mobs (*DMob1–DMob3*) does not affect spindle focusing, suggesting that *DMob4*/Phocein has a unique role in regulating microtubule dynamics. Although we did not examine mitotic defects in our mutants, we found that microtubule networks are disorganized in multiple cellular compartments, including NMJs, axons, and muscle fibers. How *DMob4* functions to stabilize microtubule networks is currently unclear, but could be mediated through several pathways. The decrease in complexity of acetylated and tyrosinated tubulin networks suggests a role for *DMob4* in organizing microtubule networks.

**References**


Delgado R, Maureira C, Oliva C, Kidokoro Y, Labarca P (2000) Size of vesicles associated with axonal microtubules. This phenotype may reflect endocytosis defects or abnormal membrane budding events from other cellular compartments, in addition to defects in axonal transport. Many proteins that are integral to endocytosis at the synapse also function in budding of vesicles from the Golgi apparatus, including Clathrin, Dynein, and Eps15 (Baillat et al., 2002; McNiven and Thompson, 2006; Soldati and Schliwa, 2006).

Regardless of the mechanism of microtubule disorganization in *DMob4*, kinase/phosphatase imbalances likely contribute. Because certain Mobs have been shown to function as Dbf-2 kinase activators and their overall three-dimensional structure is likely to be conserved, including key residues for kinase binding (He et al., 2005), it is likely that *DMob4* will also function as a kinase activator. As a component of the STRIPAK complex, which contains several serine/threonine kinases and a serine/threonine phosphatase (PP2A), loss of *DMob4* could alter the balance of STRIPAK complex activity and substrate specificity (Groudault et al., 2008). Indeed, PP2A is known to regulate the phosphorylation state of Tau and other MAPs (Sontag et al., 1996; Schild et al., 2006). Linkages between microtubule motors and their cargoes are also regulated by kinase/phosphatase switches. Jun kinases, for example, control the linkage between Kinesin and vesicular cargos (Horuchi et al., 2007). The STRIPAK complex may similarly regulate the association of Dynein with its cargoes, and Dynein is known to be differentially phosphorylated (Mische et al., 2008). Thus, removal of *DMob4* from the STRIPAK complex is likely to produce pleiotropic phenotypes as observed in *DMob4* mutants.

Our *in vivo* analysis of *DMob4* has identified a unique role for Phocein in the regulation of microtubule organization and axonal transport. Defects in axonal transport or microtubule organization have been linked to many neurodegenerative diseases, including Huntington’s disease, hereditary spastic paraplegias, amyotrophic lateral sclerosis, and Alzheimer’s disease (Duncan and Goldstein, 2006). It will be interesting to determine whether disruption of *phocein* in mammals leads to neurodegenerative disease. Additional studies of *Drosophila* Mob4 mutants should provide critical insights into how this important protein regulates axonal transport, endocytosis, and microtubule organization *in vivo*.


