Supplemental Material

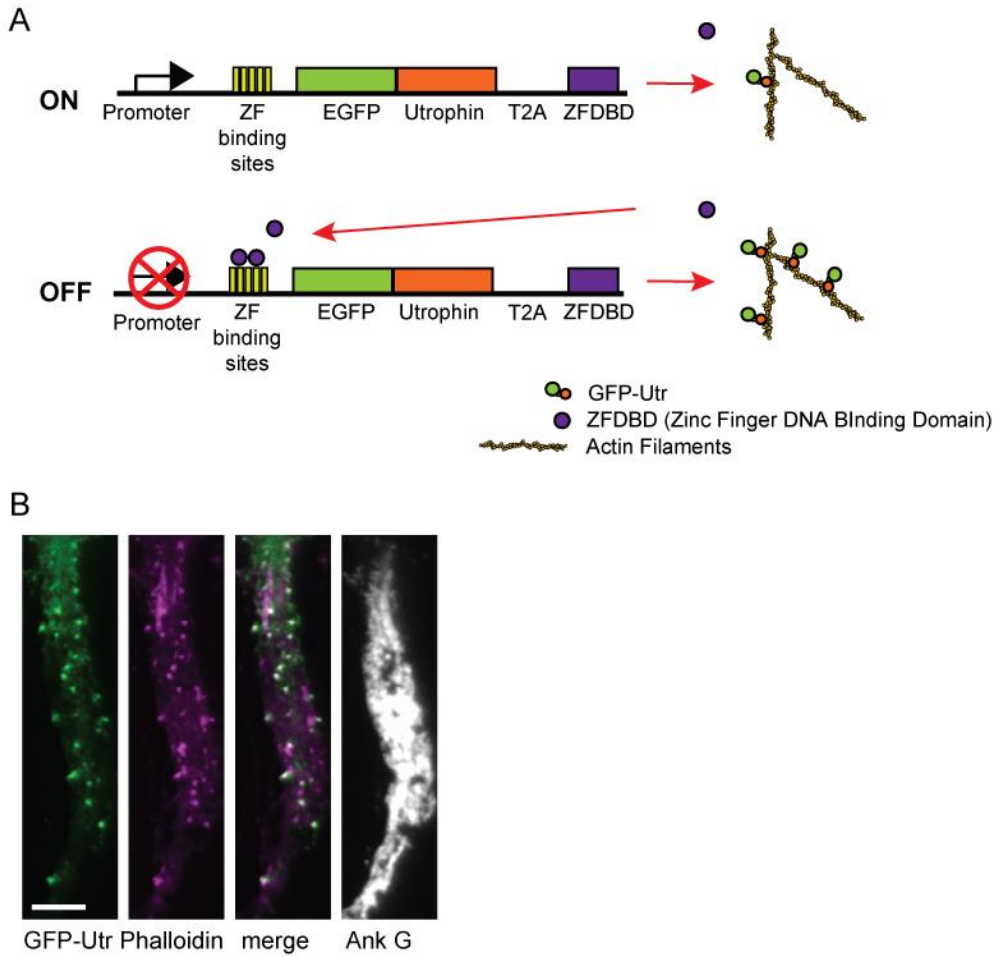

Figure S1 Regulated expression of Utrophin for labeling actin, related to Fig. 1. (**A**) Expression of Utrophin fused to GFP (GFP-Utr) and the CCR5 Zinc Finger DNA binding domain (ZFDBD) are driven by the CAG promoter. Just downstream of the transcriptional start site there are five DNA binding sites recognized by the CCR5 Zinc Finger (ZF binding site). Binding of the ZFDBD to the ZF binding site sterically hinders transcription of GFP-Utr limiting its expression. (**B**) Expression pattern of GFP-Utrophin in the proximal axon of a cortical neuron in culture (green) is highly similar to that of Phalloidin (purple), consistent with GFP-Utr faithfully labeling actin filaments. Ankyring G staining confirms that the process is an axon. Scale bar 5 μm.

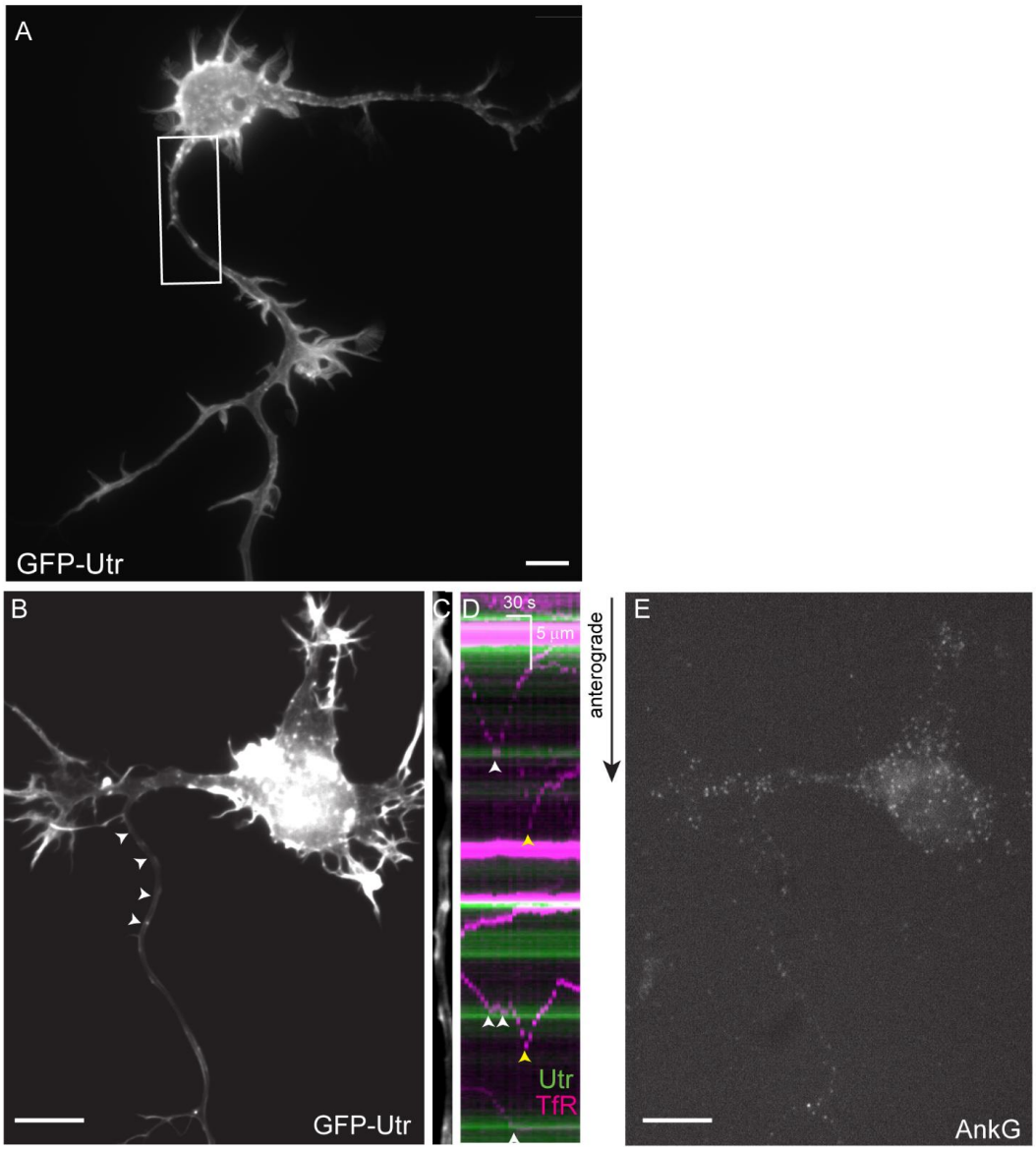

Figure S2 Vesicles carrying a dendritic protein tend to halt and reverse at sites near actin patches, related to Fig. 1. (**A**) Low magnification image of neuron whose proximal axon is shown in Fig. 1(E) (**B**) 3 DIV cortical neuron co-expressing GFP-Utr and TfR-mCherry (not shown). (**C**) Straightened axon from neuron in (B). (**D**) Kymograph of neuron in (B, C) showing GFP-Utr (green) and TfR-mCherry (purple). White arrowheads indicate places where TfR containing vesicles halted near actin patches, yellow arrowheads indicate places where halting took place away from patches. (**E**) Ankyrin G staining of neuron in (B-D) showing a lack of Ankyrin G expression. Scale bar 10 µm.

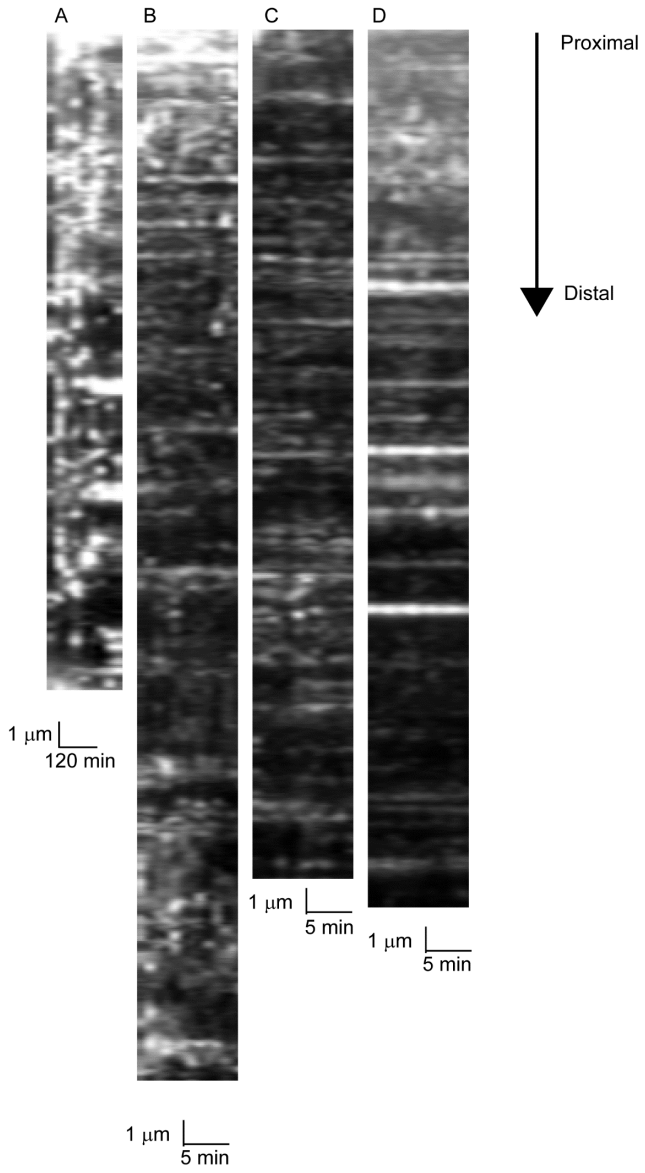

Figure S3 Kymographs of actin patches, related to Fig. 2. Kymograph of proximal axon of neurons expressing GFP-Utr. (**A**) Images taken every 30 minutes. (**B**-**D**) Images taken every 1 minute.

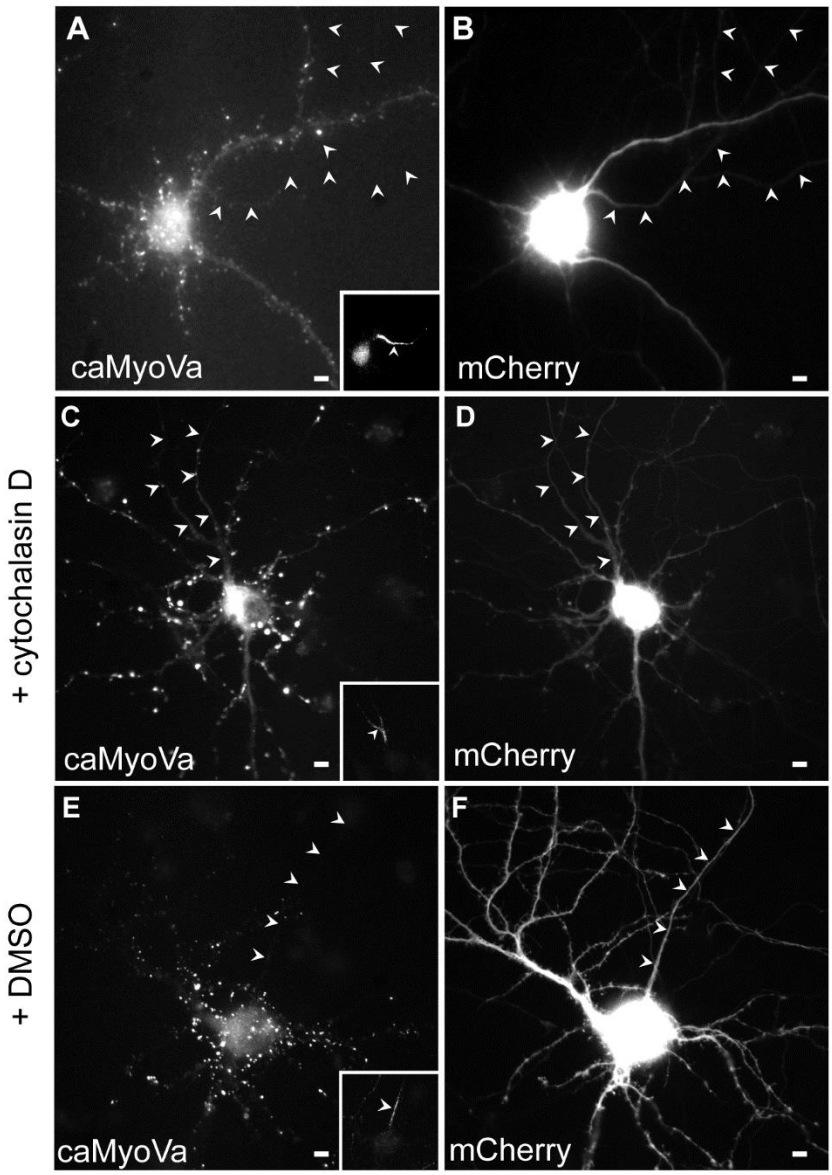

Figure S4: caMyoVa localization in the presence and absence of Cytochalasin D, related to Fig. 3. (**A**) Cortical neuron expressing caMyoVa, which is present mainly in the somatodendritic compartment in a diffuse manner (n = 10 neurons, 3 cultures). Arrowheads indicate axon. Inset shows Ankyrin G staining. (**B**) Cortical neuron in (A) expressing mCherry. (**C**) Cortical neuron in culture expresses caMyoVa in the axon in the presence of Cytochalasin D. (n = 11 neurons, 3 cultures). (**D**) mCherry expressed in the same neuron as in (C). (**E**) caMyoVa is absent from the axon in the presence of DMSO. Inset shows Ankyrin G staining. Arrowheads indicate axon (n = 9 neurons, 3 cultures). (**F**) mCherry expressed in the same neuron as in (E). Inset shows Ankyrin G staining. Arrowheads indicate axon Scale bar 5 μm.

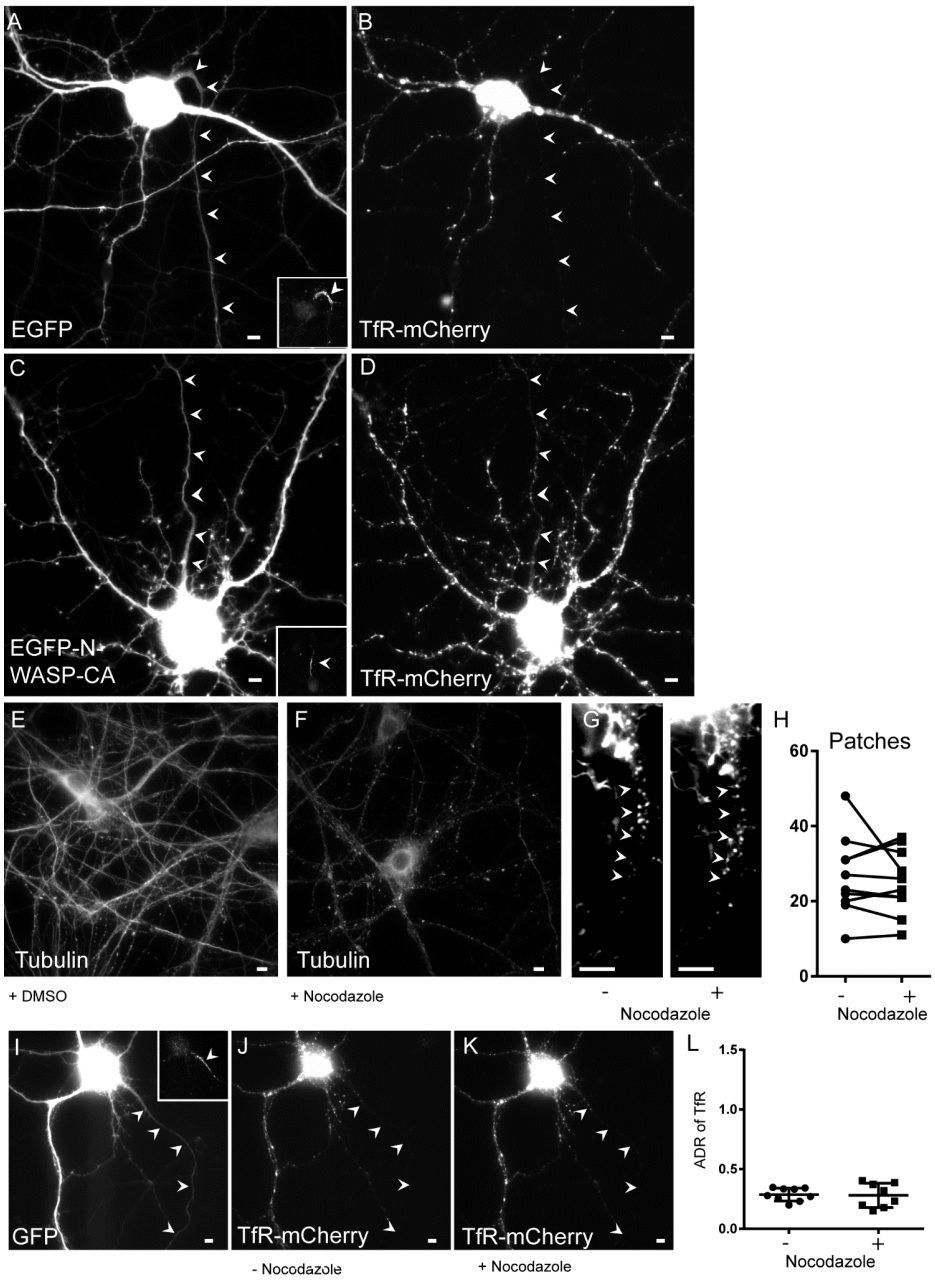

Figure S5: Effect of N-WASP-CA and Nocodazole on the number of actin patches and ADR of TfR, related to Figure 5. (**A**) Cortical neuron expressing EGFP and (**B**) TfR-mCherry, which is confined to the somatodendritic compartment. (**C**) Cortical neuron expressing EGFP-N-WASP-CA and (**D**) TfR-mCherry, which is present in the axon as well as in the somatodendritic compartment. (**E**) Image of a neuron in dissociated culture stained for tubulin after exposure to DMSO for 30 minutes shows intact microtubules. (**F**) Similar neuron to (E) exposed Nocodazole for 30 minutes shows disruption of microtubules. (**G, H**) The number of patches labeled with GFP-Utr in the proximal axon of a neuron in dissociated culture in the absence and presence of Nocodazole (n = 9 neurons, 3 cultures). (**I**) Cortical neuron expressing GFP and (**J**) TfR-mCherry prior to addition of Nocodazole. (**K**) Same neuron as in (J) following 30 minutes of exposure to Nocodazole. (**L**) ADR of TfR is not statistically different in the presence (n = 10 neurons, 2 cultures) vs. absence (n = 9 neurons, 2 cultures) of Nocodazole. Inset shows Ankyrin G staining. Arrowheads point to axon. Scale bar 5 μm.

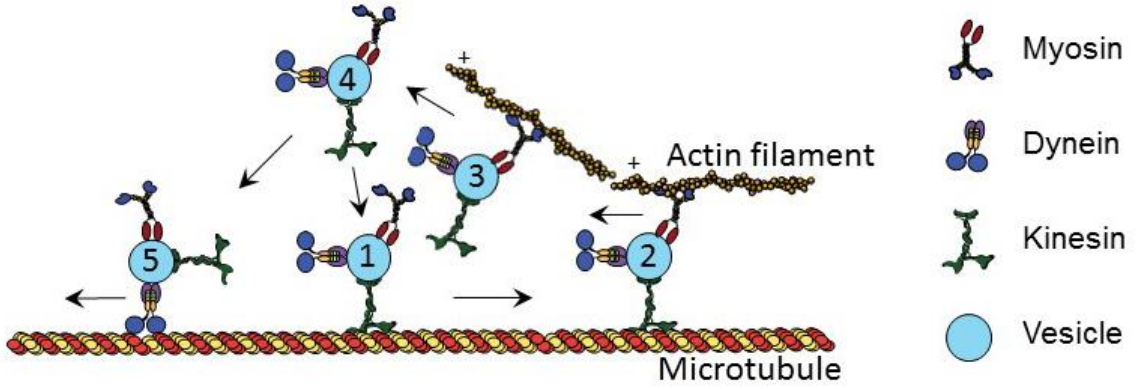

Figure S6 Schematic of proposed model for myosin/kinesin/dynein dynamics in the proximal axon, related to Fig. 1. 1. Vesicle (blue) associated with myosin and dynein is transported via kinesin anterogradely down a microtubule in the proximal axon. 2. Vesicle interacts with actin via a plus end-directed myosin causing it to halt and then reverse direction. 3. Interaction of myosin with actin leads to separation of kinesin from the microtubule. 4. Myosin moves off the end of actin causing the vesicle to float freely in the cytoplasm. Subsequently, the vesicle can interact with microtubules in one of two ways: i. It can interact via kinesin causing it to repeat steps 1-4. ii. It can interact via dynein causing it to move retrogradely on the microtubule back to the cell body (5).

Supplemental Experimental Procedures

Plasmid DNA constructs

GFP-Utr consists of the CH domain of rat Utrophin (Utr), comprising amino acids 1-261, fused downstream of EGFP with expression driven by a transcriptional regulation system developed for this application. This system consists of a CCR5 DNA binding site (Mani et al., 2005) inserted downstream of the CAG promoter adjacent to the TATA box (Figure S1) and a CCR5 zinc finger binding domain inserted after a T2A ribosome skipping sequence downstream of Utr. TfR-mCherry and TfR-GFP, which have been described previously (Al-Bassam et al., 2012; Watanabe et al., 2012) were cloned into similar vectors that containing a CMV promoter substituted for the CAG promoter. CaMyoVa-GFP/mCherry consists of amino acids 1-1096 of rat Myosin Va fused downstream of GFP/mCherry with expression driven by a CMV promoter. caMyoVI-GFP/mCherry consists of amino acids 1-993 of rat Myosin VI in a similar vector. To visualize actin in zebrafish GFP-Utr was placed into the modified expression vector pMT containing a triplicated enhancer element from the *mnx1* gene that drives expression of GFP-Utr in motoneurons (Zelenchuk and Bruses, 2011). CaMyoVa-mCherry and caMyoVI-mCherry were inserted into similar vectors to enable expression in zebrafish. These sequences in pMT are all flanked by Tol2 transposable elements (Kawakami and Shima, 1999). N-WASP-CA consists of amino acids 460-501 of the Rattus norvegicus Wiskott-Aldrich syndrome-like (Wasl).

Antibodies

The following primary antibodies at the following dilutions were used: chicken GFP (Millipore, Bellerica, MA, USA), 1:15000; mouse AnkG (Neuromab, Davis, CA, USA), 1:500; mouse HA (Covance, Princeton, NJ, USA), 1:500; rabbit AnkG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:1000; rabbit TagRFP (Evrogen, Moscow, Russia), 1:10000; mouse P16-ARC (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:200; mouse Tubulin, 1:500 (Sigma, St. Louis, MO, USA); rabbit FMN2 (Abcam, Cambridge, MA, USA), 1:2000; and Phalloidin-Alexa 594 (Thermo Fisher Scientific, Waltham, MA USA), 1:500. Secondary antibodies were conjugated to Alexa 488, 594, and 647 fluorophores (Thermo Fisher, Waltham, MA, USA).

Preparation of Cortical Neurons

Cortices from E17 Sprague-Dawley rat embryos of both sexes were dissected in 0.1 mM HEPES (Thermo Fisher, Waltham, MA, USA) supplemented with Hank's balanced salt solution (HBSS, Thermo Fisher, Waltham, MA, USA), HEPES-HBSS. The dissected cortices were trypsinized in 0.25% trypsin, 0.1 mM HEPES-HBSS for 15 min at 37˚C; the tissue was then washed 3 times in fresh HEPES-HBSS. Afterwards, the cortices were triturated to dissociate the cells. The neurons were then plated onto poly-D-lysine and laminin pre-treated 22 x 22 mm coverslips (Thermo Fisher, Waltham, MA, USA) in 6 well plates (VWR Radnor, PA, USA) at a density of $5.0x10^4$, or in 0.17 mm Delta T culture dishes (Bioptechs, Butler, PA, USA) at a density of $3.5x10^4$, in supplemented Neurobasal medium (NBM, Thermo Fisher, Waltham, MA, USA). The NBM was supplemented with 10 mL/L Glutamax, 1 mg/L gentamicin solution, 20 mL/L B-27 supplement (Thermo Fisher, Waltham, MA, USA), and 50 mL/L fetal bovine serum (Biowest, Nuaillé, France). Four hours after the neurons were plated, the medium was diluted 1:3 with serum-free supplemented NBM. After 7 DIV, the medium in the wells was diluted 1:2 with fresh supplemented NBM.
Experimental protocols were conducted according to the US National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the University of Southern California.

Calculation of patch lifetime

In order to quantitate the lifetime of actin patches a series of images were taken 1 minute apart of cortical neurons in culture expressing GFP-Utr. An image at each time point was merged with the original image so that patches that appeared in both images could be counted. The number of patches that persisted from the first image was counted for each time point and entered into GraphPad. A double exponential function calculated by GraphPad proved to be an excellent fit ($R^2 = .9979$). This calculation also yielded time constants with errors corresponding to longer- and shorter-lived patches.

Preparation and imaging of zebrafish neurons

For *in vivo* experiments in zebrafish (Danio rerio), wild-type AB, AB/TL, and Casper were used (White et al., 2008). Zebrafish were raised and nucleic acid injections were performed as described previously (Detrich et al., 2009). At the zygote stage, zebrafish were co-injected with approximately 50 – 80 pg of zebrafish plasmid DNA containing the transgene and 40 – 70 pg of *Tol2* transposase mRNA. The injected eggs were kept in egg water

overnight at 28ºC, and then screened for expression of the transgene. Only healthy-looking embryos with brightly expressed transgenes were selected using an Olympus MVX10 fluorescence microscope. At 1.5-3 dpf selected embryos were anesthetized using 0.015%–0.03% Tricaine methanesulfonate (Finquel/MS-222, Argent Labs) and were maintained in anesthetic when doing imaging. Anesthesized embryos were embedded in 1.5% SeaPlaque agarose (Lonza Inc; Allendale NJ, USA) in 30% Danieau's solution and positioned at the glass bottom (#1.5 coverslip thickness) of Lab-Tek 2-well imaging chambers (Nalge Nunc International, Penfield, NY, USA). Gender could not be determined due to the age of the fish (Uchida et al., 2002). We obtained 3D live confocal images of embryos using both a 20x/0.8NA Plan-Apochromat air objective (Carl Zeiss, Oberkochen, Germany) for low resolution images of whole fish and a 63x/1.15NA LD C-Apochromat water objective (Carl Zeiss, Oberkochen, Germany) for high resolution images of the area containing cell bodies and axon initial segments, using a Zeiss LSM 780 inverted confocal microscope. 3D reconstruction, tiling and postprocessing of images was done using ZEN (Carl Zeiss, Oberkochen, Germany), Fiji (ImageJ) and Imaris (Bitplane AG, Zurich, Switzerland) software. Experimental protocols were conducted according to the US National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the University of Southern California.

Transfection
All neuronal cultures were transfected using Ca^{2+} phosphate (Clontech) following the manufacturer's suggested protocol at 12-16 DIV.

Chemical Inhibitors
After taking images of GFP-Utr and Tfr-mCherry in imaging medium, the ARP2/3 inhibitor CK-869 (Sigma-Aldrich, St Louis, MO) at 25 μM in 0.05% DMSO, or 0.05% DMSO was added to the culture and incubated for 30 min at 37ºC with 5% CO2. Images of GFP-Utr and Tfr-mcherry of the same neurons were then obtained. Neurons were then fixed and subjected to immunocytochemistry to stain the proximal axon with Ankyrin G. Experiments to examine the effect of Nocodazole (15 μM, Sigma-Aldrich, St Louis, MO) used the same protocol. Cells expressing caMyoVa were exposed to either cytochalasin D (1 μM, Sigma-Aldrich, St Louis, MO) or DMSO immediately after transfection and incubated for 16 hours prior to imaging. Experiments examining the effects of the Formin 2 inhibitor SMIFH2 (Sigma-Aldrich Corporation, St. Louis, MO, USA) were conducted as above using SMIFH2 at 25 μM in 0.05% DMSO.

Preparation of 3 day-old neurons
In order to visualize proteins in young neurons, rat cortical neurons were prepared as described above, and then 500K neurons/reaction were pelleted to exchange the dissociation buffer with 200 μl of 1x PBS containing 0.5 μg of total plasmid DNA. The electroporation was done using the 4D-Nucleofector (Lonza, Basel, Switzerland) with the high viability setting. After application of the electrical shock the electroporated neurons were transferred into a tissue culture incubator for 10-15 min recovery before plating into the wells (at 200K-250K cells/well) containing the attachment medium (growth medium + 5% FBS). The medium was diluted 1:3 with serum-free supplemented NBM. Imaging took place at 72 hours after electroporation.

Immunostaining
Neurons were fixed in 4% paraformaldehyde in 1X PBS for 5 min and washed three times with 1X PBS for 5 min. The cells were then permeabilized and incubated in a blocking buffer (1% Bovine Serum Albumin, 5% Normal Goat Serum, 0.1% Triton X-100 in 1X PBS) for 30 minutes. After incubation with primary antibodies and the Alexa fluorophore-conjugated secondary antibodies (1:1000 dilution, Invitrogen, Carlsbad, CA, USA) for 30 min, the neurons were mounted on glass slides using Fluoromount G (Electron Microscopy Sciences, Hatfield, PA, USA).

Kymographs
All kymographs were generated using ImageJ. For kymographs of TfR vesicle movement (Figs. 1, S2), a vesicle was considered to have halted if it moved less than 1 μm for 6 seconds or more. Halting and reversing vesicles were identified by blinded observers. Images in the movies were taken at intervals of 1.5 s. Anterograde direction is facing downwards.

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