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Supplemental Data

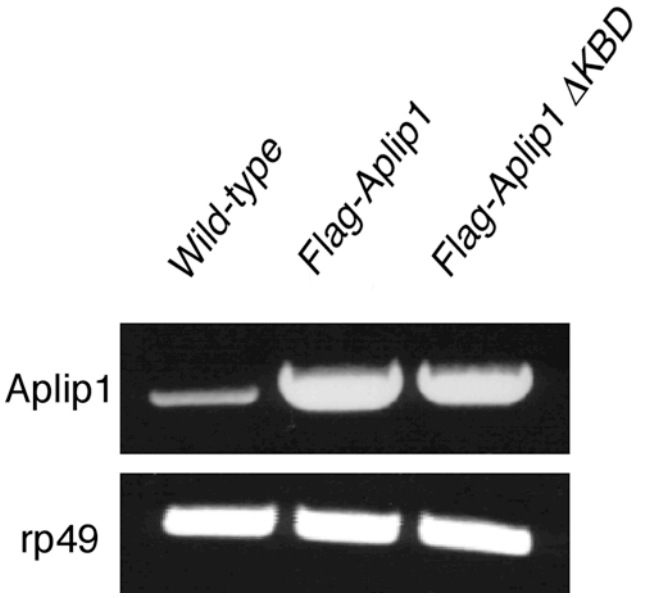

Figure S1. RT-PCR comparison of the expression levels of total *Aplip1* transcripts. Larvae carrying one copy of the *D42-Gal4* driver and one copy of either *UAS-Flag-Aplip1* or *UAS-Flag-Aplip1 ΔKBD* were raised at 25 °C and extracts were subjected to RT-PCR using *Aplip1* specific primers that avoided the KBD deleted region. Primers for ribosomal protein 49 (*rp49*) were added to provide a control band.

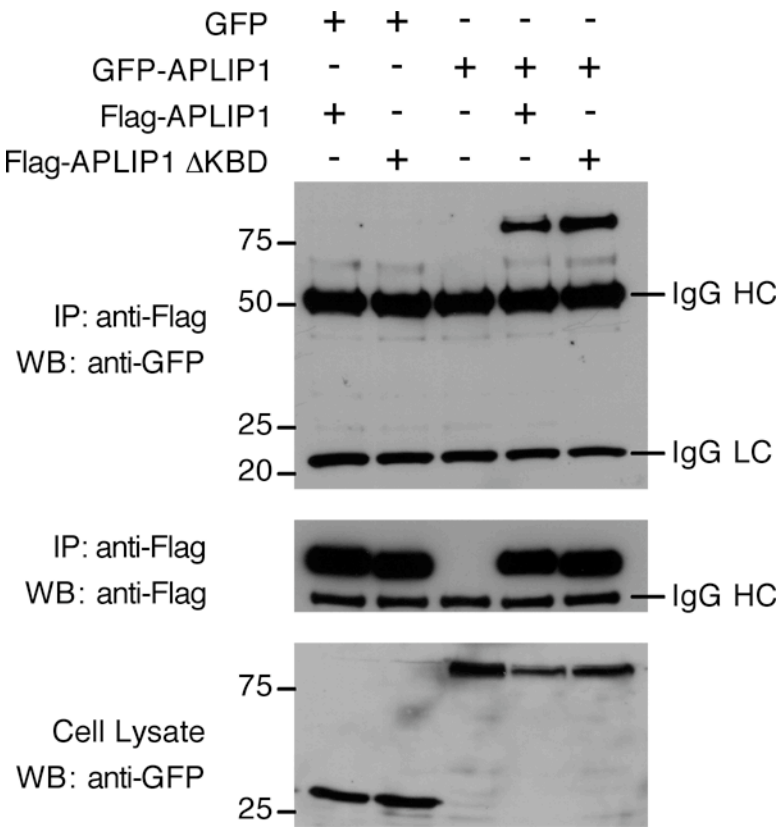

Figure S2.

Expression of GFP-APLIP1 and multimerization tests. S2 cells were transfected with the indicated epitope-tagged constructs. Cell lysates were incubated with anti-Flag antibody to immunoprecipitate Flag-APLIP1. The resulting pellets were then analyzed by western blotting using anti-GFP or anti-Flag. Note that the the GFP-APLIP1 was pulled down by both the full-length APLIP1 and the APLIP1ΔKBD, indicating that the KBD is not needed for APLIP1 multimer formation. The bottom panel demonstrates that the GFP-APLIP1 proteins were consistently present in the starting cell lysates.

Movie Legends

Movie 1. A wild-type larval neuromuscular preparation from a wandering third instar larvae that carried two copies of *UAS-GFP-nSyb* and a motor neuron driver, *D42-Gal4*. Dissection was done in Schneiders culture medium in less than 5 min, then the preparation was mounted with the exposed nerves against a coverslip and suspended over a perfusion well containing culture medium. Time-lapse imaging was done at 1 frame/sec with an Ultraview spinning disk confocal microscope within 15 min of the start of dissection. This 16.3 sec movie represents 196 sec of real time (1:12 time compression). The segmental nerves in this and the other movies are oriented with anterior to the left and posterior to the right. Hence, microtubule polarity in motor axons was primarily with minus-ends toward the left and plus-ends toward the right.

Movie 2. An *ek4/ek4* larval neuromuscular preparation that carried two copies of *UAS-GFP-nSyb* and *D42-Gal4*, prepared as explained in Movie 1. Time-lapse imaging was done at 1 frame/sec with an Ultraview spinning disk confocal microscope within 15 min of the start of dissection. This 16.7 sec movie represents 200 sec of real time (1:12).

Movie 3. A wild-type larval neuromuscular preparation that carried two copies of *UAS-mito-GFP* and *D42-Gal4* prepared as explained in Movie 1. Time-lapse imaging was done at 1 frame/sec with a Bio-rad MRC600 scanning confocal microscope within 15 min of the start of dissection. The confocal laser was used at high power for several seconds to photobleach the middle portion of the field of view, to allow clear recognition of anterograde versus retrograde transport. This 25 sec movie represents 300 sec of real time (1:12).

Movie 4. An *ek4/ek4* larval neuromuscular preparation that carried two copies of *UAS-mito-GFP* and *D42-Gal4* prepared as explained in Movie 1. Time-lapse imaging was done at 1 frame/sec with a Bio-rad MRC600 scanning confocal microscope within 15 min of the start of dissection. Photobleaching was as explained in Movie 3. This 25 sec

movie represents 300 sec of real time (1:12). The movement of the nerves was caused by larval muscle contractions.

Experimental Procedures

Drosophila strains

Fly strains were obtained from the Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu/) except for the following: *Df(3L)ru-22* was obtained from Adelaide Carpenter, *Df(3L)bab-PG*, *Df(3L)Fpa1*, and *Df(3L)Fpa2* were obtained from Frank Laski, *Df(3L)34ex5* was obtained from Joseph Gindhart, and $Klp64D^{K5}$ was obtained from Krishanu Ray. Flies carrying the mitochondrial GFP transgene $P\{w^{+mC}=UAS\text{-}mitoGFP.AP\}$ were generated as described by Pilling et al. [1].

Phenotype analysis

Third instar larvae were scored for posterior paralysis and axonal swellings as described previously [2]. Qualitative scoring of the severity of the axonal swelling phenotype as shown in Table 1 was: -, no swellings; +, some nerves had a few swellings; ++, all nerves had swellings, but parts of nerves were swelling-free; +++, all nerves had swellings; and ++++, all nerves had large swellings.

Larval tissue staining and time-lapse confocal microscopy

Sample preparation for larval tissue staining was described previously [3]. The primary antibodies and dilutions were: rabbit anti-*Drosophila* Synaptotagmin (1:750) [4], and mouse anti-Flag M2 (1:300) (Sigma). The secondary antibodies used were Alexa 488-conjugated goat anti-rabbit, and Alexa 594-conjugated goat anti-mouse (1:1000) (Molecular Probes). Live axonal organelle transport was done on dissected larval neuromuscular preparations by time-lapse fluorescence microscopy [1]. Vesicles were imaged with a Perkin Elmer Ultraview spinning disk confocal microscope. Mitochondria

were imaged with a Bio-Rad MRC 600 scanning confocal microscope. Additional experimental details are presented in the Movie legends of this Supplement.

Ligation of larval segmental nerves

To generate physical blockades of axonal transport in segmental nerves, a fine nylon fiber was tied with an overhand knot to tightly constrict wandering third instar larvae midway between head and tail. To maximize survival, the ligated animals were occasionally supplied with Schneider's insect medium (Sigma). After varying lengths of time, ligated larvae were dissected and fixed with the knot intact. The fiber was then cut, dissection was completed and specimens were immunostained as described above. The whole larva ligation approach, although quite challenging, proved easier and less damaging than direct ligation of exposed nerves in dissected animals. We presume that the benefits derive from a cushioning effect of tissues lying between the segmental nerves and the nylon fiber.

Plasmids and generation of transgenic lines

Genomic DNA containing the full-length *Aplip1* coding region plus 1.6 KB upstream and 3.4 KB downstream was inserted at the PstI site of *pCaSpeR3* [5]. RT-PCR suggested that flies transformed with this construct expressed APLIP1 at ~ 1/7 the rate of the endogenous gene. All other transformation constructs were made either in the *pUAST* [6] or in the *pUASp/EGFP-C1* [7] vectors. Full-length cDNA corresponding to *Aplip1* was obtained from Research Genetics (Clone ID: GH14842). *UAS-Flag-Aplip1* was made by PCR-amplifying the full length *Aplip1* cDNA with the addition of a Flag epitope to the 5' end and subcloning the resulting product into the XhoI-XbaI sites of *pUAST*. The KBD deletion construct was made by replacing threonine 480 with a stop codon. The P483A and P483L constructs were made by introducing appropriate codon changes using standard PCR-based mutagenesis methods. The *UAS-GFP-Aplip1* construct was made by subcloning a PCR-amplified full length *Aplip1* cDNA into the XbaI site of *pUASp/EGFP-C1*. To generate *UAS-Myc-Klc*, a full-length *Klc* cDNA (Clone ID: LD13018) was obtained from the *Drosophila* Genomics Resource Center at Indiana

University, Bloomington (http://dgrc.cgb.indiana.edu/). It was PCR-amplified with the addition of a Myc epitope to the 5' end, and subcloned into the XhoI-XbaI sites of *pUAST*. All finished constructs were sequenced using an ABI3730 and standard methods (Indiana Molecular Biology Institute). Transgenic lines were established using standard germ line transformation techniques [8].

RT-PCR

Total RNA from third instar larvae was prepared with the RNeasy Mini Kit (Qiagen). RT-PCR was performed with the SuperScript First-Strand Synthesis System (Invitrogen). The primers used for *Aplip1* were specific for the *Aplip1 ΔKBD* cDNA. The primers for *rp49* were as described [9].

S2 cell Transfection and immunoprecipitation

The Gal4-UAS system was used to transiently express recombinant proteins in *Drosophila* S2 cells as previously described [10]. Cells were co-transfected with the *UAS* constructs and a copper-inducible *metallothionein-Gal4* (*mt-Gal4*) driver using Effectine transfection reagent (Qiagen). After incubation with the transfection complex for 15 hours, expression was induced with 1 mM $CuSO_4$ for 15 hours. Cells were then harvested and lysed in CHAPS lysis buffer (PBS supplemented with 10 mM CHAPS and COMPLETE protease inhibitor cocktail, Roche) for 1 hr at 4 °C [9]. The lysate was cleared by centrifugation at 14,000 x g for 15 minutes at 4°C, and the resulting supernatant was used. Immunoprecipitations were performed with Protein G PLUS-agarose (Santa Cruz Biotechnology) bound to either mouse anti-Myc 9E10 (Developmental Studies Hybridoma Bank, University of Iowa) or mouse anti-Flag antibodies. The immunoprecipitates were processed for SDS-PAGE, and western blotting was performed with West Pico Chemiluminescent Substrate (Pierce).

Supplemental References

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