Supplemental Data:

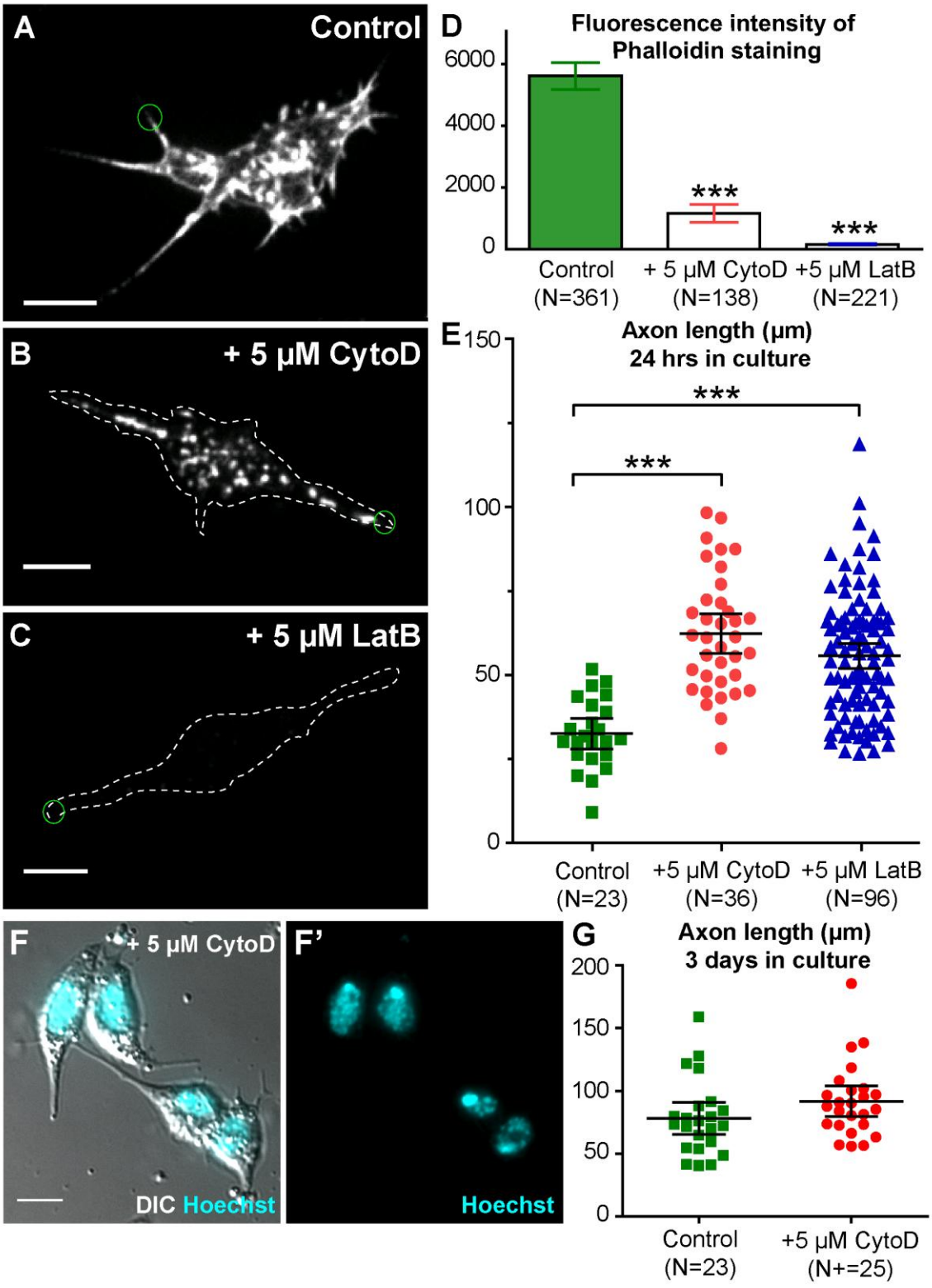

Figure S1. Actin filaments slow down axon growth in *Drosophila* cultured neurons.

(A-C) Phalloidin staining of young neurons: control neurons (A), or neurons treated with F-actin destabilizing drugs, 5 µM CytoD (B), or 5 µM LatB (C).

(D) Fluorescent intensity of Phalloidin staining at the tips of growing processes. The intensity was measured in a circle of 0.22 µm diameter and expressed in arbitrary units and plotted as an average ± 95% CI: Control=5609±440 (N=361; SEM= 224; SD=4260); +5 µM CytoD=1162±296 (N=138; SEM= 150; SD=1762); +5 µM LatB=160±21 (N=221; SEM= 11; SD=161). Unpaired t-test between control and +5 µM CytoD gives $p<0.0001$ (***); unpaired t-test between control and +5 µM LatB gives $p<0.0001$ (***).

(E) Axon length of the neurons after 24 hrs in culture. The longest neurite in each examined neurons is assumed to be the axon. Axon lengths of the three types of neurons are (95% CI for the mean): control=32.7±4.5 µm (N=23; SEM=2.2 µm; SD=10.4 µm); +5 µM CytoD=62.5±5.9 µm (N=36; SEM=2.9 µm SD=17.5 µm); +5 µM LatB =55.9±3.7 µm (N=96; SEM=1.9 µm SD=18.4 µm). Unpaired t-test between control and +5 µM CytoD gives $p<0.0001$ (***); unpaired t-test between control and +5 µM LatB gives $p<0.0001$ (***); unpaired t-test between +5 µM CytoD and +5 µM LatB gives $p=0.0613$ (not significantly different).

(F-F') DIC and Hoechst 33342 staining of 5 µM CytoD-treated neurons 1-2 hrs after plating.

(G) Axon length of the neurons after 3 days in culture. The longest neurite in each examined neurons is assumed to be the axon. Axon lengths of the two types of neurons are (95% CI for the mean): control=78.2±12.9 µm (N=23; SEM=6.2 µm; SD=29.9 µm); +5 µM CytoD=91.84±12.1 µm (N=25; SEM=5.9 µm SD=29.3 µm). Unpaired t-test between control and +5 µM CytoD gives p= 0.1166 (not significantly different).

Scale bars, 5 µm.

Related to Figure 2.

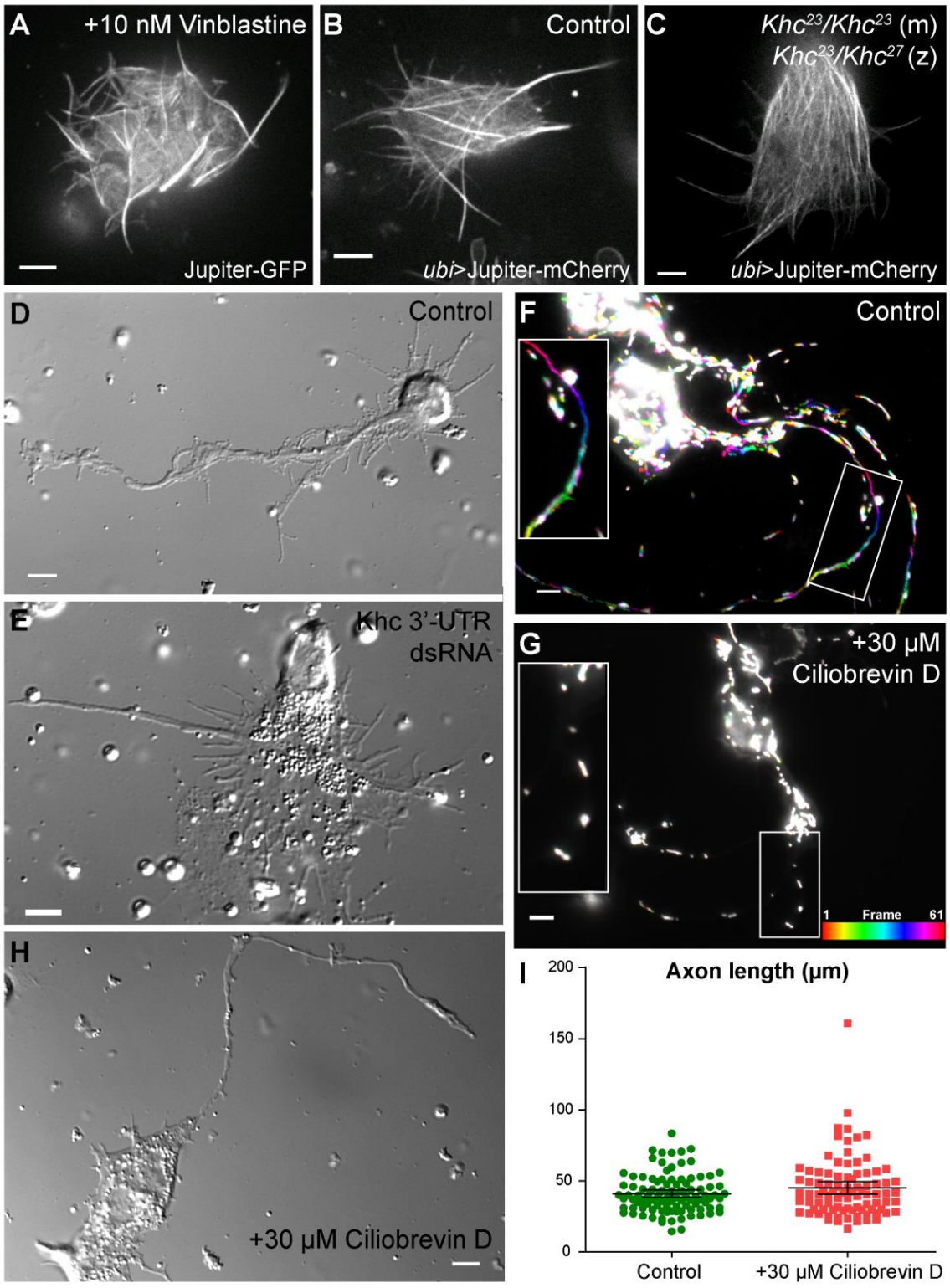

Figure S2. Khc-driven microtubule sliding drives axon growth.

(A) Jupiter-GFP labeled microtubules in in young neurons are preserved after 10 nM Vinblastine treatment.

(B-C) Khc is required for microtubule bundling. Jupiter-mCherry labeled microtubules in a control young neuron (B) and a *Khc* mutant young neuron (maternal Khc^{23}/ Khc^{23} and zygotic Khc^{23}/ Khc^{27}) (C).

(D-E) Difference in morphology between neurons from control and Khc^{27} mutant embryos. DIC images of overnight (>16 hrs) cultures. (D) control embryos; (E) Khc^{27} mutant embryos (maternal Khc^{27}/ +, zygotic Khc^{27}/ Khc^{27}) injected with Khc 3'-UTR dsRNA.

(F-G) Mitochondria movements in control (F) and 30 µM Ciliobrevin D-treated (G) neurons. Mitochondria are labeled by *D42*>Mito-GFP. A merge of 61 frames from 2-min movie in sequence, color-coded according to the bar at lower right. Due to the superposition of multiple colors, stationary mitochondria appear white, whereas moving organelles appear in multicolored paths.

(H) DIC image of a neuron treated with 30 µM Ciliobrevin D overnight (>16 hrs).

(I) Axon length of neurons after >16 hrs in culture. The longest neurite in each examined neurons is assumed to be the axon. Axon lengths of the two types of neurons are (95% CI for the mean): control=41.0±2.6 µm (N=101; SEM=1.3 µm; SD=13.3 µm); +30 µM Ciliobrevin D=45.1±4.5 µm (N=88; SEM=2.3 µm SD=21.3 µm). Unpaired t-test between control and +30 µM Ciliobrevin D gives p= 0.1115 (not significantly different).

Scale bars, 5 µm.

Related to Figures 2 and 4.

Movie S1. Mitochondria movement in a mature *Drosophila* neuron.

Time-lapse movie of the example shown in Figure 1E. A mature neuron (>16 hrs after plating) from embryos expressing *D42*>Mito-GFP and *ubi*-Jupiter-mCherry. Scale bar, 5 µm.

Movie S2. Microtubule sliding in young neurons.

Three examples of tdEOS-αtub-labeled microtubule sliding in young neurons (<3 hrs after plating). A green channel image before photoconversion preceeds each time-lapse sequence to show the overall cell shape and the photoconversion zone. Photoconversion was performed in the cell body in the first two examples (related to Figure 3A-D and 3E-H) and in the growing neurites close to the cell body in the third example. Young neurons were from embryos expressing *mat αtub+D42*>tdEOS-αtub. Scale bars, 5 µm.

Movie S3. Inhibition of tubulin polymerization does not stop microtubule movement.

Microtubule sliding in young neurons (<3 hrs after plating) treated either with 10 nM Vinblastine (1st example) or 20 nM Taxol (2nd example). Embryos expressing *mat αtub+D42*>tdEOS-αtub were used for neuronal cultures. Scale bars, 5 µm.

Movie S4. Downregulation of microtubule sliding in a mature neuron.

Neurons from embryos expressing *mat αtub+D42>*tdEOS-αtub were imaged >16 hrs after plating (related to Figure 3I-L). Scale bar, 5 µm.

Movie S5. Sliding microtubules push again the neurite tips.

Young neurons (<3 hrs after plating) from embryos expressing GFP-tagged Jupiter that labels all microtubules (sequences #1-#5). Cell membrane in sequences #2-#5 was stained by DeepRed dye. Sequences #2 and #3 correspond to Figure 3N-N'. 10 nM Vinblastine (sequences #4-#5) does not stop microtubule sliding. Scale bars, 5 µm.

Movie S6. KHC drives microtubule sliding.

Motility of microtubule labeled by mCherry-tagged Jupiter in young neurons (<3 hrs after plating). First part, control neuron (corresponds to Figure 4A); second part*, Khc* mutant (maternal Khc^{23}/Khc^{23} and zygotic Khc^{23}/Khc^{27}) (corresponds to Figure 4B); third part, neuron treated with 30 µM Ciliobrevin D (cytoplasmic dynein inhibitor). Scale bars, 5µm.

Supplemental Experimental Procedures

Fly stocks and genetics

Fly stocks are maintained on standard cornmeal food based on Bloomington recipe (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm) at room temperature. Most of the fly stocks were obtained from Bloomington stock center except for the ones listed below: Khc null allele *Khc*^{27}, Khc hypomorphic allele *Khc*^{23} and the Mitochondria marker *UAS-Mito-GFP, D42-Gal4* (gifts from Dr. W. Saxton); *UAS-Tau-GFP* and *UAS-mCD8-GFP* (gifts from Dr. M. Rolls); *w;elav-Gal4; elav-Gal4* (a gift from Dr. C. Doe); *yw, hsflp*; *ovo*^{D1}*/CyO* and *ubi-Jupiter-mCherry* (gifts from Dr. I. Palacios); UAS-EB1-GFP (a gift from Dr. S. Roger); Jupiter-GFP (ZCL2183) (Yale GFP Protein Trap Database); UASp-tdEOS-atub (made in this study: the photoconvertible tdEOS was introduced into the pUASp vector between SpeI and NotI, N-terminal of *Drosophila* α-tubulin 84B inserted between NotI and XbaI. The construct of pUASp-tdEOS-αtub was then sent to BestGene for generating transgenic flies). In order to express tdEOS-α-tub in young and mature neurons, females of *yw; UASp-tdEOS-atub; mat atub-Gal4* were crossed with males of *w; D42-Gal4*. To screen live zygotic *Khc*^{27}*/Khc*^{27} embryo, females of *w; Khc*^{27}*/CyO* were crossed with males of *w; wg*$^{[Gal-1]}$*/CyO, twist-Gal4, UAS-2XEGFP* (Bloomington stock #6662) to make the stock of *w; Khc*^{27}*/CyO, twist-Gal4, UAS-2XEGFP*. To make *Khc*^{23} germline clones with labeled microtubules, females of *w; FRT G13 Khc*^{23}*/CyO; ubi-Jupiter-mCherry* were crossed with males of *yw, hsflp; FRT G13ovo*^{D1}*/CyO*. At day 7 and 8 of development, pupae from the cross were subjected to 2 hour 37°C heat shock in

water bath. Virgins of *yw, hsflp/w; FRT G13 Khc^{23}/FRTG13 Khc^{23}; ubi-Jupiter-mCherry/+* were crossed with males of *w; Khc^{27}/CyO, twist-Gal4, UAS-2XEGFP* for collecting embryos with maternal *Khc^{23}/Khc^{23}* and zygotic *Khc^{23}/Khc^{27}*.

Drosophila **neuron culture**

The neuron preparation protocol was adapted from Mahowald lab and Lieu lab. Flies were kept in apple juice vials with fresh yeast paste for a couple days. Embryos were collected for 2 h after 1 h pre-collection, and developed at room temperature for another 4 h. Embryos were then washed in *Drosophila* embryo wash (0.4% NaCl, 0.03% TritonX-1000) and dechorionated in a fresh 1:1 solution of 95% EtOH and commercial bleach for 10min. Dechorionated embryos were washed three times in supplemented Schneider's medium (Schneider's medium supplemented with 20% Fetal bovine serum, 5 µg/ml insulin, 100 µg/ml penicillin, 100 µg/ml streptomycin and 10 µg/ml tetracycline), and disassociated gently using disposable pellet pestle grinders (Kimble chase 749520-0000 from Fisher). The disassociated mixture was centrifuged at 2500 rpm for 2 min. The pellet was resuspended in 500 µl supplemented Schneider's medium and spun down at 2500 rpm for 2 min. The final pellet was resuspended in ~100 µl supplemented Schneider's medium, and plated on Concanavalin A (ConA)-coated 25mm coverslip in 35 mm petri dish for 10 min to allow enough attachment, before the neurons on the coverslip was submerged in 2 ml supplemented Schneider's medium. The neuron culture was either imaged right away (<3 hrs after plating,

young neurons) or incubated in a humidified 25°C incubator overnight before imaging (>16 hrs after plating, mature neurons).

Embryo injection

In order to knock down maternally-loaded *Khc* mRNA in early embryos, embryos from the stock *w*; *Khc*^{27}/*CyO, twist-Gal4, UAS-2XEGFP* were collected and injected with dsRNA against Khc 3'-UTR (3.5 µg/µl) at three sites (anterior, middle, posterior) at blastoderm stage before cellularization (<30 min AEL on apple juice-agar plates) submerged in halocarbon oil 700 (Sigma H8898) at room temperature. In the experiment of rescuing Khc knockdown phenotype, Khc cDNA covering the protein coding region (pAC-KHC full length/aa 1-975, 1.5µg/µl) was co-injected with Khc 3'-UTR dsRNA (3.5 µg/µl). The injected embryos were left in the halocarbon oil in a moisture chamber at room temperature to develop for another 4-5 h before neuron preparation.

Immunochemistry

Cultured neurons were washed 3 times in PBS, and then fixed in 0.5% Glutaraldehyde for 10 min at room temperature. Samples were washed 3 times in PBS, and then quenched in fresh 1 mg/ml NaBH4 (10 min X2). Samples were washed and blocked in wash solution (0.1% TritonX-100, 1% BSA in TBS) 10 min for three times; then incubated in primary antibody for 45 min-1 h at room temperature; washed and blocked in wash solution 10 min for three times; incubated in secondary antibody (and TRITC-conjugated Phalloidin 1:1000 if

needed) for 45 min-1 h in dark at room temperature; washed and blocked in wash solution 10 min for three times; mounted in mounting media overnight in dark at room temperature overnight before imaging. Primary antibodies used in this study include: mouse anti-α-tubulin (DM1α, 1:1000), mouse anti-Futsch/22C10 (1:100, DSHB), rabbit anti-GFP (1:500, Abcam 6556), and rat anti-Elav (1:100, DSHB); secondary antibodies from Jackson ImmunoResearch (used at 1:200) include goat anti-mouse TRITC, goat anti-rabbit FITC, and donkey anti-Rat AMCA.

Microscopy

Images for immunolabeling and growth rate quantification were acquired using a Nikon U-2000 inverted microscope equipped with a Perfect Focus system (Nikon) and Coolsnap CCD (Roger Scientific), controlled by MetaMorph software (Version 7.7.5.0, Molecular Devices). To image microtubule sliding and motility, we used a Nikon Eclipse U2000 inverted stand with a Yokogawa CSU10 spinning disc confocal head and a 100 X 1.45 NA lens. Images were acquired using Evolve EMCCD (Photometrics) driven by Nikon Elements software. Photoconversion of tdEos-tagged microtubules was performed using illumination from a 100 W mercury bulb in the epifluorescence pathway filtered with a 400 nm filter and confined by a 50 μm slit in the field diaphragm position (projected as a ~3 μm bar on the sample). Images were analyzed in Fiji, and assembled in Illustrator.

Quantification of microtubule sliding

To measure microtubule sliding, we first used the IsoData Autothreshold Plug-In of Fiji to convert after-photoconversion red channel images to a binary format. After bleaching correction, we measured the fluorescence intensity outside the photoconverted area and the total fluorescence intensity in the cell 10 min after photoconversion. The ratio of fluorescence outside the photoconverted area to the total fluorescence intensity is defined as “Fluorescence outside the photoconversion zone” in Figure 3M (the column graph was generated in GraphPad Prism). Each data point in panel 3M represents an individual neuron.