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# **Cyclase-associated protein is a pro-formin anti-capping processive depolymerase of actin barbed and pointed ends**

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1 **Cyclase-associated protein is a pro-formin anti-capping processive depolymerase of actin**  
2 **barbed and pointed ends**

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8 **Key words:** Cyclase-associated protein (CAP), actin depolymerization, processive  
9 depolymerase, formin, capping protein

## 10 **Summary**

11 Cellular actin networks display distinct assembly and disassembly dynamics resulting from  
12 multicomponent reactions occurring primarily at the two ends and the sides of actin filaments [1-  
13 3]. While barbed ends are considered the hotspot of actin assembly [4], disassembly is thought  
14 to primarily occur via reactions on filament sides and pointed ends [3, 5-11]. Cyclase-associated  
15 protein (CAP) has emerged as the main protagonist of actin disassembly and remodeling – it  
16 collaborates with cofilin to increase pointed-end depolymerization by 300-fold [6, 7], promotes  
17 filament “coalescence” in presence of Abp1 [12], and accelerates nucleotide exchange to  
18 regenerate monomers for new rounds of assembly [13-15]. CAP has also been reported to  
19 enhance cofilin-mediated severing [16, 17], but these claims have since been challenged [7].  
20 Using microfluidics-assisted three-color single-molecule imaging, we now reveal that CAP also  
21 has important functions at filament barbed ends. We reveal that CAP is a processive barbed-end  
22 depolymerase capable of tracking both ends of the filament. Each CAP binding event leads to  
23 removal of about 5,175 and 620 subunits from the barbed and pointed ends respectively. We find  
24 that the WH2 domain is essential, and the CARP domain is dispensable for barbed-end  
25 depolymerization. We show that CAP co-localizes with barbed-end bound formin and capping  
26 protein, in the process increasing residence time of formin by 10-fold and promoting dissociation  
27 of CP by 4-fold. Our barbed-end observations combined with previously reported activities of CAP  
28 at pointed ends and sides, firmly establish CAP as a key player in actin dynamics.

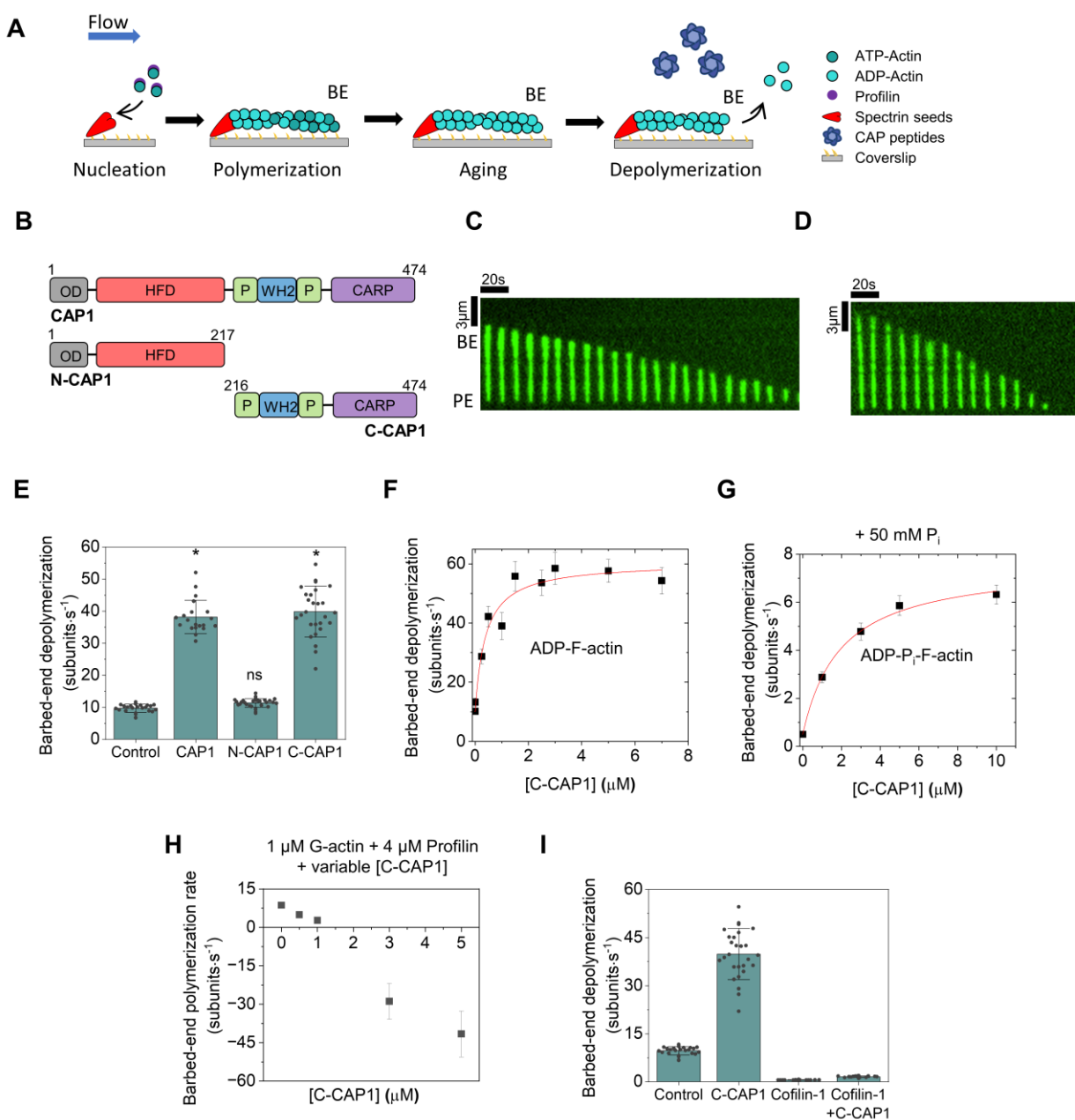
## 29 **RESULTS AND DISCUSSION**

### 30 **CAP is a barbed-end depolymerase**

31 We employed microfluidics-assisted total internal reflection fluorescence microscopy (mf-TIRF)  
32 to investigate effects of mouse CAP1 (referred to as CAP henceforth) on barbed-end  
33 depolymerization. Actin filaments with free barbed ends were elongated from coverslip-anchored  
34 spectrin-actin seeds by flowing in a solution containing Alexa-488 labelled G-actin and unlabeled  
35 profilin (profilin-actin) (Fig. 1A). The filaments were aged for 15 minutes to allow  $P_i$  release and  
36 conversion to ADP-F-actin following which they were exposed to TIRF buffer in absence or  
37 presence of CAP. While control filaments depolymerized at  $9.7 \pm 1.3$  subunits/s (su/s hereafter)  
38 ( $\pm$  sd), addition of  $1 \mu\text{M}$  CAP caused a 4-fold increase in depolymerization rate ( $38.2 \pm 5.2$  su/s)  
39 (Fig. 1C – E). Acceleration of barbed-end depolymerization by CAP is consistent with results from  
40 a recent study from the Goode lab [18]. Our observation of barbed-end depolymerization together  
41 with previous reports of 4-fold acceleration of depolymerization at pointed-ends [6, 13] establish  
42 CAP as a bona-fide depolymerase of both ends of actin filaments.

### 43 **Barbed-end depolymerization mediated by C-terminal half of CAP**

44 CAP is a modular protein whose N-terminal (N-CAP) and C-terminal (C-CAP) halves function  
45 autonomously in actin disassembly and monomer recycling (Fig. 1B) [19-21]. N-CAP consists of  
46 an oligomerization domain (OD), a helical-folded domain (HFD), and promotes pointed-end  
47 depolymerization [6, 7]. N-CAP has also been reported to bind filament sides and enhance cofilin-  
48 mediated severing [16, 17]. However, these conclusions have been challenged by a recent  
49 structural study which implied that actin-binding HFD domains of N-CAP could only be docked at  
50 the filament pointed end [7]. Consistently, the authors observed no enhancement of cofilin-  
51 mediated severing by N-CAP. C-terminal half of CAP or C-CAP consists of WH2 domain [22],  
52 CARP domain and polyproline motifs. WH2 and CARP domains bind G-actin, accelerate  
53 nucleotide exchange (from ADP to ATP) to regenerate ATP-actin monomers for new rounds of  
54 polymerization [13-15]. Lastly, while N-CAP monomers oligomerize into tetramers [23, 24] and/or  
55 hexamers [16, 17], C-CAP monomers self-assemble into dimers [15, 25].



56  
57 **Fig. 1: Mouse CAP1 accelerates actin filament barbed-end depolymerization.** (A)  
58 Schematic representation of the experimental strategy. Actin filaments with free barbed  
59 ends were polymerized by exposing coverslip-anchored spectrin-actin seeds to 1 μM G-  
60 actin (15% Alexa-488 labeled) and 4 μM profilin. The filaments were then aged for 15  
61 minutes and then exposed to 1 μM CAP (full length, N-CAP or C-CAP) or control buffer.  
62 Barbed-end depolymerization was monitored over time. BE, barbed end; (B) Domain  
63 diagram of mouse CAP1 constructs used in this study. (C) Representative kymograph of an

64 Alexa-488-labeled actin filament depolymerizing in control buffer. BE, barbed end; PE,  
65 pointed end. **(D)** Same as (C) but in the presence of 1  $\mu\text{M}$  CAP1. **(E)** Rates ( $\pm$  sd) of barbed-  
66 end depolymerization of ADP filaments in the presence of buffer (control), 1  $\mu\text{M}$  CAP1, 1  
67  $\mu\text{M}$  N-CAP1 or 1  $\mu\text{M}$  C-CAP1. \*, statistical comparison by two-sample t-test against control  
68 ( $p < 0.05$ ). ns, no evidence for significance at  $p = 0.05$ . Number of filaments analyzed for  
69 each condition (left to right): 25, 20, 30 and 29. **(F)** Rates ( $\pm$  sd) of barbed-end  
70 depolymerization of ADP filaments as a function of C-CAP1 concentration.  $N = 21$  filaments  
71 analyzed per concentration. The line is a fit to a hyperbolic binding curve. **(G)** Rates ( $\pm$  sd)  
72 of barbed-end depolymerization of ADP- $\text{P}_i$  filaments as a function of C-CAP1 concentration  
73 (same experimental strategy as in panel A was followed with the exception that filaments  
74 were maintained in 50 mM  $\text{P}_i$  throughout, see methods).  $N = 20$  filaments analyzed per  
75 concentration. The line is a fit to a hyperbolic binding curve. **(H)** Rates ( $\pm$  sd) of free barbed-  
76 end elongation in the presence of 1  $\mu\text{M}$  G-actin, 4  $\mu\text{M}$  profilin, and different concentrations  
77 of C-CAP1. Number of filaments analyzed for each condition (left to right): 20, 20, 20, 17,  
78 and 20. **(I)** Rates ( $\pm$  sd) of barbed-end depolymerization of ADP filaments in the presence  
79 of 1  $\mu\text{M}$  C-CAP1 and 5  $\mu\text{M}$  cofilin-1 (alone and together). Number of filaments analyzed for  
80 each condition (left to right): 25, 29, 20 and 20.

81 In light of previously reported depolymerization function of N-CAP, we wondered if it might also  
82 be responsible for barbed-end depolymerization by CAP. Free barbed ends of ADP-actin  
83 filaments were exposed to a solution containing either N-CAP or C-CAP. While N-CAP had no  
84 effect on barbed-ends, C-CAP accelerated their depolymerization by about 4-fold, similar to full-  
85 length CAP (Fig. 1E). Thus, the depolymerization action of CAP at the two ends is facilitated by  
86 its distinct halves i.e., N-CAP at the pointed and C-CAP at the barbed end. Additional analysis  
87 showed that C-CAP promotes depolymerization in a concentration-dependent fashion (Fig. 1F).  
88 At saturation, a maximal depolymerization rate of about 58 su/s, approximately 6-fold higher than  
89 control, was observed. These kinetics suggest that at lower C-CAP concentrations, binding of C-  
90 CAP to actin filaments might be rate-limiting.

### 91 **Effects of filament age, cofilin and polymerizable G-actin**

92 While actin networks *in vivo* can turn over in just a few seconds [26-29], depolymerization *in vitro*  
93 is orders of magnitude slower, mainly due to slow  $\text{P}_i$  release [30, 31]. We therefore wondered if  
94 C-CAP might also depolymerize unaged filaments. To prevent aging, actin filaments were  
95 maintained in TIRF buffer containing 50 mM  $\text{P}_i$ , as done previously [32]. We found that C-CAP

96 also accelerated barbed-end depolymerization of ADP-P<sub>i</sub> filaments (Fig. 1G). Notably, although  
97 average depolymerization rate of ADP-P<sub>i</sub> filaments by C-CAP was 10-fold lower than that of ADP  
98 filaments (~6 vs ~60 su/s), the fold-increase over control was almost double for ADP-P<sub>i</sub> than ADP  
99 filaments (12-fold vs 6-fold). Thus, C-CAP is capable of accelerating depolymerization of both  
100 aged and newly-assembled actin filaments.

101 While our experiments thus far were conducted in absence of G-actin, cytosol contains high  
102 amounts of assembly-competent actin monomers, majority of which are bound to profilin [2, 33,  
103 34]. We therefore investigated how presence of profilin-actin monomers affected  
104 depolymerization by C-CAP. In the presence of 1 μM profilin-actin, filament barbed ends  
105 elongated at  $8.6 \pm 0.5$  su/s (Fig. 1H). Further addition of C-CAP significantly altered this behavior.  
106 While low concentrations of C-CAP slowed net polymerization, higher concentrations induced net  
107 depolymerization. Addition of 1 μM profilin-actin to reactions containing 5 μM C-CAP led to a 30%  
108 reduction in net depolymerization rate from  $57.7 \pm 3.9$  su/s to  $41.8 \pm 8.9$  su/s. It is unclear whether  
109 this reduction results from sequestration of actin monomers by C-CAP or due to C-CAP's barbed  
110 end interaction. Notably, a known barbed-end depolymerase, twinfilin, also shows a similar  
111 behavior, both in absence (for ADP-P<sub>i</sub> filaments) and presence of polymerizable actin monomers  
112 [32].

113 We then asked if C-CAP, like N-CAP, might also synergize with cofilin [6, 7]. Supplementing C-  
114 CAP only reactions with cofilin (human Cofilin-1) almost completely extinguished barbed end  
115 depolymerization (Fig. 1I), leading to rates only slightly higher than cofilin-only reactions. Indeed,  
116 a previous study reported that cofilin's saturation of filament sides drastically reduces barbed-end  
117 depolymerization [35]. Our data suggests that C-CAP is not able to catalyze disassembly of  
118 cofilin-coated filaments. Cofilin thus has opposing effects on CAP's depolymerization abilities at  
119 the two ends of actin filaments.

## 120 **Barbed-end depolymerization by CAP is conserved and requires WH2 domains**

121 CAP is a highly conserved protein expressed across fungus, plants and vertebrates [3]. We  
122 therefore asked if barbed-end depolymerization by CAP was conserved. Similar to the  
123 mammalian homolog, the C-terminal half of *S. cerevisiae* Srv2 (C-Srv2) also accelerated  
124 depolymerization. Notwithstanding the qualitative similarity, even at high concentration (~5 μM)  
125 C-Srv2 only caused a 1.8-fold increase in depolymerization as compared to the 6-fold increase  
126 by C-CAP (Fig. 2A-C). Like N-CAP, N-Srv2 had no impact on barbed end depolymerization. Thus,  
127 combined with our previous pointed-end studies [6], our results here show that Srv2/CAP's

128 promotion of depolymerization at both filament ends is conserved between budding yeast and  
129 human homologs.

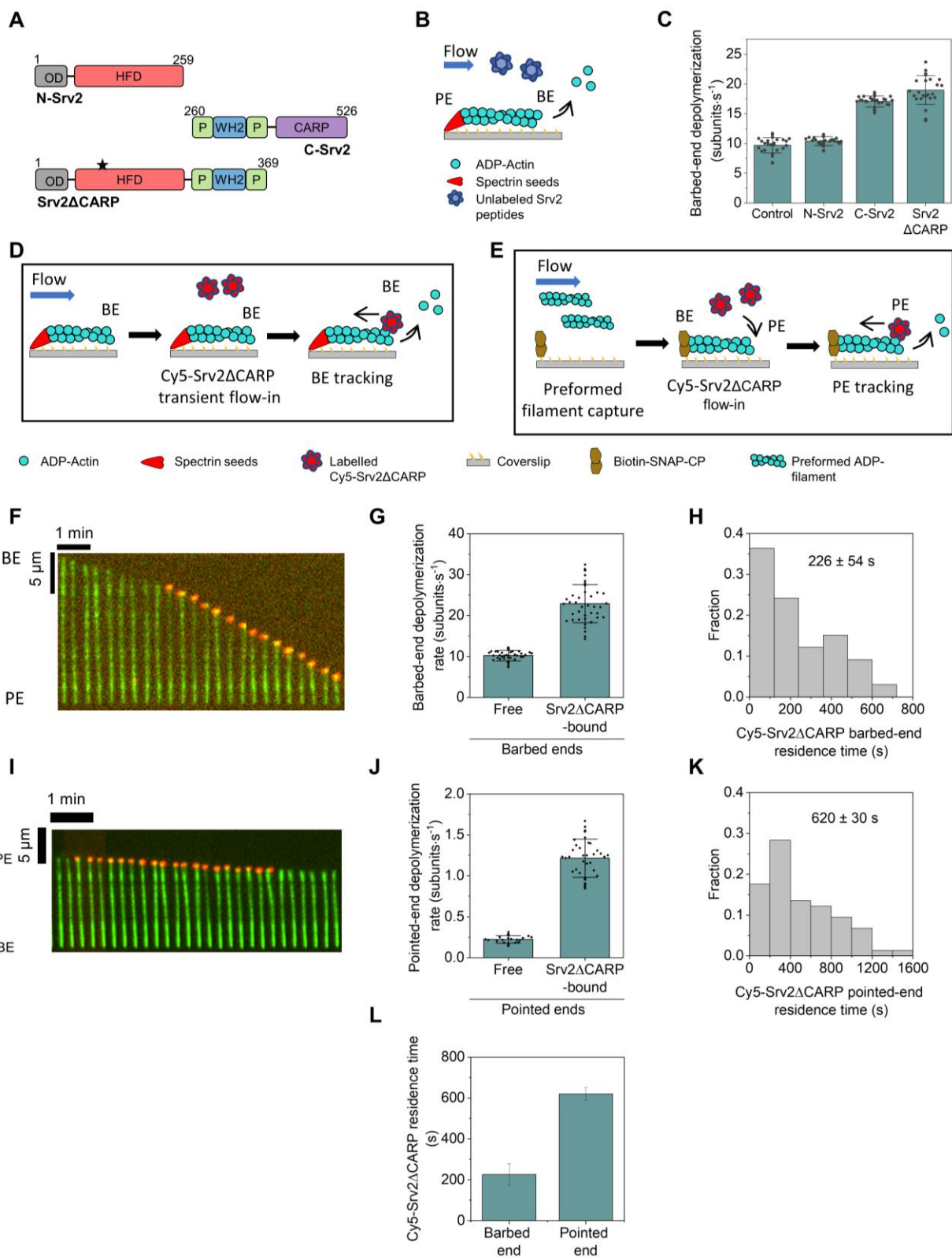
130 We then sought to determine which of the two actin-binding domains in the C-terminal half i.e.,  
131 WH2 or CARP, caused barbed-end depolymerization. We expressed and purified Srv2 $\Delta$ CARP  
132 (Fig. 2A), a new construct which contained the WH2 domain but not the CARP domain. In contrast  
133 with a recent study [18], we found that absence of CARP domain did not abolish barbed-end  
134 depolymerization (Fig. 2C). Instead, we observed that Srv2 $\Delta$ CARP and C-Srv2 promoted barbed  
135 end depolymerization equally well. Since the only actin binding motif common between the two  
136 constructs is the WH2 domain, our results imply that the WH2 domain is necessary, and CARP  
137 is dispensable for barbed-end depolymerization by Srv2/CAP.

### 138 **Srv2/CAP molecules processively track depolymerizing barbed ends**

139 To further reveal the molecular mechanism by which Srv2/CAP induces barbed-end  
140 depolymerization, we directly visualized Cy5-labelled Srv2 $\Delta$ CARP on actin filaments using  
141 single-molecule imaging. We decided to use Srv2 $\Delta$ CARP for single molecule experiments as  
142 Srv2 $\Delta$ CARP (monomers) contains only a single cysteine which can be fluorescently labelled in a  
143 residue-specific manner (Fig. 2C). In contrast, while CAP, C-CAP, and CAP1 $\Delta$ CARP monomers  
144 contain six, four and two, cysteines; Srv2 and C-Srv2 monomers contain 4 and 3 cysteines  
145 respectively. In addition, we have previously successfully used Cy5-Srv2 $\Delta$ CARP to visualize  
146 Srv2/CAP molecules on pointed-ends of cofilin-decorated filaments [6].

147 Free barbed ends of ADP-actin filaments were first transiently exposed to a flow containing Cy5-  
148 Srv2 $\Delta$ CARP (100 nM monomers) in the mf-TIRF chamber, followed by continuous exposure to  
149 TIRF buffer (Fig. 2D). We observed that Cy5-Srv2 $\Delta$ CARP molecules associated directly with actin  
150 filament barbed ends. All ends depolymerizing with a detectable Cy5 signal underwent rapid  
151 depolymerization. Consistently, the absence of Cy5 signal at the barbed end was accompanied  
152 by slow depolymerization and arrival of Cy5 signal was accompanied by initiation of rapid  
153 depolymerization (Fig. 2F,G). The depolymerization rate of Cy5-Srv2 $\Delta$ CARP-bound barbed ends  
154 was similar to maximal depolymerization seen at saturating concentrations of unlabeled  
155 Srv2 $\Delta$ CARP (Fig. 2C), suggesting that at saturating Srv2 $\Delta$ CARP, the filament end is almost  
156 continuously occupied by a Srv2 $\Delta$ CARP molecule. Each Cy5-Srv2 $\Delta$ CARP binding event on  
157 average lasted  $226 \pm 54$  seconds (Fig. 2H) and barbed ends with a visible Cy5 signal  
158 depolymerized at  $22.9 \pm 4.6$  su/s (Fig. 2G,H). Product of these two values yields the average  
159 number of subunits removed per Srv2 $\Delta$ CARP binding event,  $5,175 \pm 1,615$  subunits.





160

161

162

**Fig. 2: Srv2/CAP is an evolutionary conserved processive depolymerase of both ends of actin filaments. (A) Domain diagram of *S. cerevisiae* Srv2/CAP constructs used in this**

163 study. Black asterisk on Srv2 $\Delta$ CARP indicates the location of its single cysteine, which was  
164 used for dye-labeling. **(B)** Schematic representation of the experimental strategy. ADP-actin  
165 filaments with free barbed ends were assembled similarly as in Fig. 1A and exposed to the  
166 described Srv2 constructs. Blue colored Srv2 molecules represent unlabeled protein. **(C)**  
167 Rates ( $\pm$  sd) of barbed-end depolymerization of ADP filaments in the presence of buffer  
168 (control), 5  $\mu$ M N-Srv2, 5  $\mu$ M C-Srv2 and 5  $\mu$ M Srv2 $\Delta$ CARP. **(D)** Schematic representation  
169 of the experimental strategy for single-molecule imaging for visualizing Cy5-Srv2 $\Delta$ CARP at  
170 barbed ends. Red colored Srv2 $\Delta$ CARP molecules represent labeled protein. **(E)** Schematic  
171 representation of the experimental strategy for single-molecule imaging for visualizing Cy5-  
172 Srv2 $\Delta$ CARP at pointed ends. **(F)** Representative kymograph of an Alexa-488-labeled actin  
173 filament (green) with Cy5-Srv2 $\Delta$ CARP (red) processively tracking its barbed end. **(G)** Rates  
174 ( $\pm$  sd) of barbed-end depolymerization of free and Cy5-Srv2 $\Delta$ CARP-bound barbed ends in  
175 buffer (no free Cy5-Srv2 $\Delta$ CARP in solution). Number of filaments analyzed for each  
176 condition (left to right): 44 and 42. **(H)** Distribution of lifetimes of Cy5-Srv2 $\Delta$ CARP at barbed  
177 ends (n= 33 Cy5-Srv2 $\Delta$ CARP-bound ends). **(I)** Representative kymograph of an Alexa-488-  
178 labeled actin filament (green) with Cy5-Srv2 $\Delta$ CARP (red) processively tracking filament  
179 pointed end. **(J)** Rates ( $\pm$  sd) of barbed-end depolymerization of free and Cy5-Srv2 $\Delta$ CARP-  
180 bound pointed ends in buffer (no free Cy5-Srv2 $\Delta$ CARP in solution). Number of filaments  
181 analyzed for each condition (left to right): 25 and 40. **(K)** Distribution of lifetimes of Cy5-  
182 Srv2 $\Delta$ CARP at pointed ends (n= 74 Cy5-Srv2 $\Delta$ CARP-bound ends) **(L)** Average lifetime of  
183 Cy5-Srv2 $\Delta$ CARP at barbed and pointed ends.

184 Our single-molecule experiments imply that Srv2/CAP is a processive depolymerase of filament  
185 barbed ends. So far, *S. cerevisiae* twinfilin is the only other protein reported to processively  
186 depolymerize barbed ends [36]. Twinfilin's processive behavior has however been challenged in  
187 light of recent structural insights which implied that a twinfilin molecule might instead only cause  
188 dissociation of terminal actin subunits at the barbed end [37].

### 189 **Srv2/CAP molecules processively track depolymerizing pointed ends**

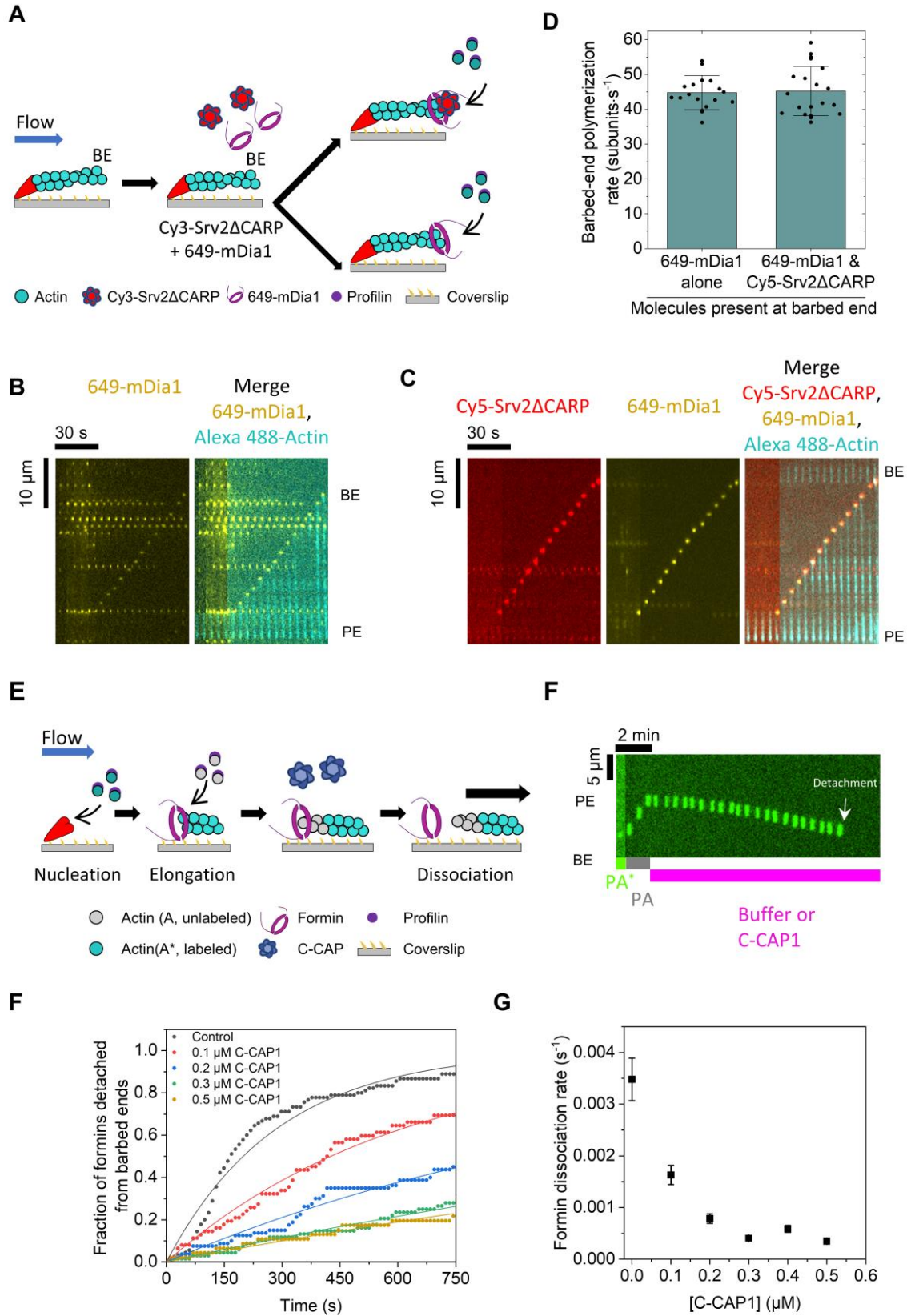
190 Two previous studies directly visualized fluorescently labelled Srv2/CAP molecules associating  
191 with pointed ends of cofilin-coated filaments [6, 7]. However, due to the transient nature of  
192 Srv2/CAP associations (~2 seconds) with pointed ends of cofilin-actin filaments, it was not  
193 possible to ascertain whether Srv2/CAP molecules translocated with depolymerizing pointed ends  
194 i.e., were processive. To address this question, we performed single-molecule imaging using Cy5-  
195 Srv2 $\Delta$ CARP. Preformed fluorescent ADP-actin filaments were captured by coverslip-anchored

196 capping protein in a mf-TIRF chamber. Upon a transient exposure to Cy5-Srv2 $\Delta$ CARP (100 nM),  
197 filaments were exposed continuously to TIRF buffer (Fig. 2E). We observed Cy5-Srv2 $\Delta$ CARP  
198 associating with filament pointed ends. All ends with a detectable Cy5 signal underwent rapid  
199 depolymerization (Fig. 2I,J). Appearance of Cy5-Srv2 $\Delta$ CARP signal was accompanied by  
200 beginning of rapid depolymerization. Consistently, the disappearance of Cy5-Srv2 $\Delta$ CARP was  
201 accompanied by resumption of slow depolymerization characteristic of free pointed ends  
202 depolymerizing in buffer (Fig. 2I). Each binding event on average lasted  $620 \pm 30$  seconds (Fig.  
203 2K, L). Pointed-ends with Cy5 signal depolymerized at  $1.2 \pm 0.2$  su/s (Fig. 2J,K), about 6-fold  
204 faster than filaments without a visible Cy5 signal. Product of these two values yields the average  
205 number of subunits removed per Srv2 $\Delta$ CARP binding event,  $743 \pm 130$  subunits. Our data thus  
206 supports the view that Srv2/CAP is a processive depolymerase of both ends of actin filaments.

207 **Srv2/CAP associates with formin-bound barbed ends and slows formin's dissociation**  
208 **without affecting elongation rate**

209 In light of direct binding of Srv2/CAP to barbed ends, we asked if Srv2/CAP might also associate  
210 with barbed ends bound to other ligands such as formin and capping protein, which either  
211 accelerate or arrest barbed assembly. We first studied formin. SNAP-tagged formin mDia1 (FH1-  
212 FH2-C) was expressed and labeled with benzylguanine functionalized fluorescent dye (649-  
213 mDia1). SNAP-tagging and labelling did not alter dimerization or actin assembly properties of  
214 formin, as seen previously [38-41].

215 Alexa-488 labeled actin filaments with free barbed ends were transiently exposed to either 649-  
216 mDia1 alone or together with Cy5-Srv2 $\Delta$ CARP, followed by a solution containing profilin-actin  
217 (Fig. 3A). In absence of Cy5-Srv2 $\Delta$ CARP, the majority of filaments only displayed 649-mDia1 at  
218 their barbed end (Fig. 3B). When 649-mDia1 and Cy5-Srv2 $\Delta$ CARP were simultaneously present,  
219 two distinct barbed-end populations were seen : 1) bound only to 649-mDia1 2) bound to both  
220 649-mDia1 and Cy5-Srv2 $\Delta$ CARP with the two molecules together tracking the elongating end  
221 (Fig. 3C). Importantly, the barbed-end elongation rate remained unchanged independent of  
222 whether formin was bound alone to barbed ends or together with Cy5-Srv2 $\Delta$ CARP (Fig. 3D).



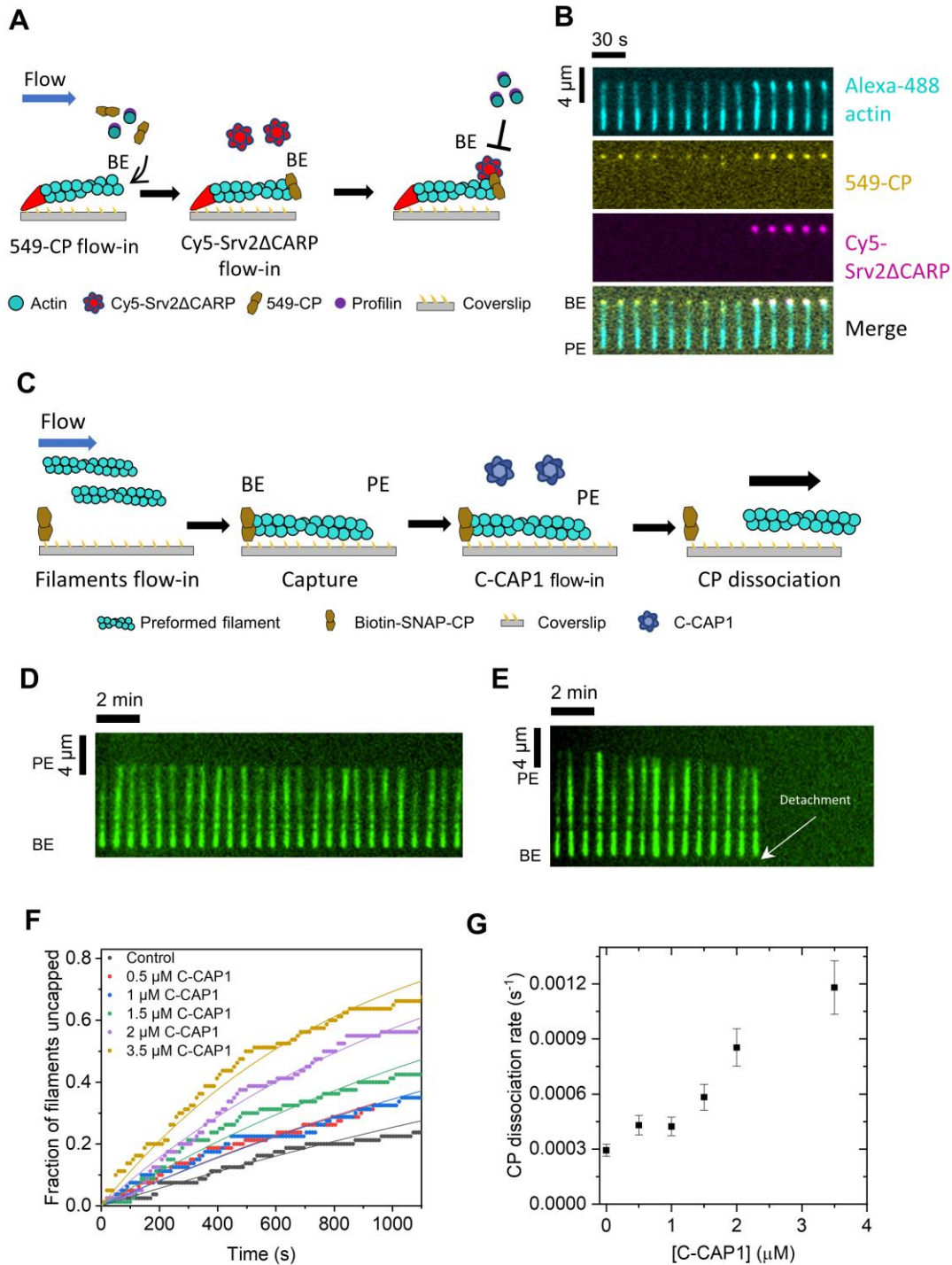
224 **Fig. 3: Srv2/CAP stabilizes barbed-end binding of formin (A)** Schematic representation  
225 of the experimental strategy. Actin filaments with free barbed ends were assembled similarly  
226 as in Fig. 1A and exposed to either 649-mDia1 alone or together with Cy3-Srv2 $\Delta$ CARP,  
227 and then exposed to 1  $\mu$ M Alexa-488 G-actin and 4  $\mu$ M profilin. Red colored Srv2 $\Delta$ CARP  
228 molecules represent labeled protein. **(B)** Representative kymograph of an Alexa-488-  
229 labeled actin filament (cyan) bound to 649-mDia1 (yellow) at the barbed end. **(C)**  
230 Representative kymograph of an Alexa-488-labeled actin filament (cyan) bound to 649-  
231 mDia1 (yellow) and Cy3-Srv2 $\Delta$ CARP (red) at the barbed end **(D)** Rates ( $\pm$  sd) of barbed-  
232 end elongation of 649-mDia-bound or 649-mDia- and Cy3-Srv2 $\Delta$ CARP-bound barbed ends  
233 elongating from 1  $\mu$ M Alexa-488 G-actin and 4  $\mu$ M profilin. Number of filaments analyzed  
234 for each condition: 20. **(E)** Schematic representation of the experimental strategy. Actin  
235 filaments were nucleated from coverslip-anchored formins by introducing a flow containing  
236 1  $\mu$ M G-actin (15% Alexa-488 labeled) and 0.5  $\mu$ M profilin. The filaments were then allowed  
237 to elongate in presence of 1  $\mu$ M unlabeled G-actin and 4  $\mu$ M profilin to ensure insertional  
238 elongation between fluorescent fragment and surface-anchored formins. These filaments  
239 were then exposed to a flow containing C-CAP in absence of profilin-actin. The survival  
240 fraction of filaments attached to formins was monitored as a function of time. Blue colored  
241 C-CAP molecules represent unlabeled protein. **(F)** Representative kymograph of a formin-  
242 anchored filament elongating from unlabeled monomers (see Fig. 3D) and then being  
243 exposed to buffer. Time point of filament detachment from formin is indicated. **(G)** Fraction  
244 of filaments attached to formin as a function of time in presence of varying concentrations  
245 of C-CAP. Experimental data (symbols) are fitted to a single-exponential function (lines) to  
246 determine formin dissociation rate. Number of filaments analyzed for each condition (from  
247 low to high C-CAP concentration): 90, 62, 80, 68, 46) **(H)** Formin dissociation rate as a  
248 function of C-CAP concentration, determined from data shown in (G).

249 We then asked if Srv2/CAP altered barbed-end processivity of formin. Since processivity is  
250 influenced by actin labelling fraction, elongation rate and presence of profilin [42], we employed  
251 an alternative strategy in (absence of profilin and G-actin) which we and others have previously  
252 used to study processivity of formin (Fig 3E) [38, 40, 42]. Actin filaments were nucleated by  
253 exposing coverslip-anchored formins to a solution containing fluorescent actin monomers and  
254 profilin (Fig. 3E). To ensure that insertional polymerization was occurring at anchored formins, a  
255 solution containing profilin and unlabeled actin monomers was introduced. As a result, fluorescent  
256 segments appeared to move along the flow as filaments elongated (Fig, 3E,F). These actin

257 filaments were then exposed to a solution containing a range of C-CAP concentrations (in  
258 absence of profilin-actin). The time-dependent disappearance of filaments from the field of view  
259 (due to their dissociation from surface-anchored formins) was analyzed to determine formin's  
260 dissociation rate from barbed ends. We found that formins remained bound to filaments for longer  
261 durations in the presence of C-CAP (Fig. 3F). Formin's dissociation rate decreased with  
262 increasing C-CAP concentration. At saturating C-CAP concentration, we observed a 10-fold  
263 increase in barbed end processivity of formin (Fig. 3G). Consistent with single-molecule analysis,  
264 C-CAP did not alter the depolymerization rate of formin-bound barbed ends. Together, our data  
265 suggests that while C-CAP stabilizes formin's association at barbed ends, it does not alter formin's  
266 effects on the polymerization/depolymerization rate.

### 267 **Srv2/CAP colocalizes with CP at barbed ends and accelerates uncapping**

268 We then sought to investigate if CAP might also alter CP's barbed-end binding. A SNAP-tagged  
269 construct of mouse capping protein (SNAP-CP) was expressed and labeled with benzylguanine  
270 functionalized green-excitable (549-CP) fluorescent dye. As seen previously, SNAP-tagging and  
271 labelling did not alter CP's interactions with the barbed-end [40, 41]. Alexa-488 labeled actin  
272 filaments with free barbed ends were transiently exposed to 549-CP in presence of profilin and  
273 G-actin (Fig. 4A). Appearance of a 549-CP signal at barbed ends coincided with filaments  
274 switching from elongating to paused state. Upon subsequent exposure to a solution containing  
275 Cy5-Srv2 $\Delta$ CARP and profilin-actin, we observed that Srv2 $\Delta$ CARP colocalized with 549-CP at  
276 barbed ends (Fig. 4B). During co-localization, filaments remained paused; no change in filament  
277 length was observed. We then asked if Srv2/CAP might influence barbed-end residence of CP?  
278 We employed a strategy similar to one used for investigating formins' processivity. Preformed  
279 fluorescently-labeled actin filaments were captured by coverslip-anchored CP in the mf-TIRF  
280 chamber (Fig. 4C). Time-dependent disappearance of filaments from the field of view was  
281 recorded and used to determine the uncapping rate. In presence of C-CAP, the uncapping rate  
282 increased linearly with C-CAP concentration (Fig D-G). Notably, although we only observed a  
283 maximum of 4-fold enhancement in CP's dissociation rate from the barbed end, given the linear  
284 nature of concentration-dependence we expect further enhancement at higher C-CAP  
285 concentrations. Our results add CAP to the list of proteins with uncapping abilities namely formin  
286 [38], CARMIL [43], twinfilin [44], cofilin [35], VopF [45].



287

288 **Fig. 4: Srv2/CAP co-localizes with CP at barbed ends and promotes uncapping. (A)**

289 Schematic representation of the experimental strategy. Actin filaments with free barbed

290 ends were assembled similarly as in Fig. 1A and were transiently exposed to 10 nM 549-

291 CP in presence of 1  $\mu$ M Alexa-488 G-actin and 4  $\mu$ M profilin. The filaments were then

292 exposed to 100 nM Cy5-Srv2 $\Delta$ CARP. Red colored Srv2 $\Delta$ CARP molecules in the schematic

293 represent labeled protein. **(B)** Representative kymograph of an Alexa-488-labeled actin  
294 filament with 549-CP and Cy5-Srv2 $\Delta$ CARP simultaneously bound to the barbed end. **(C)**  
295 Schematic representation of the experimental strategy. Pre-formed Alexa-488 labelled  
296 ADP-actin filaments were captured by coverslip-anchored biotinylated SNAP-CP anchored  
297 on the glass coverslip. Varying concentrations of C-CAP were then introduced into the  
298 chamber, and filament detachment was monitored. BE, barbed end; PE, pointed end. Blue  
299 colored C-CAP molecules represent unlabeled protein. **(D)** Representative kymograph of  
300 a CP-anchored filament in buffer **(E)** Same as (D) but with 3  $\mu$ M C-CAP **(F)** Fraction of  
301 filaments attached to CP as a function of time in presence of varying concentrations of  
302 C-CAP. Experimental data (symbols) are fitted to a single-exponential function (lines) to  
303 determine CP dissociation rate. Number of filaments analyzed for each condition: 80. **(G)**  
304 CP dissociation rate as a function of C-CAP concentration, determined from data shown in  
305 (F).

### 306 **Role of WH2 domains in barbed end effects of CAP**

307 WH2 domains have primarily been considered as actin monomer-binding motifs which promote  
308 nucleation [22, 46]. WH2-containing nucleators include SPIRE [47], Cordon Bleu [48], Leiomodin  
309 [49], VopF/VopL [50, 51], and Sca2 [52, 53]. WH2 binding to barbed ends is considered less  
310 favorable due to the flatter F-actin conformation. How then might WH2 domain of CAP interact  
311 with the barbed end? There are two possible lines of reasoning. First, although the WH2-binding  
312 site is largely obscured on subunits in the filament, the binding site is fully exposed at the barbed  
313 end [46]. Second, a high-resolution barbed-end structure recently published in a landmark study  
314 by the Dominguez lab showed that the W loop and the C-terminus of terminal actin's hydrophobic  
315 cleft undergoes a G-actin-like conformation at the barbed end [54]. These conformational changes  
316 likely play a role in barbed-end effects of WH2-containing proteins like CAP, N-WASP [55], WASP  
317 [56] and VopF [45]. To our knowledge, ours is the first report of WH2 domains facilitating  
318 processive barbed-end depolymerization.

319 In addition to binding free barbed and pointed ends, we find that CAP can also bind formin-bound  
320 and CP-bound barbed ends. Co-localization of CAP with either of these two proteins does not  
321 alter their respective individual effects on barbed-end elongation. Similar observations were  
322 previously made for budding yeast twinfilin i.e., presence of yeast Srv2 increased the processivity  
323 but not barbed-end depolymerization rate of twinfilin [36]. Interestingly, the increase in  
324 processivity of twinfilin was mediated by the N-terminal half of Srv2 and not the C-terminal half!  
325 Thus, CAP alters barbed-end residence time of elongators (formin), blockers (capping protein)



326 and depolymerases (twinfilin). In future, it will be interesting to investigate if CAP might also co-  
327 localize with formin-CP-twinfilin complex and influence the dynamics of this multiprotein barbed-  
328 end complex [40].

329 We believe our results bring new insights into how multiple proteins might co-exist at the barbed  
330 end. CP binds the barbed end primarily via interactions of its  $\beta$ - and  $\alpha$ -tentacle with hydrophobic  
331 cleft of the terminal and penultimate actin subunits [54, 57, 58]. WH2 domains also target the  
332 same hydrophobic cleft on actin [59]. Deletion of  $\beta$ -tentacle “only” weakens barbed-end affinity of  
333 CP by 300-fold in comparison to 5000-fold weakening in absence of  $\alpha$ -tentacle [58]. We speculate  
334 that destabilization of CP by CAP is primarily caused by a competition between the  $\beta$ -tentacle and  
335 the WH2 domain as reported recently [59]. This is likely further aided by splaying of the barbed-  
336 end subunits due to CP binding [54]. We believe these structural observations open the possibility  
337 for a plausible mechanism for destabilization of CP by WH2 domains, and allowing for faster  
338 uncapping by CAP. Another WH2 domain protein VopF has also been previously reported to  
339 displace CP from barbed ends [45].

340 While our manuscript was under preparation, we became aware of another study with results that  
341 partially overlap with our findings [18]. Although effects of CAP on barbed end depolymerization  
342 are qualitatively consistent between the studies, ADP-actin depolymerization rates were 4-fold  
343 faster rate in our study (~58 su/s vs 15 su/s). We believe this disparity originates from differences  
344 in the filament aging process. While we aged filaments for 15 minutes after polymerization to  
345 explicitly convert them to ADP-actin, filaments in the other study were immediately exposed to  
346 CAP following their polymerization to mimic *in vivo* conditions. As a result, the filaments likely  
347 contained a mix of ADP and ADP-P<sub>i</sub> subunits. Further, Alimov et al also report that CARP domains  
348 are required for barbed-end depolymerization. They found that a modified C-CAP construct  
349 consisting of poly-proline regions, WH2 motif and a GST tag but not the CARP domain failed to  
350 promote depolymerization. In contrast, we found that Srv2 $\Delta$ CARP which contained the WH2  
351 domain but lacked the CARP domain depolymerized barbed ends as fast as C-Srv2 which  
352 contained both WH2 and CARP. Additionally, the authors found that C-CAP promotes dissociation  
353 of formin from barbed ends. In contrast, we observe that C-CAP increased barbed-end residence  
354 time of formin by 4-fold. We further note that co-localization with CAP did not change the  
355 elongation rate of formin in our experiments.

356

## 357 **Concluding remarks and cellular implications**

358 Combining our observations with previously known activities of CAP, it depolymerizes barbed  
359 ends, accelerates cofilin-mediated pointed end depolymerization [6, 7], uncaps CP, stabilizes  
360 barbed-end binding of formin and twinfilin [36], collaborates with Abp1 to bundle filaments [12],  
361 and promotes nucleotide exchange [13-15]. In addition, a complex of lysine-acetylated actin and  
362 CAP regulates autoinhibition of formin INF2 [60]. In light of the wide diversity of effects CAP exerts  
363 on actin dynamics directly and via other proteins, it would not be an exaggeration to call CAP the  
364 “swiss army knife” of actin dynamics!

365 The phenomenon of actin “treadmilling” has formed the central bedrock of our understanding of  
366 actin dynamics for over three decades [1, 28]. “Treadmilling” entails actin filaments polymerizing  
367 at their barbed ends and depolymerizing from their pointed ends. Our discovery of barbed-end  
368 depolymerization by CAP together with previously reported barbed-end effects of twinfilin [32, 36]  
369 and pointed-end polymerization by VopF [61] further challenge the universality of actin  
370 “treadmilling”.

371 Although actin regulatory proteins have classically been studied one at a time, we now understand  
372 that in cells, they act simultaneously in multiprotein teams, often at the same site on a filament.  
373 Our results shed light on novel multicomponent activities of CAP at the barbed end i.e., promoting  
374 uncapping and stabilizing formin. It was previously shown that when present alone, CP promotes  
375 displacement of formin from the barbed end [38, 41]. However, addition of the uncapper twinfilin  
376 promoted formin-mediated actin assembly [40]. Since CAP increases twinfilin’s processivity [36]  
377 and uncaps CP[44], we speculate that in the complex intracellular milieu, CAP and twinfilin might  
378 together act as a highly potent uncapper and in turn promote formin-mediated assembly.  
379 Nevertheless, we are fully aware that future cellular studies are needed to test the physiological  
380 relevance of *in vitro* mechanisms and predictions reported here. We also believe that future Cryo-  
381 EM structural studies will be key to gaining deeper mechanistic insights on how CAP interacts  
382 with barbed ends, alone and together with formin and CP.

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389 **Author contributions:** EMT conducted experiments and analyzed data. EMT and SS prepared  
390 figures. SS designed experiments and supervised the project. SS wrote the manuscript and both  
391 authors contributed to the editing. SS acquired funding.

392 **Competing interests:** We declare no conflicts of interest.

## 393 **Methods**

### 394 **Purification and labeling of actin**

395 Rabbit skeletal muscle actin was purified from acetone powder generated from frozen ground  
396 hind leg muscle tissue of young rabbits (PelFreez, USA). Lyophilized acetone powder stored at  
397  $-80^{\circ}\text{C}$  was mechanically sheared in a coffee grinder, resuspended in G-buffer (5 mM Tris-HCl pH  
398 7.5, 0.5 mM Dithiothreitol (DTT), 0.2 mM ATP and 0.1 mM  $\text{CaCl}_2$ ), and cleared by centrifugation  
399 for 20 min at  $50,000 \times g$ . Supernatant was collected and further filtered with Whatman paper. Actin  
400 was then polymerized overnight at  $4^{\circ}\text{C}$ , slowly stirring, by the addition of 2 mM  $\text{MgCl}_2$  and 50 mM  
401 NaCl to the filtrate. The next morning, NaCl powder was added to a final concentration of 0.6 M  
402 and stirring was continued for another 30 min at  $4^{\circ}\text{C}$ . Then, F-actin was pelleted by centrifugation  
403 for 150 min at  $280,000 \times g$ , the pellet was solubilized by dounce homogenization and dialyzed  
404 against G-buffer for 48 h at  $4^{\circ}\text{C}$ . Monomeric actin was then precleared at  $435,000 \times g$  and loaded  
405 onto a Sephacryl S-200 16/60 gel-filtration column (Cytiva, USA) equilibrated in G-Buffer.  
406 Fractions containing actin were stored at  $4^{\circ}\text{C}$ .

407 To fluorescently label actin, G-actin was polymerized by dialyzing overnight against modified F-  
408 buffer (20 mM PIPES pH 6.9, 0.2 mM  $\text{CaCl}_2$ , 0.2 mM ATP, 100 mM KCl)[62]. F-actin was incubated  
409 for 2 h at room temperature with a 5-fold molar excess of Alexa-488 NHS ester dye (Thermo  
410 Fisher Scientific, USA). F-actin was then pelleted by centrifugation at  $450,000 \times g$  for 40 min at  
411 room temperature, and the pellet was resuspended in G-buffer, homogenized with a dounce and  
412 incubated on ice for 2 h to depolymerize the filaments. The monomeric actin was then re-  
413 polymerized on ice for 1 h by addition of 100 mM KCl and 1 mM  $\text{MgCl}_2$ . F-actin was once again  
414 pelleted by centrifugation for 40 min at  $450,000 \times g$  at  $4^{\circ}\text{C}$ . The pellet was homogenized with a  
415 dounce and dialyzed overnight at  $4^{\circ}\text{C}$  against 1 L of G-buffer. The solution was precleared by  
416 centrifugation at  $450,000 \times g$  for 40 min at  $4^{\circ}\text{C}$ . The supernatant was collected, and the  
417 concentration and labeling efficiency of actin was determined.

### 418 **Purification of profilin**

419 Human profilin-1 was expressed in *E. coli* strain BL21 (pRare) to log phase in LB broth at  $37^{\circ}\text{C}$   
420 and induced with 1 mM IPTG for 3 h at  $37^{\circ}\text{C}$ . Cells were then harvested by centrifugation at  
421  $15,000 \times g$  at  $4^{\circ}\text{C}$  and stored at  $-80^{\circ}\text{C}$ . For purification, pellets were thawed and resuspended in  
422 30 mL lysis buffer (50 mM Tris-HCl pH 8, 1 mM DTT, 1 mM PMSF protease inhibitors (0.5  $\mu\text{M}$   
423 each of pepstatin A, antipain, leupeptin, aprotinin, and chymostatin)) was added, and the solution

424 was sonicated on ice by a tip sonicator. The lysate was centrifuged for 45 min at 120,000 × *g* at  
425 4°C. The supernatant was then passed over 20 ml of poly-L-proline conjugated beads in a  
426 disposable column (Bio-Rad, USA). The beads were first washed at room temperature in wash  
427 buffer (10 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA and 1 mM DTT) and then washed again with  
428 2 column volumes of 10 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 1 mM DTT and 3 M urea.  
429 Protein was then eluted with 5 column volumes of 10 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA,  
430 1 mM DTT and 8 M urea. Pooled and concentrated fractions were then dialyzed in 4 L of 2 mM  
431 Tris pH 8, 0.2 mM EGTA, 1 mM DTT, and 0.01% NaN<sub>3</sub> (dialysis buffer) for 4 h at 4°C. The dialysis  
432 buffer was replaced with fresh 4 L buffer and the dialysis was continued overnight at 4°C. The  
433 protein was centrifuged for 45 min at 450,000 × *g* at 4°C, concentrated, aliquoted, flash frozen in  
434 liquid N<sub>2</sub> and stored at -80°C.

#### 435 **Purification of Cofilin-1**

436 Human Cofilin-1 was expressed in *E.coli* BL21 DE3 cells. Cells were grown in Terrific Broth to  
437 log phase at 37°C, and then expression was induced overnight at 18°C by addition of 1 mM IPTG.  
438 Cells were collected by centrifugation and pellets were stored at -80°C. Frozen pellets were  
439 thawed and resuspended in lysis buffer (20 mM Tris pH 8.0, 50 mM NaCl, 1 mM DTT, and  
440 protease inhibitors (0.5 μM each of pepstatin A, antipain, leupeptin, aprotinin, and chymostatin).  
441 Cells were lysed with a tip sonicator while being kept on ice. The cell lysate was centrifuged at  
442 150,000 × *g* for 30 min at 4°C. The supernatant was loaded on a 1 ml HisTrap HP Q column (GE  
443 Healthcare, Pittsburgh, PA), and the flow-through was collected and dialyzed against 20 mM  
444 HEPES pH 6.8, 25 mM NaCl, and 1 mM DTT. The dialyzed solution was then loaded on a 1 ml  
445 HisTrap SP FF column (GE Healthcare, Pittsburgh, PA) and eluted using a linear gradient of NaCl  
446 (20-500 mM). Fractions containing protein were concentrated, dialyzed against 20 mM Tris pH  
447 8.0, 50 mM KCl, and 1 mM DTT, flash frozen in liquid N<sub>2</sub> and stored at -80°C.

#### 448 **Purification and biotinylation of SNAP-CP**

449 SNAP-CP [41] was expressed in *E. coli* BL21 DE3 by growing cells to log phase at 37°C in TB  
450 medium, then inducing expression using 1 mM IPTG at 18°C overnight. Cells were harvested by  
451 centrifugation and pellets were stored at -80°C. Frozen pellets were resuspended in lysis buffer  
452 (20 mM NaPO<sub>4</sub> pH 7.8, 300 mM NaCl, 1 mM DTT, 15 mM imidazole, 1 mM PMSF) supplemented  
453 with a protease inhibitor cocktail (0.5 μM each of pepstatin A, antipain, leupeptin, aprotinin, and  
454 chymostatin). Cells were lysed by sonication with a tip sonicator while keeping the tubes on ice.  
455 The lysate was cleared by centrifugation at 150,000 × *g* for 30 min at 4°C. The supernatant was

456 then flowed through a HisTrap column connected to a Fast Protein Liquid Chromatography  
457 (FPLC) system. The column with the bound protein was first extensively washed with the washing  
458 buffer (20 mM NaPO<sub>4</sub> pH 7.8, 300 mM NaCl, 1 mM DTT and 15 mM imidazole) to remove non-  
459 specifically bound proteins. SNAP-CP was then eluted with 250 mM imidazole in 20 mM NaPO<sub>4</sub>  
460 pH7.8, 300 mM NaCl, and 1 mM DTT. The eluted protein was concentrated and labelled either  
461 with Benzylguanine Biotin or SNAP-surface-549 (New England Labs) according to the  
462 manufacturer's instructions. Free biotin (or dye) was removed using size-exclusion  
463 chromatography by loading the labelled protein on a Superose 6 gel filtration column (GE  
464 Healthcare, Pittsburgh, PA) eluted with 20 mM HEPES pH 7.5, 150 mM KCl, 0.5 mM DTT.  
465 Fractions containing the protein were pooled, aliquoted, snap frozen in liquid N<sub>2</sub> and stored at -  
466 80°C.

#### 467 **Purification, labeling and biotinylation of formin mDia1**

468 Mouse his-tagged SNAP-mDia1 (FH1-FH2-C) formin was expressed in *E. coli*; BL21(DE3) pLysS  
469 cells. Cells were grown in Terrific Broth to log phase at 37°C. Expression was induced overnight  
470 at 18°C by addition of 1 mM IPTG. Cells were harvested by centrifugation at 11,200 × *g* for 15 min  
471 and the cell pellets were stored at -80°C. For purification, frozen pellets were thawed and  
472 resuspended in 35 mL lysis buffer (50 mM sodium phosphate buffer pH 8, 20 mM imidazole, 300  
473 mM NaCl, 1 mM DTT, 1 mM PMSF and protease inhibitors (0.5 μM each of pepstatin A, antipain,  
474 leupeptin, aprotinin, and chymostatin)). Cells were lysed using a tip sonicator while being kept on  
475 ice. The cell lysate was then centrifuged at 120,000 × *g* for 45 min at 4°C. The supernatant was  
476 then incubated with 1 mL of Ni-NTA beads (Qiagen, USA) while rotating for 2 h at 4°C. The beads  
477 were then washed three times with the wash buffer (50 mM sodium phosphate buffer pH 8, 300  
478 mM NaCl, 20 mM imidazole and 1 mM DTT) and were then transferred to a disposable column  
479 (Bio-Rad, USA). Protein was eluted using the elution buffer (50 mM phosphate buffer pH 8, 300  
480 mM NaCl, 250 mM imidazole and 1 mM DTT). Fractions containing the protein were concentrated  
481 and loaded onto a size exclusion Superdex 200 increase 10/300 GL column (Cytiva, USA) pre-  
482 equilibrated with 20 mM HEPES pH 7.5, 150 mM KCl, 10% glycerol and 0.5 mM DTT. The eluted  
483 protein was concentrated and labelled either with Benzylguanine Biotin or SNAP-surface-649  
484 (New England Labs) according to the manufacturer's instructions. Free biotin (or dye) was  
485 removed using a Superdex 200 increase 10/300 GL column (Cytiva, USA). Fractions containing  
486 the protein were pooled, aliquoted, snap frozen in liquid N<sub>2</sub> and stored at -80°C.

487

## 488 **Purification of CAP1 and Srv2 peptides**

489 Mouse CAP1 and *S. cerevisiae* Srv2 peptides were expressed as His-tagged constructs in *E. coli*  
490 BL21 pRARE or pLysS by growing cells to log phase at 37°C in TB medium. Full-length CAP1  
491 and C-CAP contained additional SUMO and 3C cleavage sites respectively [7]. Cells were  
492 induced with 1 mM IPTG at 18°C overnight. Cells were harvested by centrifugation and pellets  
493 were stored at -80°C. Frozen pellets were resuspended in 50 mM NaPO<sub>4</sub> pH 8.0, 1 mM PMSF,  
494 1 mM DTT, 20 mM imidazole, 300 mM NaCl and protease inhibitors as described above. Cells  
495 were lysed by sonication with a tip sonicator while keeping the tubes on ice. The lysate was  
496 cleared by centrifugation at 150,000 x g for 30 min at 4°C. The lysate was incubated for two hours  
497 with Ni-NTA beads (Qiagen). Non-specifically bound proteins were removed by washing the  
498 beads with 20 mM NaPO<sub>4</sub> pH 8.0, 50 mM imidazole, 300 mM NaCl and 1 mM DTT. The bound  
499 protein was then eluted using 250 mM imidazole in the same buffer. Fractions containing the  
500 protein were concentrated and then further purified by size-exclusion chromatography using  
501 Superdex 75 Increase or Superdex 200 Increase gel-filtration columns (Cytiva, USA) equilibrated  
502 in 5 mM HEPES pH7.4, 100 mM NaCl and 1 mM DTT. Peak fractions were pooled, the protein  
503 was aliquoted, snap frozen in liquid N<sub>2</sub> and stored at -80 °C.

504 For FL-CAP1 and C-CAP1, the His-tag was cleaved prior to gel-filtration. The eluted protein from  
505 Ni-NTA beads was first dialyzed against 20 mM HEPES pH 7.4, 300 mM NaCl and 2 mM DTT,  
506 and then incubated overnight either with SUMO protease (Sigma Aldrich) or PreScission  
507 Protease. The cleaved tags were removed by incubation with Ni-NTA and/or GST beads.

## 508 **Labeling of Srv2ΔCARP**

509 For fluorescent labeling of Srv2ΔCARP, the same procedure as above was followed for unlabeled  
510 Srv2ΔCARP with the exception that 1 mM DTT in the elution buffer was replaced with 0.2 mM  
511 Tris(2-carboxyethyl)phosphine (TCEP) [63]. The eluted fractions were concentrated and  
512 incubated with at least fivefold molar excess of Cy3- or Cy5-maleimide dye (GE Healthcare,  
513 Pittsburgh, PA) for 30 min at 25 °C and additionally for 14 h at 4 °C. The excess dye was then  
514 quenched by addition of 5 mM DTT. Free dye was then separated from labeled protein using a  
515 PD-10 column with 10 mM imidazole pH 8, 50 mM KCl, 1 mM DTT and 5% glycerol. Labeled  
516 protein was concentrated. The protein was then aliquoted, snap frozen in liquid N<sub>2</sub> and stored at  
517 -80 °C.

## 518 **Microfluidics-assisted TIRF (mf-TIRF) microscopy**

519 Actin filaments were assembled in microfluidics-assisted TIRF (mf-TIRF) flow cells [62]. For all  
520 experiments, coverslips were first cleaned by sonication in Micro90 detergent for 20 min, followed  
521 by successive 20 min sonications in 1 M KOH, 1 M HCl and 200 proof ethanol for 20 min each.  
522 Washed coverslips were then stored in fresh 200 proof ethanol. Coverslips were then washed  
523 extensively with H<sub>2</sub>O and dried in an N<sub>2</sub> stream. These dried coverslips were coated with 2 mg/mL  
524 methoxy-poly (ethylene glycol) (mPEG)-silane MW 2,000 and 2 µg/mL biotin-PEG-silane MW  
525 3,400 (Laysan Bio, USA) in 80% ethanol (pH 2.0) and incubated overnight at 70°C. A 40 µm high  
526 PDMS mold with 3 of 4 inlets and 1 outlet was mechanically clamped onto a PEG-Silane coated  
527 coverslip. The chamber was then connected to a Maesflo microfluidic flow-control system  
528 (Fluigent, France), rinsed with TIRF buffer (10 mM imidazole pH 7.4, 50 mM KCl, 1 mM MgCl<sub>2</sub>, 1  
529 mM EGTA, 0.2 mM ATP, 10 mM DTT, 1 mM DABCO) and incubated with 1% BSA and 10 µg/mL  
530 streptavidin in 20 mM HEPES pH 7.5, and 50 mM KCl for 5 min. Depending upon the needs of  
531 specific experiments, spectrin-seeds, biotinylated-capping protein or biotinylated-formin was  
532 then anchored on the surface by flowing them in TIRF buffer for 5 min.

533 For ADP-P<sub>i</sub> experiments (Fig. 1G), the filaments were maintained in modified TIRF buffer (regular  
534 TIRF buffer supplemented with inorganic phosphate : 10 mM imidazole pH 7.4, 34.8 mM K<sub>2</sub>HPO<sub>4</sub>  
535 and 15.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.2 mM ATP, 10 mM DTT, 1 mM DABCO)  
536 throughout the experiment.

### 537 **Image acquisition and analysis**

538 Multi-wavelength time-lapse TIRF imaging was performed on a Nikon-Ti2000 inverted  
539 microscope equipped with a 40 mW 488 nm, 561 nm and 640 nm Argon lasers, a 60X TIRF-  
540 objective with a numerical aperture of 1.49 (Nikon Instruments Inc., USA) and an IXON LIFE 888  
541 EMCCD camera (Andor Ixon, UK). One pixel was equivalent to 144 × 144 nm. Focus was  
542 maintained by the Perfect Focus system (Nikon Instruments Inc., Japan). Time-lapsed images  
543 were acquired using Nikon Elements imaging software (Nikon Instruments Inc., Japan).

544 Images were analyzed in Fiji [64]. Background subtraction was conducted using the rolling ball  
545 background subtraction algorithm (ball radius 5 pixels). Time-lapsed images were corrected for  
546 drift using Fiji Image Stabilizer plugin. For each condition, filaments were acquired across multiple  
547 fields of view. To determine the rate of depolymerization, the in-built kymograph plugin was used  
548 to draw kymographs of individual filaments. The kymograph slope was used to calculate barbed-  
549 or pointed-end depolymerization rate of each individual filament (assuming one actin subunit  
550 contributes 2.7 nm to filament length). Data analysis and curve fitting were carried out in Microcal



551 Origin. All experiments were repeated at least three times and yielded similar results. Unless  
552 otherwise mentioned, the data shown are from one trial.

553 **Data availability:** Data supporting the findings of this manuscript are available from the  
554 corresponding author upon reasonable request.

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