# Ena/VASP Enabled is a highly processive actin polymerase tailored to self-assemble parallel-bundled F-actin networks with Fascin

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**Filopodia are exploratory finger-like projections composed of multiple long, straight, parallel-bundled actin filaments that protrude from the leading edge of migrating cells. *Drosophila melanogaster* Enabled (Ena) is a member of the Ena/vasodilator-stimulated phosphoprotein protein family, which facilitates the assembly of filopodial actin filaments that are bundled by Fascin. However, the mechanism by which Ena and Fascin promote the assembly of uniformly thick F-actin bundles that are capable of producing coordinated protrusive forces without buckling is not well understood. We used multicolor evanescent wave fluorescence microscopy imaging to follow individual Ena molecules on both single and Fascin-bundled F-actin in vitro. Individual Ena tetramers increase the elongation rate approximately two- to threefold and inhibit capping protein by remaining processively associated with the barbed end for an average of ∼10 s in solution, for ∼60 s when immobilized on a surface, and for ∼110 s when multiple Ena tetramers are clustered on a surface. Ena also can gather and simultaneously elongate multiple barbed ends. Collectively, these properties could facilitate the recruitment of Fascin and initiate filopodia formation. Remarkably, we found that Ena's actin-assembly properties are tunable on Fascin-bundled filaments, facilitating the formation of filopodia-like F-actin networks without tapered barbed ends. Ena-associated trailing barbed ends in Fascin-bundled actin filaments have approximately twofold more frequent and approximately fivefold longer processive runs, allowing them to catch up with leading barbed ends efficiently. Therefore, Fascin and Ena cooperate to extend and maintain robust filopodia of uniform thickness with aligned barbed ends by a unique mechanistic cycle.**

profilin | formin | TIRF microscopy | self organization | single molecule

The actin cytoskeleton facilitates fundamental cellular processes including division, polarization, and motility. The organization and dynamics of particular F-actin networks are determined by the coordinated action of specific subsets of actin-binding proteins with complementary biochemical properties such as sequestering, nucleating, elongating, bundling/crosslinking, and severing (1–3).

Cell motility is driven primarily by lamellipodia, protrusive structures at the cell's leading edge composed of a dendritic network of short-branched filaments produced by the rapid capping of filaments nucleated by the actin-related proteins 2 and 3 (Arp2/3) complex (4). Filopodia are exploratory finger-like projections composed of uniformly long, straight, parallel-bundled filaments that extend from lamellipodia. One current model for filopodia assembly is convergent elongation (5). Formin and/or Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) proteins gather and rapidly elongate subpopulations of lamellipodial actin-barbed ends, antagonizing inhibition by capping protein (CP), and the parallel actin-crosslinking protein Fascin aligns and bundles elongating filopodial filaments (6).

Ena/VASP proteins are large, multidomain actin-assembly factors (7, 8). The N-terminal Ena/VASP homology 1 (EVH1) domain binds FP4 motifs for proper localization (9). The central proline-rich region binds the G-actin–binding (GAB) protein profilin and SH3 domains (10–12). The EVH2 domain comprises a WH2 GAB domain (11, 13) and an F-actin–binding (FAB) domain (14). The C-terminal coiled-coil facilitates tetramerization (13, 15) and is necessary for processive actin filament elongation and proper function in vivo (11, 12, 15, 16).

The importance of Ena/VASP proteins in filopodia formation and maintenance is well established (17), and Ena/VASP's general actin-assembly properties have been defined. Most in vitro studies agree that Ena/VASP allows actin filament elongation in the presence of CP (12, 18–21), although the specific underlying mechanism(s) differ (9). Breitsprecher et al. (20) proposed that multiple mammalian or *Dictyostelium* Ena/VASP tetramers must be clustered on a surface to inhibit CP and allow long runs of processive filament elongation. Hansen and Mullins (12) proposed that individual human VASP (hVASP) tetramers protect barbed ends from CP, but only for very short processive runs (∼1.4 s = 40 subunits) (12). Furthermore, how Ena/VASP proteins and Fascin assemble robust filopodia composed of bundled filaments of uniform length is not known.

The namesake Enabled (Ena), the sole *Drosophila* family member, was identified as a mutant ameliorating defects caused by loss of the tyrosine kinase *Abl* (22). Ena regulates many morphogenetic processes; for example, *ena* mutants display defects in axon guidance (23–25) and reduced filopodia length and number during dorsal closure; these defects disrupt the correct pairing of contralateral cells during epithelial zippering (26). Conversely, Ena overexpression induces filopodia assembly (26).

## Significance

**Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) proteins are required for the formation and maintenance of filopodia, finger-like projections at the leading edge of migrating cells that are composed of parallel actin filaments bundled by Fascin. We imaged individual fluorescently labeled *Drosophila* Ena molecules on both single and Fascin-bundled actin filaments in vitro. Ena stimulates actin assembly by remaining continuously associated with the barbed end and increasing the elongation rate by approximately two- to threefold. Remarkably, the frequency and length of Ena's processive runs are enhanced on filaments within a Fascin bundle, which drives a positive feedback cycle that allows the assembly of uniformly thick filopodia-like F-actin bundles composed of multiple filaments with aligned ends.**

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We used single- and two-color total internal reflection fluorescence microscopy (TIRFM) to observe the assembly of both single and Fascin-bundled filaments in the presence of *Drosophila* Ena. We discovered that Ena possesses a unique combination of actin assembly properties that in combination with Fascin drive the self-organization and maintenance of robust filopodia-like bundles composed of coherently elongating actin filaments of uniform length.

## Results

**Enabled Accelerates Actin Monomer Assembly.** A poorly conserved linker separates *Drosophila* Ena's N-terminal EVH1 domain and C-terminal proline-rich and EVH2 domains (Fig. S1*A*). We purified full-length Ena [Ena(FL)] and versions lacking the linker [Ena(ΔL)] or the entire N terminus [Ena(ProEVH2)] (Fig. S1 *A* and *B*). We initially assessed Ena's effects on the assembly of Mg-ATP-actin monomers in bulk pyrene actin assays. All three constructs accelerated spontaneous actin assembly in a similar concentration-dependent manner (Fig. S1 *C* and *D*). Ena(ΔL) and Ena(ProEVH2) were used for subsequent experiments because of significantly higher protein yields. We then used TIRFM to visualize Ena's effect on the assembly of 1.5-μM Mg-ATP-actin monomers (33% Oregon green-actin) (Fig. S1*E* and Movie S1). Compared with actin only (Fig. S1*E*, *Upper*), 125-nM Ena(ProEVH2) initiated rapid assembly of a complex bundled F-actin network (Fig. S1*E*, *Lower*), displaying a range of filament characteristics (nucleation, bundles, buckles, increased elongation rate) requiring further characterization to parse out Ena's diverse actin assembly properties.

**Ena Enhances Barbed-End Elongation.** We assessed Ena's effect on the barbed ends of individual actin filaments via single-color TIRFM (Fig. 1). At high concentrations of Ena(ProEVH2) (25 nM), several revealing filament behaviors are observed (Fig. 1*A*, *Upper* and Movie S2). First, all filament barbed ends (red arrowheads) elongate at an elevated rate of ∼30 subunits/s, threefold faster than controls (Fig. 1*B*). Second, short filaments (filament b) often appear on the sides of preexisting filaments (filament a) and subsequently assemble into bundles. Third, filaments buckle (Fig. 1*A*, arrow) as they elongate from barbed ends that are temporarily adsorbed to the coverslip, suggesting that an individual Ena unit remains continuously associated with an elongating barbed end for tens of seconds. Buckling is a hallmark of processive elongation of the barbed ends of filaments from formin dimers attached to a surface (27, 28) but previously had been observed only in Ena/VASP proteins densely clustered on beads (20, 29).

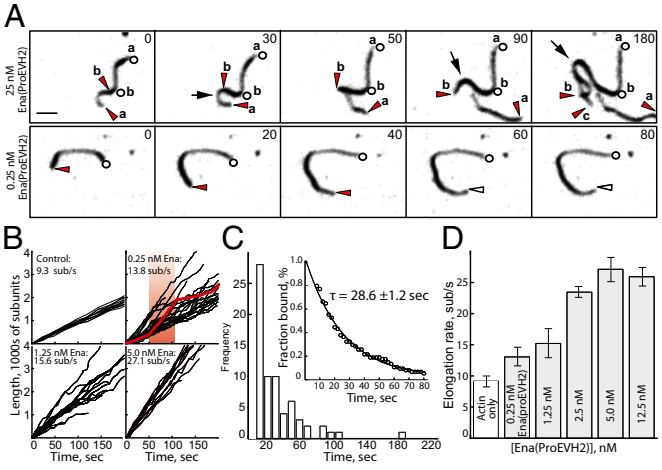


**Fig. 1.** Ena's effect on actin filament barbed-end dynamics. Single-color TIRFM of 1.5-μM Mg-ATP-actin (33% Oregon green-actin). (*A*) Time-lapse (in seconds) of 25-nM (*Upper*) or 0.25-nM (*Lower*) Ena(ProEVH2). Red arrowheads indicate rapidly elongating barbed ends; white arrowheads indicate a slowly elongating barbed end; circles indicate pointed ends; and arrows indicate buckled filaments. (Scale bar: 2 μm.) (*B*) Length of individual filaments over time in the absence (*Upper Left*) or presence of 0.25-, 1.25-, and 5.0-nM Ena(ProEVH2). Average elongation rates are indicated. The red region indicates a period of fast elongation on the red trace. (*C*) Histogram showing the duration of fast elongation runs for 0.25-nM Ena(ProEVH2). (*Inset*) A single exponential fit of fraction bound over time. Average processivity equals ∼29 s. (*D*) Dependence of the average barbed-end elongation rate on the concentration of Ena(ProEVH2). Error bars indicate SEM; $n \geq 10$ filaments from two or more movies.

At 100-fold-lower Ena(ProEVH2) concentrations (0.25 nM), individual filament barbed ends exhibit bimodal behavior (Fig. 1*A*, *Lower* and Movie S3). Barbed ends switch between elongating at the rapid rate observed at high Ena concentrations (∼25 subunits/s) and the control rate (∼10 subunits/s). Periods of rapid elongation sometimes correspond with filament buckling when barbed ends are adsorbed to the coverslip (Movie S3, arrow). Tracing individual filaments over time at low (0.25 nM) and intermediate (1.25 nM) Ena(ProEVH2) concentrations confirmed that barbed ends switch between elongating at control and ∼2.5- to threefold accelerated rates (Fig. 1*B*). We interpret the fast periods as single processive runs by Ena (width of red shaded region in Fig. 1*B*). With this assumption, fitting a single exponential decay to a plot of fraction bound over time revealed an average processive run time of 28.6 s, allowing the addition of ∼700 G-actin subunits (Fig. 1*C*). When the Ena(ProEVH2) concentration increases, the average barbed-end elongation rate saturates at ∼25 subunits/s because all barbed ends are associated with Ena (Fig. 1 *B* and *D*).

Ena/VASP proteins contain a conserved Pro region that binds the actin monomer-binding protein, profilin (Fig. S1*A*) (10–12), although the reported effects of profilin on diverse Ena/VASP proteins range from negligible (12, 20) to only a modest increase in the elongation rate (18, 21). Combining pyrene and TIRFM assays, we found Ena(ΔL) increases the elongation rate approximately two- to threefold with either actin or actin bound to *Drosophila* profilin (Chickadee) (Fig. S2). Therefore, physiological profilin-actin is suitable for Ena-mediated processive elongation, but profilin does not enhance Ena's activity significantly as it does for formins (7). Utilization of profilin-actin by Ena/VASP proteins likely requires both the Pro-rich and GAB domains, although the mechanism is not clear (12, 30). Together, these data demonstrate that Ena enhances barbed-end elongation and suggest it acts processively.

**Ena Tetramers Are Highly Processive Actin Polymerases.** To test our hypothesis that individual Ena units remain processively associated with elongating barbed ends and to determine the oligomeric state of the processive units, we made fluorescently labeled SNAP-549-Ena(ΔL) for two-color TIRFM. The majority of SNAP-549-Ena(ΔL) elutes from a gel filtration column as a tetramer (Fig. S3 *A* and *B*) that behaves equivalently to unlabeled Ena(ΔL) in pyrene assays (Fig. S3*C*). In TIRFM assays with low (0.05 nM) concentrations of SNAP-549-Ena(ΔL), individual filaments switch between control (∼14 subunits/s) and approximately threefold faster (∼37 subunits/s) elongation rates (Fig. 2 *A*–*C* and Movies S4 and S5), identical to the bimodal behavior of unlabeled Ena. SNAP-549-Ena(ΔL) processively tracks barbed ends during fast elongation (Fig. 2 *A* and *B*, red arrowheads), coinciding with buckling filaments when SNAP-549-Ena(ΔL) is adsorbed to the coverslip (Fig. 2*B*, white arrow). Single exponential decay fits to plots of the fraction of Ena-bound filament barbed ends over time revealed two different processive behaviors (Fig. 2*D*). Processive runs average 9.2 s ($n$ = 134, 64% of events) when SNAP-549-Ena(ΔL) is in solution (Fig. 2*A*), whereas runs average 62.5 s ($n$ = 76, 36% of events) when SNAP-549-Ena(ΔL) is adsorbed to the coverslip (Fig. 2*B*). Thus, Ena is a highly processive actin polymerase.

To determine Ena(ΔL)'s oligomeric state, we photobleached soluble and adsorbed SNAP-549-Ena(ΔL) spots that were processively

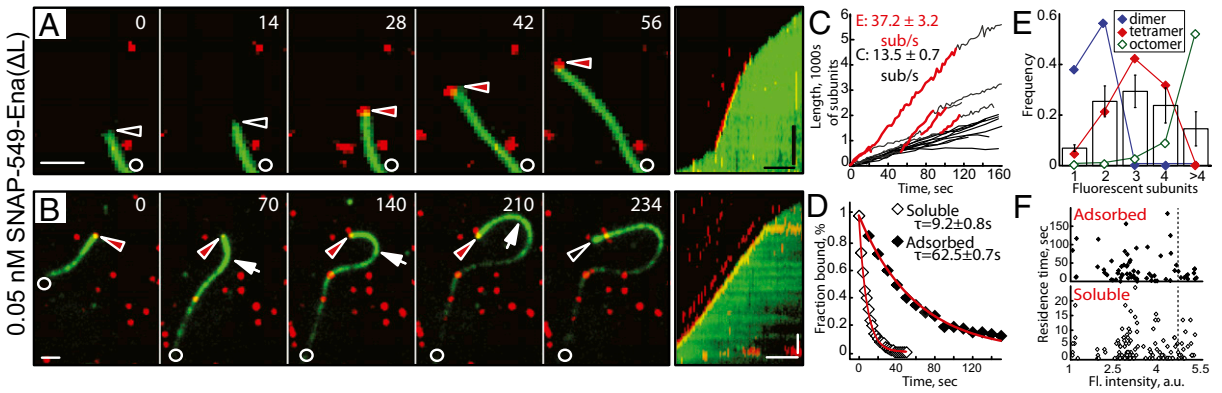


**Fig. 2.** Ena tetramers are highly processive actin polymerases. Two-color TIRFM showing 1.5-μM Mg-ATP-actin (15% Oregon green-actin) assembled with 0.05-nM SNAP-549(red)-Ena(ΔL). (*A* and *B*, *Left*) Merged time-lapse micrographs (in seconds). (Scale bar: 2 μm.) Arrowheads indicate free (black) or SNAP-549-Ena(ΔL)–associated (red) barbed ends. (*A* and *B*, *Right*) Merged kymographs of filament length (*y* axis; scale bar: 2 μm) over time (*x* axis; scale bar: 40 s). (*A*) Processive SNAP-549-Ena(ΔL) in solution. (*B*) Processive SNAP-549-Ena(ΔL) adsorbed to the coverglass. The filament buckles (white arrow). (*C*) Traces of individual filaments over time with free (black traces) or SNAP-549-Ena(ΔL)–associated (red traces) barbed ends. (*D*) Exponential fits of fraction bound over time for soluble and adsorbed processive events. (*E*) Histogram showing photobleaching steps for SNAP-549-Ena(ΔL) processive spots. Diamonds represent predicted photobleaching distributions based on a binomial model where the measured subunit-labeling stoichiometry is 0.75. (*F*) Dependence of the SNAP-549-Ena(ΔL) processive run length on its fluorescence intensity in solution or adsorbed to a surface. Dashed lines indicate high fluorescence intensity corresponding to Ena units larger than single tetramers.

associated with barbed ends. We found that 84% bleach in four or fewer steps, consistent with a tetramer (based on ~74% labeling efficiency; Fig. 2*E*); 16% bleach in more than four steps, suggesting that a dimer of Ena tetramers also can track barbed ends. However, plotting the dependence of soluble and adsorbed SNAP-549-Ena(ΔL) residence times on fluorescence intensity revealed that processive run length does not seem to depend on oligomerization of Ena tetramers (Fig. 2*F*). Conversely, *Dictyostelium* and human VASP were reported to elongate barbed ends processively only when clustered on beads (20, 29). Therefore, we examined whether clustering greater amounts of SNAP-biotin-Ena(ΔL) on Quantum dots (Qdot) adsorbed to the coverslip further increases Ena's processive run length (Fig. S4 and Movie S6). At high Ena:Qdot ratios, nucleation events often are observed from Qdots (Fig. S4*A*, *Inset*), and the average Ena processive run length increases twofold, to ~110 s (Fig. S4*B*). Thus, fly Ena does not require clustering for processivity. Clustering multiple Ena tetramers may increase the processive run length modestly, although it is difficult to differentiate between the contributions of adsorption and clustering in experiments with beads (20) and Qdots.

**Ena Gathers and Elongates Multiple Barbed Ends.** Actin filament barbed ends can converge in lamellipodia and elongate into extending filopodia (5), possibly mediated by Ena/VASP proteins (16, 19). One-color TIRFM with Ena(ΔL) revealed the coalescence of individual barbed ends that subsequently elongate together (Fig. 3 *A*–*D* and Movies S7 and S8). The elongation rates of the two colocalized barbed ends (~33 subunits/s) match the maximum rate of individual barbed ends associated with an Ena tetramer (Fig. 3 *C* and *D*). Furthermore, two-color TIRFM with labeled SNAP-549-Ena(ΔL) showed that colocalized barbed ends often separate into distinct Ena puncta (Fig. 3*E* and Movie S9).

**Behavior of Ena-Bundled Filaments.** Ena/VASP proteins often are associated with bundled actin filaments in structures such as filopodia and stress fibers, and Ena/VASP bundles F-actin in vitro (Fig. S1*E*) (13, 18). We used single- and two-color TIRFM to characterize bundles produced by intermediate to high Ena concentrations (Fig. S5). At a concentration of 62.5 nM, Ena (ProEVH2) produces robust F-actin bundles (Fig. S5*A* and Movie S10). Bundles of both polarities (62% parallel, 38% antiparallel) are identifiable, because brighter barbed ends are easily visualized (Fig. S5 *A* and *B*, arrowheads). Trailing barbed ends elongate more slowly than leading barbed ends but still elongate more rapidly than actin-only controls (Fig. S5*C*). At a concentration of 12.5 nM, SNAP-549-Ena(ΔL) also bundled filaments (Fig. S5 *D* and *E* and Movies S11 and S12). SNAP-549-Ena(ΔL) interacts with the sides of individual filaments transiently, although their barbed ends are always bound by Ena (Fig. S5 *D* and *E*, *Insets*). Conversely, SNAP-549-Ena(ΔL) rapidly accumulates all along both parallel and antiparallel bundles (Fig. S5 *D* and *E*). Therefore, high concentrations of Ena bundle F-actin, and Ena preferentially associates with the sides of bundled filaments.

**Reconstituting Filopodia-Like F-Actin Bundles.** We next investigated how Ena contributes to filopodia-like networks composed of parallel actin filaments bundled by Fascin (6). We used two-color TIRFM to observe actin assembly with low concentrations (0.1 nM) of SNAP-549-Ena(ΔL) on parallel F-actin bundles made by Fascin. SNAP-549-Ena(ΔL) can processively track the aligned barbed ends (arrowheads) of multiple coherent

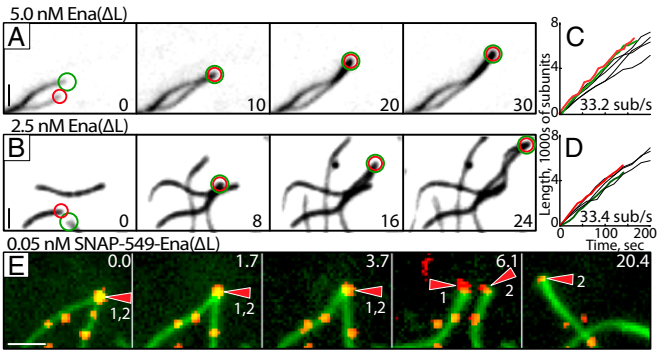


**Fig. 3.** Ena gathers and processively elongates multiple barbed ends simultaneously. (*A* and *B*) Single-color TIRFM montages (in seconds) from reactions containing Ena(ΔL) and 1.5-μM Mg-ATP actin (33% Oregon Green-actin). Circles indicate barbed ends that colocalize and elongate together. (*C* and *D*) Plots over time of the length of individual filaments with barbed ends colocalizing with another barbed end (red and green traces) or elongating alone (black traces). (*E*) Merged two-color TIRFM montages (in seconds) showing an oligomer of SNAP-549-Ena(ΔL) tetramers associated with two gathered barbed ends (red arrowheads) that then separate.

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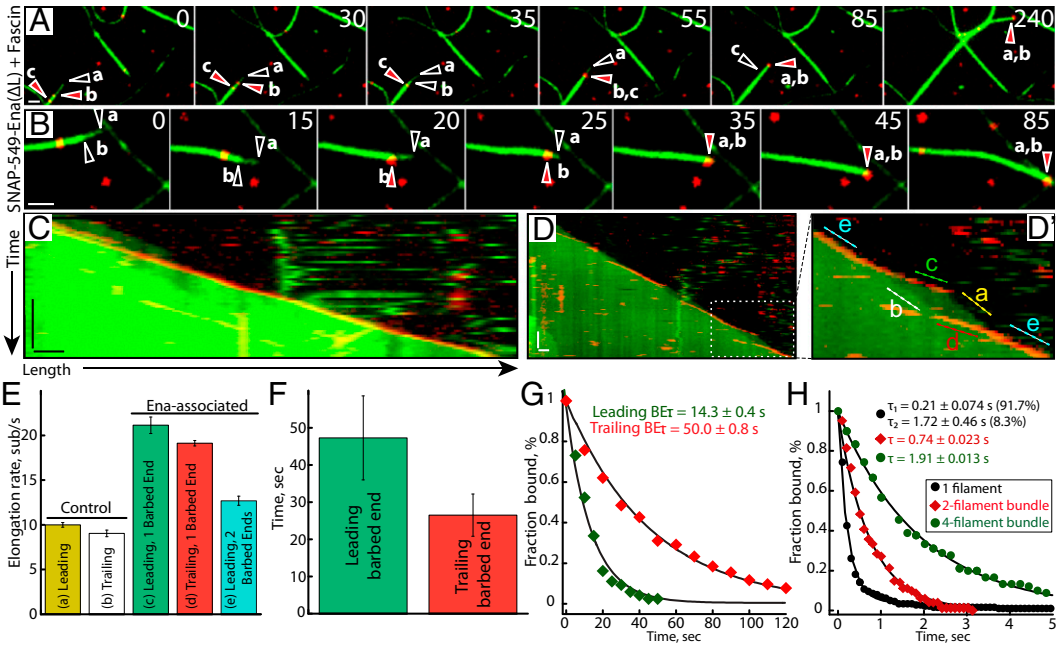


**Fig. 4.** Ena behavior on Fascin-bundled actin filaments. Two-color TIRFM of 1.5-μM actin (15% Oregon green-actin) assembled in the presence of 0.05-nM SNAP-549-Ena(ΔL) and Fascin. (*A* and *B*) Merged time-lapse micrographs (in seconds) with 400-nM (*A*) or 50-nM (*B*) Fascin. Arrowheads indicate free (black) or SNAP-549-Ena(ΔL)–associated (red) barbed ends. (Scale bars: 2 μm.) (*C*–*D′*) Kymographs of filament length (*y* axis; scale bar: 2 μm) over time (*x* axis; scale bar: 100 s). (*C*) Bundle in *A*. (*D*) Bundle in *B*. (*D′*) Zoomed view of the dashed box in *D*. Dashed lines with letters mark different elongation rates quantified in *E*. (*E*) Average elongation rates of leading and trailing barbed ends in Fascin-bundled actin filaments that are either free or associated with SNAP-549-Ena(ΔL). Free leading (yellow bar) and trailing (white bar) actin filaments, Ena-associated leading (green bar) and trailing (red bar) actin filaments, and Ena simultaneously associated with two leading filaments (blue bar). Error bars indicate SEM, $n \geq 10$ filaments. (*F*) Average time between two Ena processive runs on leading ($n = 32$) or trailing ($n = 20$) barbed ends. Error bars indicate SEM. (*G*) Single exponential fit of fraction bound over time for leading (green trace, $n = 65$) or trailing (red trace $n = 105$) Ena-associated barbed ends. (*H*) Exponential decay fits to fraction bound over time on the side of single filaments (black trace, $n = 177$), two-filament bundles (red trace, $n = 81$), or four-filament bundles (green trace, $n = 90$).

filaments bundled by 500-nM Fascin (Fig. 4 *A* and *C* and Movie S13), although specific Ena properties were difficult to investigate because of the high filament number.

Reducing the Fascin concentration to 50 nM resolved individual events; remarkably, in two-filament bundles (Fig. 4 *B* and *D* and Movie S14), trailing barbed ends (Fig. 4*B*, arrowhead b) repeatedly catch up with the leading barbed end (Fig. 4*B*, arrowhead a) and then both ends coelongate. A kymograph revealed a cycle (Fig. 4 *D′*, *a*–*e*) in which the differences in (*i*) elongation rates (Fig. 4*E*), (*ii*) the frequency of Ena-mediated processive runs (Fig. 4*F*), and (*iii*) the length of processive runs (Fig. 4*G*) allow Ena-associated trailing barbed ends to catch up with the leading barbed end. In general, barbed ends elongate faster when associated with Ena, whether trailing or not (Fig. 4*E*). Ena-associated single barbed ends grow approximately twofold faster than free barbed ends and 1.5-fold faster than two barbed ends simultaneously associated with a single Ena tetramer. Importantly, the time between Ena dissociation and the next Ena association is approximately twofold less for trailing barbed ends (26.5 s, $n = 20$) than for leading barbed ends (47.3 s, $n = 33$) (Fig. 4*F*), and Ena is approximately fivefold more processive when associated with a trailing barbed end ($\tau = 50.0$ s, ~950 subunits) than with a with leading barbed end ($\tau = 14.3$ s, ~300 subunits; Fig. 4*G*). Therefore, based on direct measurements of the on ($k_+$) and off ($k_-$) rates, the affinity of Ena for trailing barbed ends ($k_+ = 230 \pm 100$ μM/s, $k_- = 0.02$/s, $K_d = 0.09$ nM) is ~10-fold higher than for leading barbed ends ($k_+ = 120 \pm 90$ μM/s, $k_- = 0.09$/s, $K_d = 0.8$ nM).

The differences between Ena's actin-assembly properties on trailing and leading barbed ends induce a cycle that promotes the robust coalignment of the barbed ends of multiple actin filaments at the leading end of uniformly thick Fascin-mediated bundles that could be capable of producing filopodia with coordinated protrusive force. Fig. 4 *D′*, *a*–*e* provides an example. Ena initially elongates two barbed ends simultaneously at an intermediate rate (Fig. 4 *D′*, *e*). One barbed end subsequently loses Ena and trails behind (Fig. 4 *D′*, *b*), and the Ena-associated barbed end moves ahead by elongating ~2.0-fold faster (Fig. 4 *D′*, *c*). Ena dissociates from the leading barbed end in ~15 s, slowing the elongation (Fig. 4 *D′*, *a*). The trailing barbed end quickly picks up new Ena tetramer and rapidly catches up to the free leading barbed end (Fig. 4 *D′*, *d*), returning the bundle to the initial state of uniform thickness with two adjoined leading Ena-associated barbed ends (Fig. 4 *D′*, *e*). This cycle likely is driven by individual Ena tetramers, because the average fluorescence intensity of SNAP-549-Ena(ΔL) remains constant on single and shared barbed ends (Fig. S6) and because shared barbed ends elongate at an intermediate rate.

To investigate why trailing barbed ends pick up a new Ena tetramer twice as quickly as leading barbed ends, we investigated how well Ena interacts with the sides of filaments. We compared the dwell times of diffusing SNAP-549-Ena(ΔL) on single filaments and on two- and four-filament Fascin bundles, using two-color TIRFM (Fig. 4*H* and Movie S15). Ena tetramers interact with the sides of single filaments only transiently (mean $\tau = 0.21$ s), with occasional longer-lived events ($\tau = 1.71$ s, ~8.0%). However, Ena's mean dwell time increases significantly as the number of filaments in bundles increases to two ($\tau = 0.74$ s) or four ($\tau = 1.91$ s). Therefore, the increased dwell time on the sides of bundled filaments may help explain why trailing barbed ends are more likely than leading barbed ends to encounter a new Ena tetramer.

**Ena Protects Filament Barbed Ends from CP.** Ena/VASP proteins weakly antagonize CP in solution (12, 18, 19), and some Ena/VASP family proteins must be clustered on a surface to prevent capping (20). However, our TIRFM experiments revealed that individual

Ena tetramers are highly processive. We initially explored these differences by measuring Ena's affinity for barbed ends using seeded pyrene assays. Both Ena(ΔL) and Ena(ProEVH2) increased the barbed-end elongation rate of preassembled F-actin seeds in a concentration-dependent manner (Fig. 5 *A* and *B*). Fitting the dependence of the assembly rate on concentrations of Ena (ΔL) or Ena(ProEVH2) tetramers revealed apparent $K_d$s for barbed ends of ∼1 nM (Fig. 5*B*), ∼10-fold lower than measured for hVASP tetramers (∼9 nM) (12). CP normally blocks barbed-end elongation of F-actin seeds. Like other Ena/VASP proteins (12, 20), Ena(ΔL) enhances barbed-end elongation in the presence of saturating CP (Fig. 5*C*). In fact, Ena requires ∼15-fold less protein to achieve assembly rates similar to those reported for other Ena/VASP proteins in the presence of CP (18, 19), suggesting that Ena is a more effective CP antagonist. Furthermore, two-color TIRFM imaging revealed that SNAP-549-Ena(ΔL) allows the rapid elongation of preassembled F-actin seeds in the presence of CP, resulting in significantly longer filaments (Fig. S7 and Movie S16). Therefore, processive barbed-end association allows Ena to promote actin filament assembly by (*i*) increasing the elongation rate, (*ii*) gathering multiple barbed ends, and (*iii*) antagonizing CP.

## Discussion

**Ena Is a Tunable Processive Actin Polymerase and an Effective Anticapper.** We discovered that *Drosophila* Ena possesses an important set of complementary properties well suited to assembling long, straight, rapidly elongating, bundled actin filaments that are protected from CP (Fig. 5 *D*, *a*–*c*). Individual Ena tetramers remain processively associated with elongating barbed ends while increasing the elongation rate approximately two- to threefold. Processive elongation allows Ena to protect filaments robustly from saturating concentrations of CP. Importantly, Ena's processive run length (residence time) is modulated depending upon whether it is in solution (∼10 s), adsorbed to a surface (∼60 s), associated with trailing Fascin-bundled actin filaments (∼50 s), or clustered on a surface (∼110 s). Both individual and clustered Ena tetramers can gather and simultaneously elongate multiple barbed ends. Ena also interacts with the sides of filaments, and this interaction is strengthened when multiple filaments are closely apposed in Fascin- or Ena-generated bundles.

Although the general actin-assembly properties of Ena/VASP family proteins are conserved, particular reaction rates vary significantly (12, 18–21, 29). For example Mena, EVL, and hVASP increase the elongation rate by only 1.5- to 2.0-fold, whereas *Dictyostelium* VASP (DdVASP) increases elongation sevenfold (12, 20, 29). Furthermore, processive run lengths are quite different. DdVASP reportedly is not processive in solution (20, 29), whereas hVASP takes processive runs of 1.4 s, approximately sevenfold shorter than those of Ena (9.2 s) (12). As a result, *Drosophila* Ena confers increased protection of barbed ends from CP. Processive run lengths and the corresponding anticapping activity of DdVASP and hVASP are increased dramatically when clustered on beads (20), and the same may be modestly true for Ena. We hypothesize that different Ena/VASP isoforms are tailored for particular roles, and that clustering, Fascin bundling, and immobilization at the membrane modulate Ena/VASP processive run lengths.

**Mechanism of Ena Processivity.** Processive barbed-end elongation is likely driven by coordination between the FAB and GAB domains from all four peptides in an Ena tetramer. FAB domains bind the sides of actin filaments, and GAB domains bind G-actin or bind F-actin with lower affinity (12). The FAB domain of a FAB/GAB pair tethers the tetramer near the barbed end, and the GAB domain contributes its bound monomer (11). Before this FAB/GAB pair dissociates, any of the three other FAB/GAB pairs in the tetramer can bind to the adjacent protomer and add a monomer.

We hypothesize that longer processive runs on trailing barbed ends in Fascin-bundled filaments are possible because Ena FAB

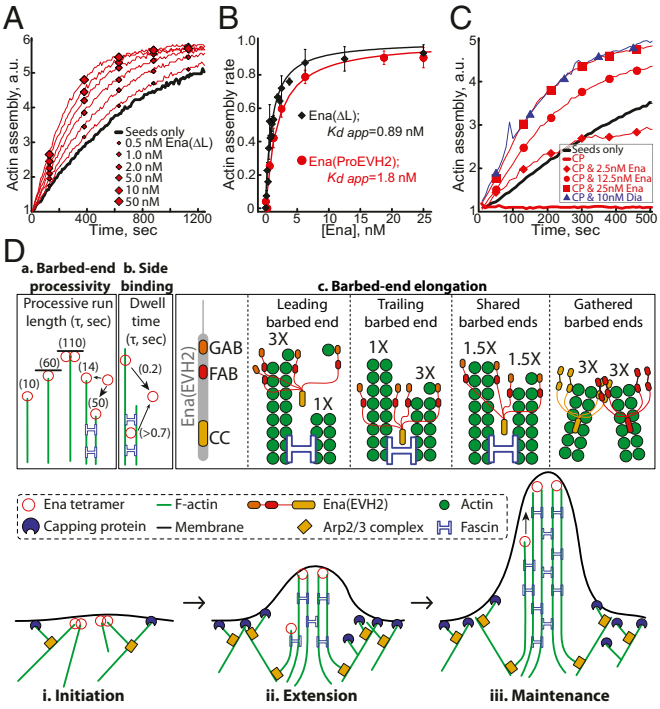


**Fig. 5.** Ena protects barbed ends from CP. (*A*–*C*) Seeded elongation: 0.25-μM Mg-ATP G-actin (10% pyrene-actin) added to barbed ends of 0.5-μM preassembled F-actin seeds. (*A*) Time course of actin elongation from seeds alone (thick black curve) or in the presence of indicated Ena(ΔL) concentrations (red curves). (*B*) Dependence of initial assembly rates on Ena concentration, revealing apparent barbed-end equilibrium dissociation constants: Ena(ΔL), 0.89 nM (black trace); Ena(ProEVH2),1.8 nM (red trace). Error bars indicate SEM, $n = 4$. (*C*) Time course for seeded assembly alone (thick black curve) or seeds with 4-nM CP alone (thick red curve); with 4-nM CP plus the indicated concentrations of Ena(ΔL) (traces with red diamonds, circles, and squares); or formin Dia(FH1FH2) (trace with blue triangles). (*D*) Cartoon models of Ena's tunable actin-assembly properties (*a*–*c*) and filopodia formation (*i*–*iii*). (*D*, *a*) Ena takes longer processive runs when adsorbed to a surface (τ = 60 s), when multiple Ena tetramers are clustered (τ = 110 s), or when on a trailing Fascin-bundled filament (τ = 50 s) than when in solution (τ =10–14 s). Ena associates with Fascin-bundled trailing barbed ends approximately twofold faster than with leading barbed ends (arrows). (*D*, *b*) Ena remains associated with the sides of bundled F-actin longer than with single filaments. (*D*, *c*) Regardless of context, Ena-associated individual barbed ends elongate two to three times faster than free barbed ends, and shared (barbed ends elongate 1.5 times faster than free barbed ends. (*D*, *i*) Barbed ends generated by Arp2/3 complex are gathered and protected from CP by Ena. (*D*, *ii*) Elongating Ena-protected filaments are bundled by Fascin, which enhances Ena-mediated processive elongation. (*D*, *iii*) Filopodia filaments remain uniformly long because Ena processively elongates Fascin-bundled trailing F-actin barbed ends more often and for longer durations.

domains that are not associated with the barbed end can interact with adjacent filaments in the bundle (Fig. 5 *D*, *c*), thereby preventing tetramers from diffusing away and increasing the likelihood that they reassociate with the barbed end at very fast timescales. This idea is supported by increased Ena dwell times on the sides of filaments as a function of bundle size (Fig. 4*H*). Similarly, processive run lengths are approximately sixfold longer (60 s) when Ena is absorbed to a surface (Fig. 2*D*); the adsorption may mimic the physiological state of Ena association with an adjacent filament in a bundle or with a membrane via its EVH1 domain.

Interestingly, when two barbed ends become closely apposed in a Fascin-mediated bundle, a single Ena tetramer can track both barbed ends (Figs. 4*D′* and 5 *D*, *c*), a scenario previously predicted (11). When Ena runs on two barbed ends, the elongation rate of both filaments drops from ∼2.5-fold to ∼1.5-fold

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faster than control filaments (Fig. 4*E*). On a single barbed end all four GAB/FAB pairs likely contribute to elongation, whereas only two GAB/FAB pairs contribute to each barbed end when two filaments share a single tetramer. The elongation rate also may drop because increased coordination is required for a single Ena tetramer to be positioned correctly to add actin monomers onto different barbed ends.

**Ena- and Fascin-Mediated Assembly of Filopodia-Like Networks.** The combination of Ena's diverse actin-assembly properties and Fascin-mediated F-actin bundling is tailored to initiate, extend, and maintain filopodia-like F-actin networks of uniform thickness without jagged ends (Fig. 5 *D*, *i–iii*). For filopodia initiation (Fig. 5 *D*, *i*), Ena associates with and gathers multiple new filament ends, protecting them from CP. We suspect the Arp2/3 complex nucleates filaments that are remodeled by Ena/VASP and Fascin to build filopodia by convergent elongation (5). Ena may be one protein facilitating the junctions observed by electron microscopy between barbed ends at lamellipodia of migrating cells (5). It will be important to add Arp2/3 complex to our reconstitution studies, as has been done with formin (31). In most cases multiple Ena tetramers gather barbed ends (Fig. 3*E*), suggesting that the clustering of membrane-associated Ena/VASP-binding proteins such as IRsp53 (32) or lamellipodin (33) will facilitate Ena/VASP-mediated barbed-end gathering.

Robust filopodia-like networks can be extended (Fig. 5 *D*, *ii*) and maintained (Fig. 5 *D*, *iii*) by a unique mechanistic cycle mediated by Ena and Fascin. By remaining processively associated with elongating barbed ends, Ena allows filaments to become longer by both increasing the elongation rate and antagonizing CP. Multiple Ena-associated filaments then are well suited for bundling by Fascin, which in turn promotes the ability of trailing Ena-associated barbed ends to catch up with the filopodia tip. Meanwhile, Ena quickly dissociates from leading barbed ends after only moderate processive runs (~10 s) (Fig. 4*G*), slowing their elongation. Because Ena remains associated with the sides of Fascin-bundled filaments approximately fivefold longer than with individual filaments (Fig. 4*H*), trailing barbed ends pick up a new Ena tetramer approximately twice as fast as leading barbed ends (Fig. 4*F*). Trailing Ena-associated barbed ends thus catch up with leading barbed ends because (*i*) Ena increases the elongation rate, and (*ii*) Ena's processive runs on Fascin-bundled trailing barbed ends are approximately fivefold longer (50 s) (Fig. 4*G*). At high Fascin concentrations, Ena processively surfs on multifilament bundles for extremely long runs that can last minutes (Fig. 4*A*), likely enhancing protection from CP. Fascin therefore appears to be a multifunctional hub for filopodia formation. In addition to bundling F-actin and positively modulating Ena's actin-assembly properties, Fascin also collaborates with the formin Daam1 to bundle F-actin (34).

## Materials and Methods

TIRFM images were collected at 1- to 4-s intervals with an iXon EMCCD camera (Andor Technology) using an Olympus IX-71 microscope fit with through-the-objective TIRF illumination. Photobleaching TIRFM images were collected continuously (10–20 frames/s) in the 561-nm channel, with the 488-nm shutter opened every 20–40 frames to visualize actin. Mg-ATP-actin (15–33% Oregon Green-actin) was mixed with 2× TIRF buffer [1×: 10 mM imidazole (pH 7.0), 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 50 mM DTT, 0.2 mM ATP, 50 μM $CaCl_2$, 15 mM glucose, 20 μg/mL catalase, 100 μg/mL glucose oxidase, and 0.5% (400 centipoise) methylcellulose] and Ena constructs (with or without 3.0-μM profilin, 50-nM Fascin, or 500-nM Fascin) and was transferred to a flow cell for imaging at 23 °C. For two-color TIRFM, we cyclically imaged Oregon-green actin (one frame, 488-nm excitation for 50 ms) and SNAP-Ena (one frame, 561-nm excitation for 50 ms).

Additional materials and methods can be found in *SI Materials and Methods*.

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