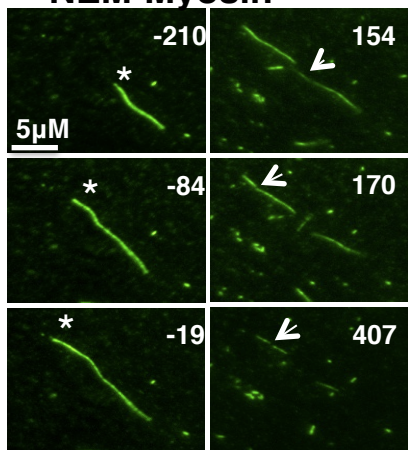
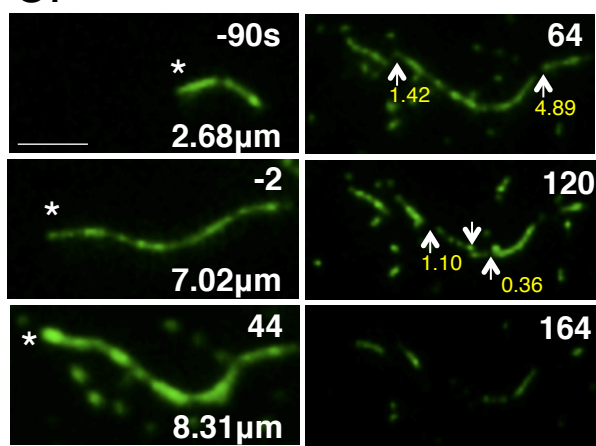


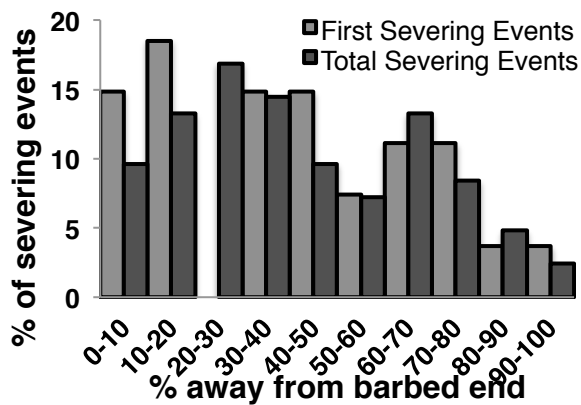
### A. NEM-Myosin



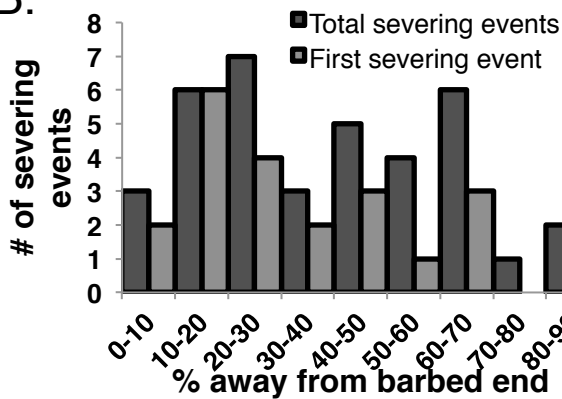
### C. mPEG-silane



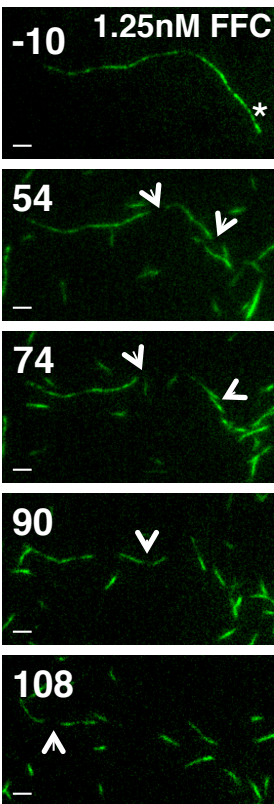
### D.



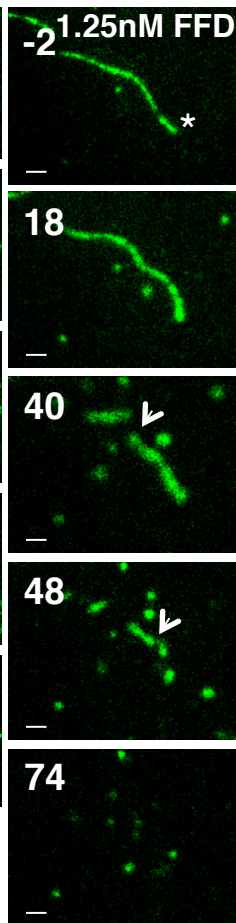
### B.



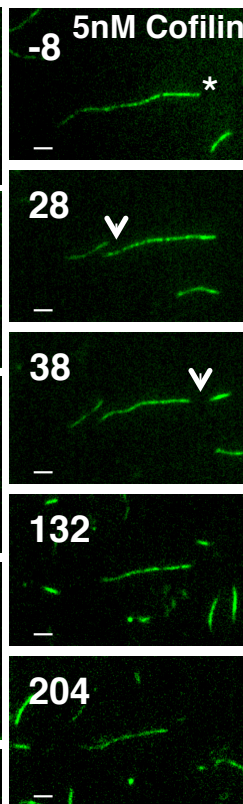
### E.



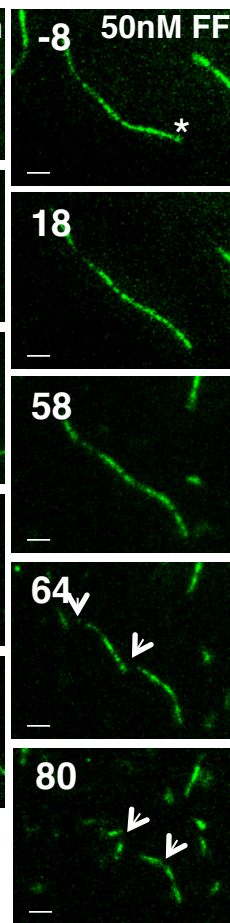
### F.



### G.



### H.



### I.

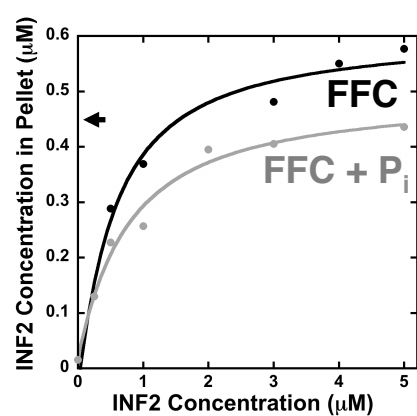


FIGURE S1.

## Figure S1. INF2 severing and binding characteristics (related to Figure 1)

- A. TIRF microscopy montage of INF2-FFC (10nM) with A488-labeled actin (20% A488-actin, starting 1  $\mu$ M total actin) on NEM-myosin treated slides. Negative times represent filament elongating in absence of INF2, asterisk indicates barbed end, and arrows indicate severing events. . Actin monomers removed upon INF2 addition at time 0. Scale bar 5 $\mu$ m.
- B. Histogram of severing event positions quantified from timecourses similar to A. Total of 22 filaments (59 severing events) were quantified with an average filament length of 15.6 $\mu$ m, range 9.9 $\mu$ m- 22.4 $\mu$ m at time = 0. Barbed end fragments were measured as a percentage of total filament length.
- C. TIRF microscopy montage of INF2-FFC (10nM) severing actin (20% A488-actin, 1  $\mu$ M starting actin concentration) on mPEG-silane treated slides. Negative times represent filament elongating in absence of INF2. Actin monomers removed upon INF2 addition at time 0. Asterisk indicates barbed end, arrows indicate severing events, severing position noted ( $\mu$ m from barbed end). Scale bar 2 $\mu$ m.
- D. Histogram of severing event positions quantified from timecourses similar to C. Total of 17 filaments (84 severing events), were quantified with an average filament length of 7.6  $\mu$ m, range 4.2 $\mu$ m- 14.8 $\mu$ m at time = 0. Barbed end fragments were measured as a percentage of total filament length.
- E. TIRF microscopy montages of INF2-FFC (1.25nM) with A488-labeled actin (20% A488-actin, starting 1  $\mu$ M total actin) on DDS/F127 treated slides. Representative examples from quantification of severing rate in figure 1G. Negative times represent filament elongating in absence of INF2, asterisk indicates barbed end, and arrows indicate severing events. Actin monomers were removed upon INF2 addition at time 0. Scale bar 2 $\mu$ m.
- F. As in (A) but with INF2-FFD (1.25nM)
- G. As in (A) but with cofilin (5nM)
- H. As in (A) but with INF2-FF (50nM)
- I. Cosedimentation assay using phalloidin-stabilized filaments (0.5  $\mu$ M) incubated 5 min with indicated concentration of INF2-FFC in the absence or presence of 10mM phosphate. Arrow indicates concentration of actin in the pellet (0.45  $\mu$ M).

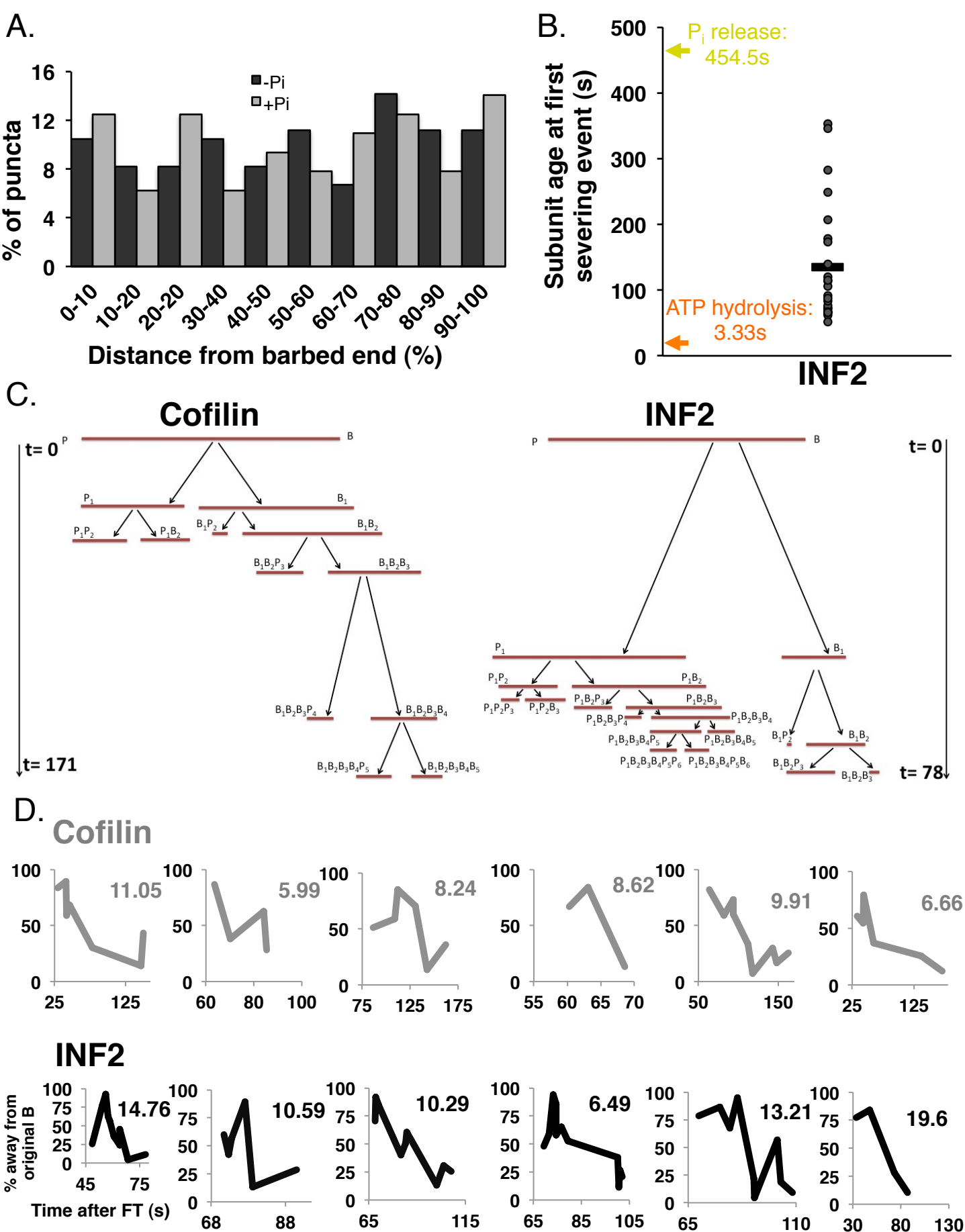


FIGURE S2.

**Figure S2. Quantification and comparison of INF2-FFC and cofilin-mediated severing (related to Figure 2)**

- A. Histogram of initial GFP-INF2-FFC puncta binding locations along filament +/- phosphate.  $n = 64$  and  $134$  puncta ( $7$  and  $16$  filaments) in the absence or presence of phosphate, respectively. Puncta were analyzed prior to severing and/or saturation, for  $21.1-45.3$ s or  $15.4-28.3$ s in the absence or presence of phosphate, respectively. Average filament length  $12.6\mu\text{m}$  and  $11.9\mu\text{m}$  (range  $5.8-24.2\mu\text{m}$  and  $6.3-19.6\mu\text{m}$ ) in the absence and presence of phosphate respectively.
- B. Quantification of subunit age at first severing event. Subunit ages were determined from filament elongation rate and location along filament where subunit was located at time of severing event ( $n=25$  filaments). For comparison, subunits that should have undergone ATP hydrolysis and phosphate release are denoted in orange and yellow (respectively). These values are based on expected rates from Blanchoin and Pollard 2002 Biochemistry, Carlier et al. 1998 FEBS letters.
- C. Additional examples of tree maps diagramming severing events with time (sec) on the y-axis, from Figure 2E.  $t=0$  denotes time of cofilin or INF2-FFC addition, B and P denote barbed and pointed ends respectively. Severed filament fragments are named based on severing history.  $6.7$  (cofilin) and  $14.8$  mm (INF2) filament represented, time and sizes drawn to scale.
- D. Individual filaments monitored for severing position as a function of time after addition of INF2 or cofilin (six filaments for each). Y-axis values represent distance away from original filament barbed end, where total filament length = 100%. Starting filament lengths given in  $\mu\text{m}$  on each graph.

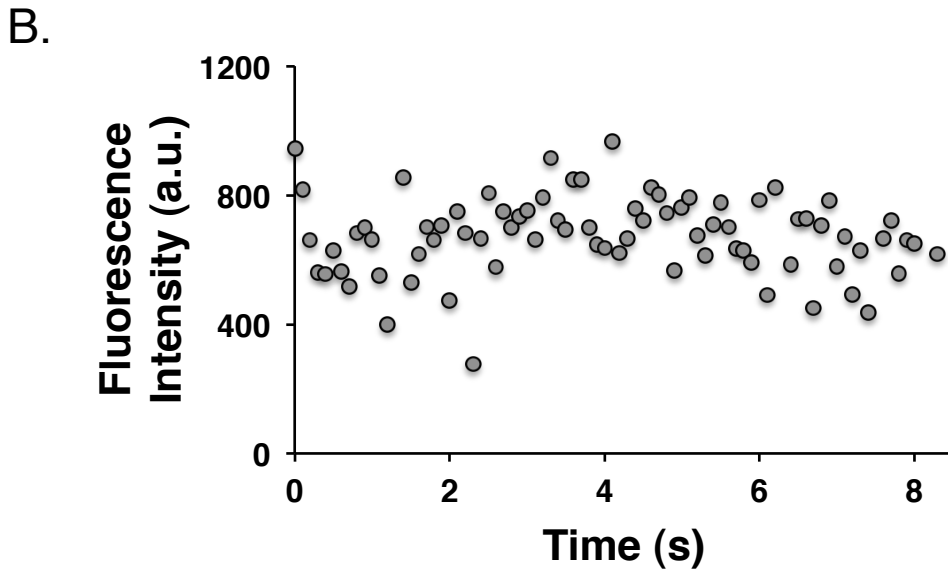
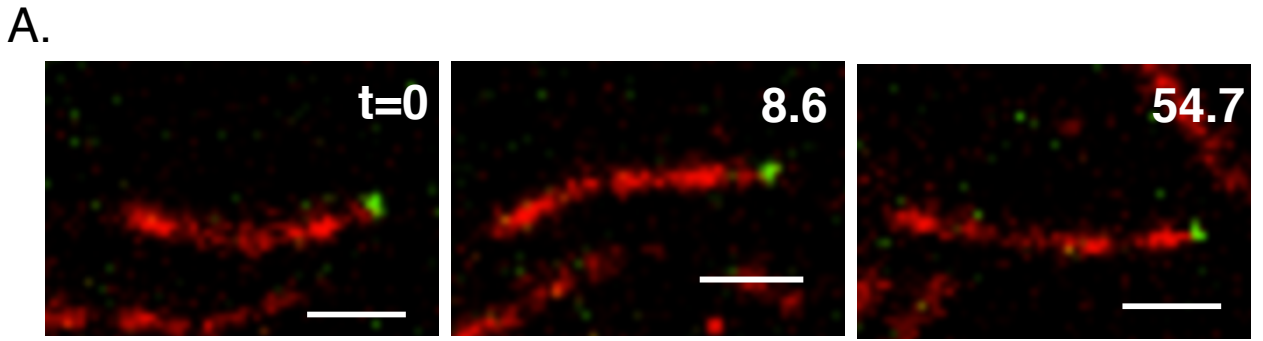


FIGURE S3.

**Figure S3. INF2 processivity on filament barbed ends and calibration of single GFP fluorescence (related to Figure 3).**

- A. Two-color TIRF microscopy montage of actin monomers (1  $\mu$ M, 20% TAMRA) mixed with GFP-INF2-FFC (1 nM) on DDS/F127-treated slides. INF2 is processively bound to elongating barbed end. Time is in sec. Scale bar 2  $\mu$ m.
- B. Distribution of fluorescence intensity values for a SINGLE GFP-INF2 punctum at a filament barbed end quantified over time (83 frames, 8.3s, corresponding to filament in Figure 3C, right panel).

**Table S1. INF2 binding affinities and stoichiometries from pelleting assays (related to Figure S1)**

	<b>K<sub>d</sub> (μM)</b>	<b>Actin Binding Stoichiometry (INF2/Actin)</b>
<b>FFC</b>	0.46	1.13
<b>FFC + P<sub>i</sub></b>	0.66	0.91
<b>FF</b>	2.8	0.8

**Movie S1. GFP-INF2-FFC binding and severing actin filament.** GFP-INF2 FFC (10nM) was added to TAMRA-actin filaments (1 $\mu$ M, 20% TAMRA initially) after allowing for polymerization for 7 minutes. GFP-INF2 puncta bind and then sever throughout filament. Movie corresponds to montage in Figure 1C and represents images taken 25s after GFP-INF2 FFC addition and actin monomer washout for a duration 54s. Dual-color simultaneous images were taken every 100ms and condensed to a 27s movie. Barbed end is on right, scale bar is 2 $\mu$ m.

**Movie S2. GFP-INF2-FFC + 10mM phosphate binding actin filament.** GFP-INF2 FFC (100nM) + 10mM P<sub>i</sub> was added to TAMRA-actin filaments (1 $\mu$ M, 20% TAMRA initially) after allowing for polymerization for 7 minutes. GFP-INF2 puncta bind throughout the filament until saturation. Movie corresponds to montage in Figure 1D and represents images taken 10s after GFP-INF2 FFC + P<sub>i</sub> addition and monomer washout for a duration of 35s. Dual-color simultaneous images were taken every 100ms and condensed to a 17s movie. Barbed end is on right, scale bar is 2 $\mu$ m.

**Movie S3. A488-Cofilin binding and severing actin filament.** A488-Cofilin (5nM) was added to TAMRA-actin filaments (1 $\mu$ M, 20% TAMRA initially) after allowing for polymerization for 7 minutes. A488-cofilin vectorially binds and severs aged filament regions. Movie corresponds to montage in Figure 1E and represents images taken for 40s immediately after A488-Cofilin addition and actin monomer washout. Dual-color simultaneous images were taken every 100ms and condensed to a 20s movie. Barbed end is on right, scale bar is 2 $\mu$ m.

**Movie S4. A488-Cofilin + 10mM phosphate addition to actin.** A488-Cofilin (100nM) + 10mM P<sub>i</sub> was added to TAMRA-actin filaments (1 $\mu$ M, 20% TAMRA initially) after allowing for polymerization for 7 minutes. A488-cofilin binding to filaments in the presence of 10mM P<sub>i</sub> is dramatically reduced. Movie corresponds to montage in Figure 1F and represents images taken for 57s immediately after A488-Cofilin + P<sub>i</sub> addition and actin monomer washout. Dual-color simultaneous images were taken every 100ms and condensed to a 28s movie. Barbed end is on right, scale bar is 2 $\mu$ m.

**Movie S5. Close-up of GFP-INF2-FFC puncta before and after a severing event- barbed end only.** GFP-INF2 FFC (10nM) was added to TAMRA-actin filaments (1 $\mu$ M, 20% TAMRA initially) after allowing for polymerization for 7 minutes. A single GFP-INF2 punctum binds at a severing site, and then severs with punctum localization after severing event at newly formed barbed end only. Movie corresponds to montage in Figure 4C, right panel and represents images taken 25s after GFP-INF2 FFC addition for 14s immediately after GFP-INF2 FFC addition and actin monomer washout. Dual-color simultaneous images were taken every 100ms and condensed to a 9.8s movie. Barbed end is on right, scale bar is 2 $\mu$ m.

**Movie S6. Close-up of GFP-INF2-FFC puncta before and after a severing event- barbed and pointed end.** GFP-INF2 FFC (20nM) was added to TAMRA-actin filaments (1 $\mu$ M, 20% TAMRA initially) after allowing for polymerization for 7 minutes. A single GFP-INF2 punctum binds at severing site, and then severs with puncta localization after severing event at both newly formed barbed and pointed ends. Movie corresponds to montage in Figure 4C, left panel and represents images taken 24s after GFP-INF2 FFC addition and monomer washout for a duration of 8s. Dual-color simultaneous images were taken every 100ms and condensed to a 5.6s movie. Barbed end is on right, scale bar is 2 $\mu$ m.



## **Supplemental Experimental Procedures**

### ***DNA Constructs***

Human INF2 ORF clone (CAAX variant, catalog number SC313010) was obtained from OriGene Technologies, Inc (Rockville, MD). INF2-FFC (amino acids 469-1249), INF2-FFD (amino acids 469-1015), and INF2-FF (469-941) were amplified using a GC-rich PCR system (Roche Applied Science) and subcloned into pGEX-KT vector [1] with a modified tobacco etch virus protease site for bacterial expression as a glutathione S-transferase fusion protein. GFP-fusions were made by cloning in PCR amplified INF2 into a pGEX-KT vector modified with eGFP. The GFP contained the A206K mutation, which reduces self-association dramatically [2].

### ***Protein Preparation and Purification***

All formin constructs were expressed in Rosetta2 E. coli (Stratagene Inc) as GST fusion proteins, following procedures used previously [3]. The constructs used were INF2-FFC (human CAAX variant, amino acids 469-1249), INF2-FF (human, 469-941), INF2-FFD (human, 469-1015), GFP-INF2-FFC, GFP-INF2-FF, FMNL3-FH2 (mouse), and mDia1-FH2 (mouse). Briefly, expression was induced in log phase cultures with 0.5 mM IPTG at 16°C. After expression, extracts were passed over glutathione-Sepharose, cleaved with tobacco etch virus protease to elute the formin construct from GST, and further purified by ion exchange chromatography on SourceQ and/or gel filtration by Superdex200. All proteins were stored at -80°C in K50MEIDT. Rabbit skeletal muscle actin was purified from acetone powder [4] and labeled with pyrenylidoacetamide [5], Alexa 488-succinimidyl ester (Invitrogen A20000) or TAMRA NHS ester (Invitrogen C1171) [6]. Both labeled and unlabeled actin were gel-filtered on

Supderdex200 and stored in G buffer at 4°C. Cofilin (budding yeast, D34C mutant) and Alexa488-labeled cofilin (maleimide link to cysteine 34) were expressed and purified as described previously [7].

### ***Buffers***

The following buffers were used frequently: G-buffer (2 mM Tris, pH 8, 0.5 mM DTT, 0.2 mM ATP, 0.1 mM CaCl<sub>2</sub>, and 0.01% NaN<sub>3</sub>), G-Mg buffer (same as G-buffer but with 0.1 mM MgCl<sub>2</sub> instead of CaCl<sub>2</sub>), 10× K50MEI (500 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM EGTA, and 100 mM imidazole, pH 7.0), 10× Na50MEI (same as 10× K50MEI but with 500 mM NaCl instead of KCl), and polymerization buffer (G-Mg buffer plus either 1× K50MEI or 1× Na50MEI and 0.5 mM thesit (the common name for the detergent nonaethylene glycol monododecyl ether (Sigma, P-9641), which was included to minimize protein adhesion to the tube/well). Polymerization buffer with 1× Na50MEI was used for pelleting assays because dodecyl sulfate precipitates as the potassium salt. Protein storage buffer (K50MEIDT) was composed of 1xK50MEI, 1mM DTT, 0.02% thesit. The TIRF buffer contained: 1xKMEI, 100mM DTT, 0.2mM ATP, 15mM Glucose, 0.5% Methyl Cellulose, 0.01mg/mL catalase (Sigma C3515), 0.05mg/mL glucose oxidase (Sigma G6125), 0.1% BSA). In cases of phosphate addition, 10 mM NaPO<sub>4</sub> pH 7.0 was added from a 500 mM stock.

### ***TIRF Microscopy***

Glass flow chambers were prepared from either VWR microcover glass (24x60mm No 1.5) and Gold Seal Rite-On micro slides (3x1 inch) or VWR microcover glasses (22x22mm and 18x18mm No 1.5) to hold 10μL volume. Three distinct methods for coverglass preparation were

used, described in turn below. Method 1: NEM-myosin. Flow chambers were formed then treated with 10nM N-ethylmaleimide treated rabbit skeletal muscle myosin (Cytoskeleton, Inc MY02) [8] for 1 minute. Chambers were then washed with high salt, BSA containing buffer (1% BSA, 50mM Tris, pH7.5, 600mM NaCl) to disassemble myosin aggregates, followed by low salt, BSA containing buffer (1% BSA, 50mM Tris, pH 7.5, 150mM NaCl) prior to use. Method 2: mPEG-Silane. Coverglasses were washed with acetone (30'), 96% ethanol (10'), RBS 35 detergent (Thermo 27952; 2 hours), and then water. Slides were dried with compressed airflow, incubated overnight with 5K mPEG-silane (Laysan Bio) at 1mg/mL in 96% ethanol, and then rinsed with 96% ethanol, followed by water. Chambers were washed with low salt, BSA containing buffer prior to use. Method 3: DDS/F127. Coverglasses were silanized using the procedure adapted from Gell et al. [9]. Briefly, coverglasses were washed in acetone (50'), ethanol (10'), water (1'), then incubated in Pirhana Solution (1:2 ratio of 30% H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>SO<sub>4</sub>) for one hour. Glasses were then rinsed with water, 0.1M KOH, then water again and dried with inert gas before silanization. Glasses were silanized over night in a 0.0025% solution of dichlorodimethyl silane (Sigma 85126) in chloroform, then washed with methanol, dried with inert gas, and stored in clean sealed containers. For silanized cover glasses, chambers were incubated with 1% Pluronic F127 (Sigma P2443) in BRB80 buffer (80mM PIPES/KOH, pH 6.9, 1mM EGTA, 1mM MgCl<sub>2</sub>) for 1 minute, then equilibrated in TIRF buffer.

For severing assays, 2μM actin (20% A488-labeled) in G-buffer was mixed with 2xTIRF buffer, flowed into the flow chamber, and polymerized for 7 minutes with monitoring on a Nikon Eclipse TE-2000 microscope with Nikon D-eclipse C1 488nm laser. At 7 min, 10μL formin or cofilin diluted in 1xTIRF buffer was flowed into the chamber, simultaneously washing out the

remaining actin monomers. Images were acquired every 2s with TIRF objective (60x 1.49 N.A.) and a Roper Cool-Snap camera.

### ***Two-color Simultaneous TIRF Microscopy***

For nucleation assays, unlabeled actin monomers were mixed with 20% TAMRA-labeled actin monomers in G-buffer, then diluted with 2xTIRF buffer without or with GFP-INF2 construct and flowed into the flow chamber. The filaments were visualized as quickly as possible (45-85s) on a Nikon Eclipse Ti-E inverted microscope with perfect focus and 488nm and 561nm laser. For severing assays, 2 $\mu$ M actin (20% TAMRA-labeled) in G-buffer was mixed with 2xTIRF buffer, flowed into the flow chamber, and polymerized for 7 minutes with monitoring. At 7 min, 10 $\mu$ L GFP-formin or A488-cofilin diluted in 1xTIRF buffer was flowed into the chamber, simultaneously washing out the remaining actin monomers. Simultaneous two-color images were acquired every 100ms with TIRF objective (60x 1.49 N.A.) and two iXON Ultra 897 cameras.

### ***Image Analysis and Quantification of GFP-INF2 molecules per punctum***

Images were processed and analyzed using Nikon NIS-Elements Software. For the determination of number of INF2 molecules per GFP punctum, single filament image sequences were background subtracted individually due to the variation in fluorescence intensity values throughout the field of view. Fluorescence intensity measurements were taken over 5-10 frames within 1-2 minutes of image sequence per diffraction-limited GFP punctum. Fluorescence intensity values were normalized to punctum area and averaged. Number of GFP-INF2

molecules per punctum was determined by calibrating punctum fluorescence intensity values at the barbed end to represent 2 GFP-INF2 molecules (1 dimer).

### ***Co-sedimentation Assays***

Actin (4 $\mu$ M) was polymerized for 1h at 23°C in polymerization buffer (G-Mg buffer plus 1xNaMEI), followed by addition of 2.5x molar excess phalloidin. This actin stock was diluted to desired concentration in polymerization buffer in the absence or presence of formin, in polycarbonate 7x20mm centrifuge tubes (Beckman 343775) to a final volume of 200 $\mu$ L. Filaments were pipetted using cut pipette tips to minimize shearing. After 5 min at 23°C, samples were centrifuged at 80,000 rpm for 20 minutes at 4°C in a TLA-100.1 rotor (Beckman). 100 $\mu$ L of supernatant was removed and mixed with SDS-PAGE sample buffer. Pellets were washed briefly with 200 $\mu$ L 1xNaMEI, and then resuspended in 125 $\mu$ L SDS-PAGE sample buffer. Pellets were analyzed by Coomassie-stained SDS-PAGE and ImageJ software.

### ***Negative Staining Electron Microscopy***

To make negatively stained grids, actin (bare or INF2-FFC-decorated) samples are applied to EM grids with continuous carbon in 2.5  $\mu$ L aliquots. In each grid, the applied sample is allowed to absorb to the grid for one minute, then blotted with a filter paper and stained by 6  $\mu$ L 2.5% uranyl acetate solution for two minutes. Prepared grids are imaged in an FEI Tecnai F20 microscope operated at 200keV acceleration. Images are taken with a Tietz F415 CCD camera (TVIPS) at 50,000 nominal magnification, giving a calibrated pixel size of 2.216 Å. Filaments from the CCD frames are manually selected with EMAN (ref: <http://www.ncbi.nlm.nih.gov/pubmed/10600563>) *helixboxer* module. Helical 3D reconstructions

are conducted with IHRSR (doi: 10.1016/j.jsb.2006.05.015) with EMAN as the reconstruction engine. Figures are prepared with UCSF Chimera (<http://www.ncbi.nlm.nih.gov/pubmed/15264254>).

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