Formins are a conserved group of proteins that nucleate and processively elongate actin filaments. Among them, the formin homology domain–containing protein (FHOD) family of formins contributes to contractility of striated muscle and cell motility in several contexts. However, the mechanisms by which they carry out these functions remain poorly understood. Mammalian FHOD proteins were reported not to accelerate actin assembly in vitro; instead, they were proposed to act as barbed end cappers or filament bundlers. Here, we show that purified Drosophila Fhod and human FHOD1 both accelerate actin assembly by nucleation. The nucleation activity of FHOD1 is restricted to cytoplasmic actin, whereas Drosophila Fhod potently nucleates both cytoplasmic and sarcomeric actin isoforms. Drosophila Fhod binds tightly to barbed ends, where it slows elongation in the absence of profilin and allows, but does not accelerate, elongation in the presence of profilin. Fhod antagonizes capping protein but dissociates from barbed ends relatively quickly. Finally, we determined that Fhod binds the sides of and bundles actin filaments. This work establishes that Fhod shares the capacity of other formins to nucleate and bundle actin filaments but is notably less effective at processively elongating barbed ends than most well-studied formins.

Formins are a major, conserved group of proteins known for their ability to both nucleate new actin filaments and remain processively associated with fast-growing barbed ends via their formin homology 2 (FH2) domains. While bound to the barbed end, many formins accelerate elongation, using their proline-rich FH1 domains to recruit profilin-bound actin monomers to the barbed end. In addition to these classic activities, many formins have additional effects on actin filaments, such as severing, bundling, or cross-linking to microtubules (1). Animals have seven formin families, which share the same domain structure, including the highly conserved FH2 domains. Importantly, formins differ in their actin assembly activities and modes of regulation, allowing them to fulfill distinct cellular roles.

The formin homology domain–containing protein (FHOD) family of formins has two mammalian isoforms, FHOD1 and FHOD3, which assemble contractile actin structures in several contexts. FHOD1 is widely expressed and assembles stress fibers that contribute to the adhesion, spreading, and motility of numerous cell types (2–8). FHOD3 has also been implicated in the motility of some cancers (9, 10), but its expression is predominantly restricted to striated muscle (11, 12). In cardiomyocytes, FHOD3 localizes to the sarcomere and is required for its assembly and maintenance (12–15), whereas FHOD1 distinctly localizes to costameres and intercalated discs (16, 17). Changes in expression level and polymorphisms of both FHOD1 and FHOD3 are associated with cardiomyopathies (12, 17–20).

The mechanisms by which FHOD family members function remain poorly understood. Unlike other formins, purified FHOD1 and FHOD3 were shown to slow, rather than accelerate, actin assembly in vitro and have therefore been proposed to act as actin cappers or bundlers (13, 21). In contrast, the cell biology data suggest that FHOD1 and FHOD3 function as actin nucleators in vivo. Across a wide range of cell types, expression of constitutively active FHOD1 or FHOD3 is sufficient to induce the formation of stress fibers (2–5, 13). Furthermore, FHOD3 is required for sarcomere assembly following latrunculin washout (12). These data are most suggestive of actin nucleation, although FHOD1 and FHOD3 might instead function by stabilizing or reorganizing existing actin filaments.

Drosophila melanogaster has a single FHOD family member, referred to here as Fhod (it is also known as Fhos or knittrig). The Fhod gene is alternatively spliced to produce eight different isoforms, which maintain constant FH1 and FH2 domains but alter their regulatory N termini and C-terminal tails. The role of FHOD proteins in cell motility and contractility is well conserved in Drosophila, because Fhod contributes to motility in macrophages and tracheal tip cells (22), sarcomere organization in striated muscle (23, 24), and cardiac contractility (18).

Here, we show that purified Drosophila Fhod and human FHOD1 accelerate actin assembly by nucleation. The nucleation activity of FHOD1 is restricted to cytoplasmic actin, whereas Drosophila Fhod potently nucleates both cytoplasmic...
and sarcomeric actin isoforms. *Drosophila* Fhod remains processively associated with the barbed end, where it slows elongation in the absence of profilin and allows elongation, at rates similar to actin alone, in the presence of profilin. Although Fhod does not accelerate barbed-end elongation, we find that Fhod protects barbed ends from capping protein with a characteristic run length of ~2 μm. Fhod additionally binds tightly to the sides of filaments and bundles filaments together.

**Results**

**Fhod accelerates actin assembly**

We purified the C-terminal half of *Drosophila* Fhod isoform A, encompassing the FH1 domain, FH2 domain, and C-terminal tail (Fig. 1, *A* and *B*). This isoform is sufficient to rescue viability in Fhod null flies (22), and its C terminus is identical to that of isoform H, which rescues sarcomere organization in indirect flight muscle (23). We first tested the effect of Fhod on the assembly of *Acanthamoeba* actin in bulk pyrene assays; Fhod accelerates actin assembly in the presence of profilin (Fig. 1C).

To further compare Fhod to characterized formins, we introduced two classical mutations, I966A and K1112A, in conserved residues of the FH2 domain (25). The I966A mutation almost completely abolished activity, whereas the K1112A mutation markedly reduced, but did not eliminate, activity (Fig. 1D). We also tested the ability of Fhod to promote actin assembly in the presence of profilin, which binds most actin mono-
not rabbit skeletal actin. alone or in the presence of 8 nM
results are consistent with the more restricted localization of
in the presence rabbit skeletal actin (Fig. 2, nucelated both actin isoforms, with only slightly lower activity
inability to nucleate this isoform. In contrast,
that FHOD1 does interact with rabbit skeletal actin, despite its
inhibited over the first 1000 s (21). The pyrene trace suggests
results. Indeed, human FHOD1 did not nucleate rabbit skeletal
assembly rates from
actin from rabbit skeletal muscle (13, 21). Because formin activ-
(Fig. 2A). Previous work with both FHOD1 and FHOD3 used
FHOD1 accelerated actin assembly in our hands, albeit weakly
was reported to inhibit actin assembly (21). Surprisingly,
FHOD1 accelerated actin assembly in our hands, albeit weakly
(Fig. 2A). Previous work with both FHOD1 and FHOD3 used actin from rabbit skeletal muscle (13, 21). Because formin activity
can depend on the actin isoform,4 we asked whether our use
actin, but not rabbit skeletal actin. A, assembly of 2 μM A. castellanii actin (10% pyrene-labeled) alone or in the presence of 40 nM human FHOD1. B, assembly of 2 μM actin from Acanthamoeba or rabbit skeletal muscle (10% pyrene-labeled) alone or in the presence of 40 nM human FHOD1. C, assembly of 2 μM actin from Acanthamoeba or rabbit skeletal muscle (10% pyrene-labeled) alone or in the presence of 8 nM Drosophila Fhod. D, quantification of actin assembly rates from C. The data are means ± standard deviation from three independent experiments.

Figure 2. Human FHOD1 accelerates assembly of Acanthamoeba actin, but not rabbit skeletal actin. A, assembly of 2 μM A. castellanii actin (10% pyrene-labeled) alone or in the presence of 40 nM human FHOD1. B, assembly of 2 μM actin from Acanthamoeba or rabbit skeletal muscle (10% pyrene-labeled) alone or in the presence of 40 nM human FHOD1. C, assembly of 2 μM actin from Acanthamoeba or rabbit skeletal muscle (10% pyrene-labeled) alone or in the presence of 8 nM Drosophila Fhod. D, quantification of actin assembly rates from C. The data are means ± standard deviation from three independent experiments.

Figure 3. Fhod does not accelerate barbed-end elongation. A, actin elongation from preformed seeds. Final conditions were 0.25 μM F-actin seeds (~0.4 nM barbed ends), 0.5 μM G-actin (10% pyrene-labeled), and 1.5–48 nM Fhod. Fhod slows barbed-end elongation in a dose-dependent manner. B, quantification of elongation rates from A. Elongation rates were measured as the initial slope over the first 90 s, relative to the slope of actin alone. The data are the means ± standard deviation from three independent experiments. The binding curves show the best fit to the average values. C, actin elongation from preformed seeds, as in A, with 1.5 μM S. pombe profilin. Fhod does not slow elongation in the presence of profilin. D, quantification of elongation rates from C. The data are the means ± standard deviation from three independent experiments. E, actin elongation from preformed seeds. Final conditions were 0.25 μM F-actin seeds (~0.4 nM barbed ends), 0.5 μM G-actin (10% pyrene-labeled), and 40 nM human FHOD1. Fhod slows barbed-end elongation. F, actin elongation from preformed seeds, as in E, with 1.5 μM S. pombe profilin. FHOD1 allows barbed-end elongation at rates similar to actin alone.

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Because we did not observe clear evidence of processive elongation by FHod, we used several additional assays to verify and characterize the interaction between FHod and barbed ends. We first verified barbed end binding in bulk barbed end depolymerization assays. The dose dependence gives us an additional measure of the affinity between FHod and barbed ends. FHod inhibited actin depolymerization with a $K_d$ of 5 nM (Fig. 4, C and D), similar to our measurement from the seeded elongation assays. We then used actin reannealing assays, in which two colors of sheared actin filaments were mixed and allowed to reanneal. FHod inhibited actin reannealing, indicating that it binds barbed ends and can remain bound on the timescale of minutes (Fig. 4C). Thus FHod binds barbed ends tightly, like other formins, but does not accelerate elongation, unlike most formins.

**FHod antagonizes capping protein**

We measured the ability of FHod to antagonize capping protein, which binds tightly to barbed ends and prevents elongation. In bulk seeded elongation assays, 6 nM capping protein was sufficient to completely abolish actin elongation. FHod abrogated this effect when added to F-actin seeds prior to capping protein (Fig. 5A). We fit these data to a competition binding equation to determine that FHod has an apparent $K_d$ of 7 nM for growing barbed ends (Fig. 5B), consistent with our previous measurements. Recent evidence suggests that formins can antagonize capping protein not only by passive competition for the barbed end but also by binding capped barbed ends and actively displacing capping protein (29, 30). However, filaments did not grow when capping protein was added before FHod (data not shown), indicating that the actin elongation we observed was due to FHod processively protecting the barbed end and not due to FHod actively displacing capping protein from barbed ends.

We used TIRF microscopy to observe competition between FHod and capping protein on individual filaments. We incubated seeds with FHod prior to adding capping protein and actin monomers and then measured how long FHod could protect the growing barbed ends. Consistent with our bulk assays, we found that barbed ends were completely capped by capping protein in the absence of FHod but were able to grow in the presence of FHod. Because the vast majority of filaments were capped by the time we could start imaging (2–3 min after the start of polymerization), we measured filament lengths in static images taken 5 min after addition of actin monomers (Fig. 5, C and D). By fitting the filament lengths to a single exponential curve, we determined that FHod has a characteristic run length of 2 μm (Fig. 5E). This provides us with an approximate measure of FHod processivity, with two assumptions: 1) capping protein binds to barbed ends as soon as FHod dissociates, and 2) capping protein does not cap barbed ends that are already bound by FHod. The first assumption is consistent with the strong affinity (~0.4 nM) of capping protein for barbed ends. However, the ability of capping protein to bind mDia1-bound barbed ends and displace mDia1 (29, 30) suggests that capping protein might also bind FHod-bound barbed ends, which would make our measurement of FHod processivity an underestimate.
**Figure 5.** *Fhod* antagonizes capping protein. A, actin elongation from preformed seeds with a range of *Fhod* concentrations added before capping protein. Final conditions were 0.25 μM *F-actin* seeds (~0.4 nM barbed ends), 0.5 μM *G-actin* (10% pyrene-labeled), 1.5 μM *S. pombe* profilin, ± 6 nM mouse capping protein and 1.5–48 nM *Fhod*. B, quantification of elongation rates from *A*, measured as the initial slope over the first 90 s relative to the slope of actin alone. *Fhod* antagonizes capping protein, allowing elongation. Data with *Fhod* and capping protein were fit to a competition binding model to determine the affinity of *Fhod* to barbed ends. The data and reported *Kd* are means ± standard deviation from four independent experiments. The binding curve shows the best fit to the average values. C, observation of actin elongation (white) from preformed seeds (green) with *Fhod* added before capping protein. Final conditions were 5 nM *F-actin* seeds (1% biotinylated, labeled with Alexa Fluor 647-phalloidin), 1 μM *G-actin* (10% Alexa Fluor 594-labeled), 5 μM *S. pombe* profilin, ± 2 nM *Fhod*, and 6 nM capping protein. Images were taken 5 min after initiation of polymerization. Scale bars, 10 μm. D, quantification of filament lengths from C. The data represent the amount of elongation from preformed seeds (*n* > 150 for each condition). At least five fields of view from one (actin alone) or two (all other conditions) flow chambers were analyzed for each condition. In conditions with capping protein, no box is visible because over 75% of seeds did not elongate. E, exponential fit of filament lengths in the presence of both *Fhod* and capping protein from *D*, excluding seeds that did not elongate (*n* = 69 filaments from two flow chambers). *Fhod* has a characteristic run length of 2.0 μm.

**Discussion**

Here, we show that *Drosophila* *Fhod* shares the classic activities of formin, actin nucleation and processive elongation, with the additional capacity to bundle actin filaments. Our observation of actin assembly with both *Drosophila* *Fhod* and human FHOD1 contrasts substantially with the previous reports that mammalian FHOD proteins slow actin assembly *in vitro* (13, 21). We resolved these conflicting results for FHOD1 by showing that FHOD1 nucleates actin from *Acanthamoeba* effectively but does not nucleate actin from rabbit skeletal muscle. Our group previously reported a similar preference for the formin Delphilin, which nucleates cytoplasmic actin isoforms much more effectively than actin from rabbit skeletal muscle. Although Delphilin is expressed exclusively in neurons and therefore unlikely to encounter sarcomeric actin isoforms, FHOD1 is expressed in both muscle and non-muscle cells. In cardiomyocytes, FHOD1 is largely excluded from the sarcomere, instead localizing primarily to the costamere and intercalated disc (16, 17). Therefore, the inability of FHOD1 to nucleate sarcomeric actin might be important to its function in this context.

The use of rabbit skeletal actin in previous work is unlikely to explain why FHOD3 did not nucleate *in vitro*, because FHOD3...
is expressed predominantly in striated muscle and required for sarcomere assembly. Given the conserved role of FHOD family members in striated muscle, *Drosophila* Fhod and mammalian FHOD3 likely share a common mechanism in assembling sarcomeric actin. Although we find the cellular data with FHOD3 most suggestive of nucleation (12, 13), it remains possible that FHOD proteins instead stabilize or bundle filaments that are polymerized by a different actin nucleator. Flies carrying the I966A mutation, which abolishes nucleation and barbed end binding while retaining bundling activity, have relatively mild defects in sarcomere organization (23), supporting the possibility that the side binding and bundling activities of Fhod are more critical than nucleation or barbed end binding.

We did not observe evidence of accelerated barbed-end elongation with either Fhod or human FHOD1. This is not unprecedented, because some formins such as *Drosophila* Daam (31) and mouse FMNL1 (32) either slow barbed-end elongation or leave the elongation rate unchanged in the presence of profilin. We expect that both the FH1 and FH2 domains contribute to the inability of Fhod to accelerate barbed-end elongation. The slow barbed-end elongation in the absence of profilin is suggestive of an FH2 domain that spends most of the time in a closed conformation, similar to Cdc12 (33). The addition of profilin restores the elongation rate to that of actin alone, indicating that the FH1 domain has some ability to recruit profilin-actin but perhaps not as effectively as the FH1 domains of other formins. The effectiveness of polyproline tracks in the FH1 domain depends on the number of prolines and their distance from the FH2 domain (34, 35). The polyproline tracks of the Fhod FH1 domain are located relatively far from the FH2 domain, with the closest track (PPPMMP) located 31 residues from the FH2 domain. For comparison, the weak elongator Cdc12 has its closest polyproline track 26 residues away from the FH2 domain, whereas the closest polyproline tracks of the strong elongators Bni1 and mDia1 are only 22 and 16 residues away, respectively.

We approximate that Fhod has a characteristic run length of 2 μm, which is equivalent to a dissociation rate of ~0.01 s⁻¹ based on the elongation rate of 8 subunits/s. This dissociation rate is an order of magnitude faster than mDia1 and several orders of magnitude faster than mDia2, Bni1, Cdc12, and Capu (26, 36), which does not fit the general trend of faster elongation rates, resulting in faster dissociation rates (34). We observed evidence of Fhod protecting barbed ends only when experiments were performed in a tube, i.e. reannealing assays and when Fhod was incubated with seeds and actin monomers in a tube prior to introducing the mixture onto the surface. This suggests that the surface hinders Fhod processivity, making our measurement of Fhod processive elongation activity an underestimate. However, that Fhod is sensitive to conditions that do not perturb the processivity of other formins may indicate that processive elongation is not a critical activity of Fhod. FHOD family members generally localize to the relatively short actin filaments found in stress fibers and the sarcomere, which likely do not require accelerated barbed end growth. Therefore, Fhod nucleation and bundling activities might be more important in these contexts. We found that Fhod is indeed a potent actin bundler; its affinity of 0.18 μM for sides of actin filaments is comparable with the formins Fus1 (37) and AFH1 (38) and an order of magnitude stronger than mDia1 (39), Daam (31), and Capu (26). Alternatively, it is possible that Fhod accelerates actin elongation in vivo through collaborations with other proteins; for example, CLIP-170 was recently shown to augment the processive elongation of mDia1 (40).

### Experimental procedures

**Protein expression, purification, and labeling**

cDNA for *Drosophila* Fhod isoform A (SD08909, obtained from the Drosophila Genomics Resource Center) and human FHOD1 (generous gift from T. Iskratsch) were used as templates to clone C-terminal constructs into a modified version of the pET-15b plasmid with an N-terminal His₆ tag. Point mutations were generated by site-directed mutagenesis as described (41). *Drosophila* Fhod constructs were transformed in Rosetta (DE3) cells (Novagen), which were grown in 1 liter of Terrific Broth supplemented with 100 mg/liter ampicillin and 32 mg/liter chloramphenicol. Expression was induced at an OD of 0.6–0.8 by adding 0.25 mM isopropyl β-D-thiogalactoside and shaking overnight at 18 °C. The cells were harvested by centrifugation, washed in PBS, and flash frozen in liquid nitrogen. Human FHOD1 was expressed in Rosetta 2 (DE3) cells induced with 0.5 mM isopropyl β-D-thiogalactoside as above.

Cell pellets expressing *Drosophila* Fhod were thawed in extraction buffer (10 mM MOPS, pH 7, 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF, 2 μg/ml DNasel). All subsequent steps were performed on ice or at 4 °C. The cells were lysed by microfluidizing, cleared by centrifugation at 20,000 × g for 20 min, and then purified using a HitrapSP-FF cation exchange column (GE Life Sciences) with a gradient of 0.3–1 M NaCl over 16 column volumes. Pooled fractions were diluted at least 6-fold into 10 mM Tris, pH 8, 1 mM DTT and further purified on a MonoQ anion exchange column (GE Life Sciences) with a gradient of 40–500 mM NaCl over 50 column volumes. Peak fractions were exchanged into storage buffer (10 mM Tris, pH 8, 150 mM NaCl, 1 mM DTT, 0−20% glycerol), centrifuged at 20,000 × g for 20 min, flash frozen in liquid nitrogen, and stored at −80 °C.

Cell pellets expressing human FHOD1 were resuspended in extraction buffer (50 mM sodium phosphate, pH 8, 300 mM NaCl, 1 mM βME) supplemented with PMSF and DNaseI and lysed as above. Clarified lysates were nutated with 1 ml of Talon resin (Clontech) per liter of culture for 1 h. Resin was washed with 20 column volumes of extraction buffer, followed by 20 column volumes of wash buffer (50 mM sodium phosphate, pH 7, 300 mM NaCl, 1 mM βME). Resin was washed twice for 30 min each with 20 column volumes of wash buffer supplemented with 10 mM MgCl₂ and 5 mM ATP. FHOD1 was eluted with 200 mM imidazole in wash buffer. Eluted protein was dialyzed into 10 mM MOPS, pH 7, 200 mM NaCl, 1 mM DTT, and then run on a Mono S cation exchange column (GE Life Sciences) with a gradient of 0.2–1 M NaCl over 32 column volumes. Peak fractions were dialyzed overnight into storage buffer (10 mM Tris, pH 8, 150 mM NaCl, 1 mM DTT), centrifuged at 20,000 × g for 20 min, flash frozen in liquid nitrogen, and stored at −80 °C. Actin assembly activity remained stable after freeze-thaw or up to 4 days on ice.

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**Fhod nucleates actin**

_Drosophila_ Fhod protein binding determinations were determined using the absorbance at 280 nm with an extinction coefficient of 122,840 M⁻¹ cm⁻¹ (ProtParam), which was verified by comparing the absorbances of native and denatured protein. The concentration of human FHOD1 was determined by quantitative Sypro Red staining. All _Drosophila_ Fhod and human FHOD1 concentrations are reported in terms of dimer concentrations.

_Acanthamoeba castellani_ actin was purified (42) and labeled with pyrene iodoacetamide (42), Alexa Fluor 594 succinimidyl ester (43), or EZ-link maleimide-PEG2-biotin (Thermo Scientific) (44) according to published protocols. Unlabeled and pyrene-labeled rabbit skeletal actin were kindly provided by the Reisler laboratory (University of California, Los Angeles). _Schizosaccharomyces pombe_ profilin was purified as described (32) on a polyproline column provided by the Reisler laboratory. The concentration was determined using the absorbance at 280 nm with an extinction coefficient of 1.63 OD/mg/ml (45). Mouse capping protein was purified as described (46).

**Pyrene assays**

Pyrene assays were performed essentially as described (47) on an Infinite 200 Pro plate reader (Tecan). In all assays, Fhod was diluted in storage buffer before addition to polymerization buffer to improve stability. The concentration of barbed ends was calculated from the slope (obtained by linear regression over 90 s) using the equation [be] = elongation rate/(kₐ[G-actin] − k₋), where kₐ = 11.6 μM⁻¹ s⁻¹, and k₋ = 1.4 s⁻¹ (48).

For seeded elongation assays, actin filaments were sheared by passing three times through a 24-gauge needle and then aliquoted into each well of a microplate. Proteins were added to the seeds and incubated for 2–4 min at room temperature; in experiments with both Fhod and capping protein, capping protein was added 2–4 min after addition of Fhod. Seeds and additional proteins in KMEH (10 mM HEPES, pH 7, 1 mM EGTA, 50 mM KCl, 1 mM MgCl₂) were added to magnesium-actin to initiate actin elongation. Elongation rates were determined by linear regression over the first 90 s and normalized against the rate of actin alone in each experiment. For experiments without capping protein, the affinity of Fhod for barbed ends was determined by fitting the data to the simplified binding equation, where

\[
CF = \frac{[Fhod]}{[Fhod] + K_d} \times \frac{[CP]}{K_a R_0 + [be]} \times a + b
\]

where \( r \) is the normalized elongation rate, \( K_d \) is the affinity of capping protein for barbed ends (0.4 nM, measured in a seeded elongation assay in the absence of Fhod), \( K_a \) is the affinity of Fhod for barbed ends, and \( R_0 \) is the concentration of free barbed ends when \([Fhod] = 0\). The total concentration of barbed ends was calculated from the initial slope of the polymerization trace for actin alone as described above.

For depolymerization assays, 1 μM F-actin (70% pyrene-labeled) was incubated for at least 15 min at room temperature and then depolymerized by diluting 10-fold in 1 × KMEH with additional proteins. The depolymerization rate was determined by linear regression over the first 90 s. The affinity of Fhod for barbed ends was determined by fitting the data to the simplified binding equation as above.

**TIRF microscopy**

In nucleation assays, assembly of 2 μM actin was initiated by the addition of KMEH with or without Fhod. After 5 min, actin was removed from the reaction and stabilized by diluting 10-fold in 1 × KMEH containing Alexa Fluor 488-phalloidin. Actin was incubated with phalloidin for 10 min, diluted 20-fold in 1 × KMEH supplemented with 100 mM DTT, spotted on a poly-L-lysine-coated coverslip, and imaged. All steps were performed as delicately as possible with cut pipette tips to minimize shearing.

For elongation experiments, biotinylated coverslips were prepared as follows. Coverslips were rinsed three times in MilliQ water, placed in 2% Hellmanex (Hellma Analytics) at 60–65 °C for 2 h, and then rinsed another five times in MilliQ water. Once dry, the coverslips were silanized with (3-glycidoxypropyl)trimethoxysilane for 1 h in a hybridization oven. Unreacted (3-glycidoxypropyl)trimethoxysilane was removed by rinsing three times with acetone. Coverslips were then PEGylated with a mixture of methoxy-PEG-NHS and biotin-PEG-NHS as described (47).

Flow chambers of ~15 μl were assembled on the slide using strips of double-sided tape. Flow chambers were prepared with the following steps: 1) block with 25 μl of 1% Pluronic F-127 (Sigma), 50 μg/ml casein, in PBS, for 2 min; 2) 25 μl of 1 × KMEH; 3) 25 μl of 40 mM streptavidin in 1 × KMEH; 4) 25 μl of 1 × TIRF buffer (1 × KMEH, 0.5% methylcellulose (400 cP, Sigma), 50 mM DTT, 0.2 mM ATP, 20 mM glucose); 5) 50 μl of magnesium-actin and additional proteins to be assayed, in 1 × TIRF buffer supplemented with 5 μM F-actin seeds (1% biotinylated, stabilized with Alexa Fluor 647-phalloidin), 250 μg/ml glucose oxidase, 50 μg/ml catalase, and 50 μg/ml casein. Fhod was incubated with seeds for at least 30 s prior to addition of magnesium-actin; in experiments with both Fhod and capping protein, Fhod was incubated with seeds for 15 s prior to addition of capping protein, and magnesium-actin was added after an additional 30 s.

To determine the characteristic run length of Fhod on barbed ends in the presence of capping protein, 1 − cumulative frequency was treated as the fraction of filaments that were still elongating at a particular length. The data were fit to the exponential equation, \( 1 - cf = e^{-l/\lambda} + a + b \), where \( cf \) is the cumulative frequency, \( l \) is the filament length, and \( \lambda \) is the characteristic run length.

Reannealing assays were conducted essentially as described (50) using Alexa Fluor 488- or rhodamine-labeled phalloidin-actin, sheared by passing three times through a 27-gauge needle. The final concentrations were 250 nM F-actin and 10 nM Fhod. The samples were diluted 50-fold, spotted on poly-L-lysine-coated coverslips, and imaged. In all experiments, actin filaments were visualized on a DM16000 TIRF microscope (Leica) with an HCX PL APO.
phalloidin-stabilized actin filaments (final concentration, 5 μM) with varying concentrations of phalloidin-stabilized F-actin for 30 min at room temperature. Samples were centrifuged at 90,000 rpm for 25 min in a TLA-100 rotor. Pellets were concentrated by resuspending in one-fourth the original volume. The fraction of Fhod that pellets in the absence of F-actin was calculated by adjusting for the 4-fold concentration of pellets with resuspension. The fraction of Fhod bound to F-actin was calculated by adjusting for the gels were stained with SyproRed. The amount of Fhod in each fraction was quantified using QuantityOne software, the gels were stained with SyproRed. The amount of Fhod in the pellet, and the affinity of Fhod for F-actin was determined by fitting the data to the binding equation, \( \theta = \frac{[\text{F-actin}]}{[\text{F-actin}] + K_d} \ast a + b. \)

For low-speed cosedimentation, Fhod was incubated with phalloidin-stabilized actin filaments (final concentration, 5 μM) for 1 h at room temperature and then centrifuged at 12,000 × g for 15 min. The amount of actin in the supernatants and pellets was quantified by Coomassie-staining SDS-PAGE gels.

Author contributions—A. A. P., Z. A. O. D., A. P. v. L., and M. E. Q. designed the experiments. A. A. P., Z. A. O. D., A. P. v. L., and K. V. B. performed the experiments. A. A. P. and M. E. Q. wrote the manuscript with input from all authors.

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