The actin-binding protein cortactin promotes the formation and maintenance of actin-rich structures, including lamellipodial protrusions in fibroblasts and neuronal dendritic spines. Cortactin cellular functions have been attributed to its activation of the Arp2/3 complex, which stimulates actin branch nucleation, and to its recruitment of Rho family GTPase regulators. Cortactin also binds actin filaments and significantly slows filament depolymerization, but the mechanism by which it does so and the relationship between actin binding and stabilization are unclear. Here we elucidated the cortactin regions that are necessary and sufficient for actin filament binding and stabilization. Using actin cosedimentation assays, we found that the cortactin repeat region binds actin but that the adjacent linker region is required for binding with the same affinity as full-length cortactin. Using total internal reflection fluorescence microscopy to measure the rates of single filament actin depolymerization, we observed that cortactin–actin interactions are sufficient to stabilize actin filaments. Moreover, conserved charged residues in repeat 4 were necessary for high-affinity actin binding, and substitution of these residues significantly impaired cortactin-mediated actin stabilization. Cortactin bound actin with higher affinity than did its paralog, hematopoietic cell-specific Lyn substrate 1 (HS1), and the effects on actin stability were specific to cortactin. Finally, cortactin stabilized ADP–actin filaments, indicating that the stabilization mechanism does not depend on the actin nucleotide state. Together, these results indicate that cortactin binding to actin is necessary and sufficient to stabilize filaments in a concentration-dependent manner, specific to conserved residues in the cortactin repeats, and independent of the actin nucleotide state.

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1 To whom correspondence should be addressed: Depts. of Molecular Biophysics and Biochemistry and Neuroscience, Yale University, 333 Cedar St., New Haven, CT 06520. Tel.: 203-785-5624; Fax: 203-785-7979; E-mail: anthony.koleske@yale.edu.

Actin filament networks provide mechanical support as well as contractile forces essential for directed cell motility and cell and tissue morphogenesis (1, 2). Proteins that regulate actin polymerization, stability, and branching are critical for cells to form diverse actin-rich subcellular structures from a common pool of monomers (3, 4). The actin-binding protein cortactin promotes the formation and maintenance of actin-rich structures in diverse cell types. Cortactin is essential for dynamic protrusions of lamellipodia (5–7), maturation of invadopodia in invasive cancer cells (8–11), integrity of endothelial cell adherens junctions (12, 13), and maintenance of dendritic spine structure in neurons (14, 15). Cortactin functions in cellular structures have been predominantly attributed to its binding and activation of the Arp2/3 complex, which stimulates actin filament branching (16–18), as well as its participation in Rho family GTPase signaling (11, 19, 20). The necessity of cortactin Arp2/3 complex activation in cell protrusion and motility depends on cellular context (21–24), suggesting that cortactin has underappreciated, Arp2/3 complex–independent functions in actin-rich structures.

In addition to its roles in Arp2/3 complex activation and cell signaling, cortactin has direct effects on actin filament stability. Cortactin employs 6.5–37-amino acid “cortactin repeats” to bind actin filaments (25). These cortactin repeats and its N-terminal acidic (NTA)2 domain mediate proper cortactin localization in fibroblasts and neurons (5, 15). EM has revealed a 23-Å-resolution structure of cortactin-bound actin filaments (26), but the cortactin repeat region is natively unfolded in solution (27), limiting any high-resolution structural details of the cortactin repeat fold or the cortactin–actin interaction interface. Previous analyses of cortactin truncation or deletion mutants indicate that cortactin repeat 4 is necessary for actin binding, lamellipodial protrusion, and exosome secretion (5, 6, 28) and that cortactin splice variants lacking repeats 5 and 6 have reduced abilities to bind actin and support cell migration (29). In vitro, cortactin-bound actin filaments depolymerize significantly slower than actin filaments alone, and this effect depends on the concentration of cortactin in solution (30). However, it is not known whether cortactin-mediated actin stabilization requires the NTA domain, proline-rich domain, or Src homo-

2 The abbreviations used are: NTA, N-terminal acidic (domain); SH3, Src homology 3; TIRF, total internal reflection fluorescence; CR, cortactin repeat; ANOVA, analysis of variance; mW, milliwatt; GST, glutathione S-transferase; TEV, tobacco etch virus; HABA, 4′-hydroxyazobenzene-2-carboxylic acid.
Cortactin–actin interactions stabilize actin filaments

The cortactin repeat region binds actin, and the affinity is increased by a 77-amino acid adjacent linker.

We used cosedimentation assays to identify the regions of cortactin that mediate high-affinity binding to actin filaments. Cortactin bound actin with a $K_d$ of $0.79 \pm 0.16 \mu M$, similar to previous measurements (Fig. 1, C, F, and G) (25, 31). The cortactin fragment containing cortactin repeats (CRs) 1–6 (CR16) bound actin with significantly lower affinity ($K_d = 2.7 \pm 0.3 \mu M$, $p < 0.0001$) compared with full-length cortactin (Fig. 1, E–G, and Figs. S1). A cortactin construct containing CR16 and the adjacent 77-amino acid linker region, CR16L, bound to actin with a $K_d$ of $0.77 \pm 0.19 \mu M$, similar to full-length cortactin (Fig. 1, D, F, and G).

We also tested which repeats within the CR region (Fig. 2, A and B) mediate actin binding. Only trace amounts of CR 1–4 bound to actin in the pellet, even when present at 12 \mu M, suggesting that this region binds actin weakly, if at all (Fig. 2, C, F, and G). CR 3–6 bound actin, albeit with significantly lower affinity than CR16. We could not saturate filaments with CR 3–6 and can only place a limit on the affinity of $K_d \approx 7.0 \mu M$ (Fig. 2, D, F, and G). CR 3–6 with the adjacent linker region, CR36L, bound actin filaments with a higher affinity ($K_d = 2.6 \pm 0.7 \mu M$) (Fig. 2, E–G), but this affinity was significantly lower than that of CR16L ($p < 0.0001$). Our data indicate that six cortactin repeats and the adjacent segment are sufficient to recapitulate high-affinity actin binding similar to full-length cortactin.

Our measurements also revealed the stoichiometry of each cortactin protein that saturated binding to actin filaments. Full-length cortactin bound at a ratio of 1 cortactin:5 actin monomers, as published previously (Fig. 1G) (31, 32). CR16L bound at a ratio of 1 CR16L:2.5 monomers, and CR36L bound at almost a 1:1 molar ratio with actin monomers (Figs. 1G and 2G).

Figure 1. The cortactin repeats bind actin, and the affinity is increased by the adjacent linker. A and B, domain architecture and purified recombinant protein of cortactin and truncations containing the cortactin repeat region (CR16 and CR16L). Purified proteins were separated by SDS-PAGE and visualized with Coomassie Blue R250. C–E, cosedimentation assays measuring binding of cortactin, CR16, and CR16L to actin filaments. The supernatant ($S$, 27% of total) and pellet ($P$) were analyzed by SDS-PAGE and Coomassie Blue staining, with quantification of cortactin and actin in the pellet using densitometry. Experiments were performed in triplicate at nine concentrations for cortactin and six concentrations for CR16 and CR16L (F and G). Densitometry values of cortactin:actin bound are plotted versus the concentration of cortactin to determine a dissociation constant ($K_d$) and maximum stoichiometry ($B_{\text{max}}$). Each individual replicate is shown. A t test was performed between cortactin and CR16L, $p < 0.0001$.
Cortactin–actin interactions stabilize actin filaments

Differences in cortactin:actin stoichiometry have been observed in the presence of other actin- and cortactin-interacting proteins, like the Abl2 tyrosine kinase (31). Here, all cortactin fragments lacking the NTA, proline-rich, and SH3 domains bound actin filaments at higher stoichiometry than full-length cortactin. Removal of the flanking NTA and SH3 domains may remove a steric hindrance to CR–actin interactions.

Cortactin inhibits actin filament depolymerization

We next sought to determine the minimal domains in cortactin capable of stabilizing actin filaments (30) by measuring fluorescent actin filament depolymerization rates in vitro using TIRF microscopy. In the initial phase of our work, we noted frequent pausing during filament depolymerization. Although this phenomenon has been ascribed to photoinduced cross-linking under some circumstances (33), we observed pausing under even very low laser illumination (0.015 mW/100 ms), and pausing frequency did not scale with illumination intensity. We reasoned that pauses may result from the tethering of biotinylated actin to streptavidin linkages on the coverslip. To circumvent this limitation, we first grew biotinylated actin filament seeds tethered to the coverslip via streptavidin. We then grew nonbiotinylated untethered barbed ends from these seeds with 0.24 μM actin monomers for 15 min, washed out free actin monomers, and monitored filament shortening rate (Fig. 3A). Consistent with other studies of untethered filaments (34–36), filaments shortened with fewer pauses. The slow growth of untethered barbed ends for 15 min provided several half-lives of phosphate release from ADP–P_i–bound subunits in the filament (35, 37).

We used this single-filament approach to measure the impact of cortactin on actin stability. Following actin monomer washout, control actin filaments grown slowly from ATP–actin monomers depolymerized at a rate of −5.8 ± 1.5 subunits/s (Fig. 3, B–D), a rate very close to that reported previously for ADP–actin depolymerization using TIRF microscopy (38). Inclusion of 1 μM cortactin slowed actin filament shortening to −1.1 ± 0.3 subunits/s. Based on its K_p, cortactin is bound at 56% of its saturation density, about 1 cortactin:9 actin subunits at this concentration. In 0.15 μM cortactin, where cortactin is bound at 16% saturation, actin depolymerized at a rate corresponding to actin alone. At an intermediate concentration, 0.35 μM, at which cortactin is bound at only 30% saturation, the rate of depolymerization was intermediate (~2.5 ± 0.4 subunits/s) (Fig. 3E and Movie S1). In all, observation of untethered barbed ends reveals that cortactin stabilizes actin in a concentration-dependent manner and that it can function even at subsaturating concentrations.

Cortactin–actin interactions are necessary and sufficient to stabilize filaments

We next tested which cortactin domains were necessary and sufficient for actin filament stabilization using truncated cortactin proteins (Fig. 4, A and B). The cortactin fragment lacking the Arp2/3 complex–binding NTA domain (cortΔNTA) stabilized actin filaments when present at 1 μM (shortening rate of
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Figure 3. Cortactin decreases the rate of actin depolymerization from untethered barbed ends. A, conditions for TIRF microscopy measures of actin depolymerization at untethered barbed ends. Actin monomers (1 μM, 0.1% biotin-labeled, 33% Oregon Green–labeled) were polymerized for 7 min before non-tethered barbed ends (0.24 μM, 0% biotin-labeled, 33% Oregon Green–labeled) were grown for 15 min, with actin-binding proteins present for the last 5 min of growth. B, depolymerization of actin in the absence of actin monomers or cortactin. Some filament ends grew from seeds that were not visible at the resolution of the microscope but nonetheless were anchored at the point of the seed. In the example shown, an untethered barbed end grew from a non-visible seed (unfilled red arrowhead) with an adjacent seed also present (filled red arrowhead) and that cortactin truncations with lower affinity for actin filaments are less able to slow depolymerization. We also found that 1 proline-rich, and SH3 domains of the protein are not necessary degree as full-length cortactin, demonstrating that the NTA, which binds filaments with the same affinity as full-length cortactin. We also tested whether the cortactin paralog HS1 could stabilize actin filaments (Fig. 3C). In the absence of actin, monomers and cortactin filaments shrink over time. The yellow arrowhead marks the position of the pointed end, whereas the blue arrowhead marks the position of the barbed end. The kymograph was generated with the JFilament ImageJ plugin. D, depolymerization of actin filaments is quantified by linear regression of depolymerization events. Quantification begins one frame preceding filament shortening. E, cortactin stabilizes actin filaments in a concentration-dependent manner. Rates of depolymerization are from three to five chambers for each condition (16 chambers for control) with 8–12 filaments analyzed per chamber. Analysis was performed grouping by both chamber and all filaments, plotting mean ± S.D. One-way ANOVA was used to control rates and varying concentrations of cortactin (**, p < 0.01; ****, p < 0.0001; N.S., not significant).

−1.4 ± 0.6 subunits/s). Likewise, a cortactin fragment lacking the proline-rich and SH3 domains (cort∆ProSH3) also slowed filament depolymerization (−1.4 ± 0.1 subunits/s) (Fig. 4C). Both mutants slowed filament depolymerization to the same degree as full-length cortactin, demonstrating that the NTA, proline-rich, and SH3 domains of the protein are not necessary to stabilize actin. We also found that 1 μM CR16L fragment, which binds filaments with the same affinity as full-length cortactin, was sufficient to stabilize actin filaments (−1.5 ± 0.4 subunits/s depolymerization rate), whereas CR16, which has a lower affinity for actin, had a greatly reduced ability to stabilize actin (−3.5 ± 1.2 subunits/s) (Fig. 4C). Together, these results demonstrate that cortactin binding to actin is sufficient to stabilize actin filaments and that cortactin truncations with lower affinity for actin filaments are less able to slow depolymerization.

To test whether the effects on actin stability were specific to cortactin, we tested whether the cortactin paralog HS1 could bind and stabilize actin filaments (Fig. 5A). HS1, which only contains 3.5 actin-binding repeats, bound actin with significantly lower affinity than cortactin (Kd ≈ 4.4 μM, Fig. 5, B and C). When present at 1 μM in TIRF microscopy studies of depolymerization, HS1 did not affect actin depolymerization, in notable contrast to the stabilizing effects of all cortactin CR-containing constructs at the same concentration (Fig. 5D). At this concentration, HS1 binds with density 1 HS1:11 actin monomers, and we demonstrate that 0.35 μM cortactin slows actin depolymerization when bound 1 cortactin:16 actin monomers. Thus, although cortactin has concentration-dependent effects on actin stability, HS1 does not stabilize actin filaments when bound at a similar density.

Substitutions in conserved, charged regions in repeat 4 disrupt cortactin–actin interactions and actin filament stabilization

Previous work demonstrated that deletion of CR4 reduces/disrupts cortactin binding to actin in vitro (5) and its ability to support cell protrusion and exocytosis (6, 39). In addition, substitutions that reduce the net positive charge in cortactin repeats 3 and 4 decrease binding (28, 40). We tested whether specific substitutions of well-conserved charge clusters or sites of covalent modifications within repeats 3 and 4 impacted actin binding (Fig. 6A). Replacement of residues 181–184 (KTEK) or 188–190 (QKD) in the context of CR16L with stretches of alanine did not impact binding affinity relative to CR16L (Kd (KTEK/AAAA) = 0.51 ± 0.07 μM; Kd (QKD/AAA) = 0.81 ±
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A and B, domain architecture and purified recombinant protein of cortactin truncations lacking the proline-rich and SH3 domains (cort\DeltaProSH3, residues 1–401) and NTA domain (cort\DeltaNTA, residues 83–546). C, cortactin binding actin is sufficient to stabilize actin filaments. The cortactin truncations cort\DeltaProSH3 and cort\DeltaNTA decrease the rate of actin depolymerization with no statistically significant difference from full-length protein. CR16L, which binds actin with the same affinity as full-length cortactin, also stabilizes filaments to the same degree as full-length cortactin when present at 1 \muM. CR16, which has reduced binding affinity, stabilizes filaments but to a significantly lesser degree than full-length cortactin. Cortactin and control data are replicated from Fig. 3 for clarity. CR16L(DKS/AAA) did not stabilize filaments grown from ADP–actin independently of filament aging, ATP hydrolysis, and phosphate release (Fig. 8). ADP–actin filaments depolymerized at a rate of –7.1 filaments/s, corresponding well with rates extrapolated from EM measurements of actin filaments (42). This measurement was not statistically significantly different from the control filaments observed after aging ATP–actin, indicating that the events observed from aged filaments were predominantly from ADP-bound barbed ends. Cortactin stabilized ADP–actin filaments from depolymerization to a similar extent as aged ATP-actin (depolymerization rate, –1.8 ± 0.6 subunits/s) and ATP-actin filaments measured previously (30). These data indicate that cortactin effects on filament depolymerization are not due to decreased rates of ATP hydrolysis or phosphate release, which would be expected to stabilize actin.

Discussion

Cortactin promotes the formation and maintenance of actin-rich structures, including lamellipodia, invadopodia, and dendritic spines (5–11, 14, 15). Cortactin binds and activates the Arp2/3 complex and signals through other actin-binding proteins (43) and small GTPases (11, 19, 20). Here we demonstrate that interactions between cortactin and actin filaments are sufficient to promote actin stability. Although the CR region is sufficient to bind actin, a 77-amino acid adjacent linker is necessary to achieve the high-affinity binding of cortactin. Cortac-
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that the adjacent linker directly contacts actin filaments to contribute to high-affinity binding. Alternatively, this linker portion might support folding of the CR and its proper binding to filaments. In this work as well as that of others (26), we demonstrate changes in binding stoichiometry between the isolated CR and actin depending on the regions flanking the cortactin repeat region.

We measure actin stability here by observing depolymerization of untethered barbed ends from actin filaments (Figs. 3 and 4). Previous work has observed slow depolymerization of untethered actin filaments before the disassembly rate increased over time (35). Those studies used a microfluidic chamber to observe depolymerization during the washout step, labeled actin with Alexa 488 on lysine, and observed depolymerization immediately after filament growth with 2 μM actin monomers. Here, there was a delay in imaging (~90 s) while actin monomers were washed out of the chamber, during which time any residual ADP-Pi subunits at the barbed end may have dissociated, resulting in the linear depolymerization events we see. Instead of observing filaments immediately after polymerization in ATP–actin, growing actin filaments for 15 min at 0.24 μM provided several half-lives for Pi release, which increased the uniformity and rates of depolymerization relative to our previous studies (30). These aged ATP–actin filaments depolymerize at a similar rate as ADP–actin, strongly suggesting that the depolymerization events we measured after 15 min of slow untethered growth were from ADP-bound barbed ends. ADP–actin filaments depolymerize in our study at rates identical to those observed previously in EM measures of untethered filaments (42), confirming a lack of interference from coverslip interactions. To our knowledge, these are the first measurements of depolymerization of untethered actin filaments labeled with Oregon Green 488 on Cys-374. It is possible that, in addition to aging filaments and delayed imaging after washout, differences in the dye label could impact actin depolymerization rates.

Cortactin stabilizes ADP–actin as well as filaments grown from ATP–actin (Fig. 8), ruling out ATP hydrolysis and phosphate release as potential mechanisms of cortactin filament stabilization. Cortactin stabilizes ADP–actin despite a reduced affinity for filaments relative to ATP–actin. Based on the affinity of cortactin for ADP–actin ($K_d = 3.9 \mu M$) (6), 1 μM cortactin should only have reached 20% binding saturation on ADP–actin, again indicating that cortactin stabilizes actin at subsaturating concentrations.

Substitutions that reduce cortactin affinity for actin impair its ability to stabilize actin filaments. We demonstrate that substitution of the well-conserved residues Asp-207 through Ser-209 reduces actin binding and actin stabilization by cortactin (Fig. 6 and 7). These residues undergo serine phosphorylation in a cellular phosphoproteomics assay (41) and are cross-linked to actin when incubated with filaments (44). Substitution of serine 209 with alanine or aspartic acid disrupts actin binding, indicating that the serine side chain may have a specific effect on actin binding independent of phosphorylation or charge. CR16 alone binds actin with reduced affinity relative to CR16L and cortactin, and this reduced binding correlates with a diminished effect on actin stability. Although these trunca-
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A sequence of cortactin repeats 3 and 4 showing sites where substitutions were generated with PCR-based mutagenesis in the context of CR16L, which fully recapitulates binding of full-length cortactin. Mutations targeted well-conserved clusters of positive charge that also are sites of posttranslational acetylation or phosphorylation in cells. B–D, cosedimentation assays measuring actin filament binding of CR16L constructs with substitutions at residues 181–184 (KTEK), 188–190 (QKD), or 207–209 (DKS) to alanine. See Fig. 1 for conditions. S, supernatant; P, pellet. E and F, densitometry values of CR16L mutants:actin in cosedimentation experiments were plotted versus the concentration of cortactin to determine a dissociation constant (Kd). All points are shown for three replicates. CR16L(DKS/AAA) reaches the same binding density as other mutants but has significantly reduced binding (p < 0.01, Student’s t test) compared with CR16L(KTEK/AAA) and CR16L(QKD/AAA), which bind with the same affinity as WT CR16L. G, DKS substitutions in CR16L disrupt actin stabilization. Actin depolymerization rates for three experiments with control, cortactin, and CR16L data are replicated from Figs. 3 and 4 for clarity. Analysis was performed grouping both by chamber and all filaments, plotting mean ± S.D. One-way ANOVA; *, relative to controls; #, relative to CR16L(DKS/AAA); N.S., not significant; ****, p < 0.0001; #, p < 0.05; ##, p < 0.01; ####, p < 0.0001.

Figure 6. Substitutions in conserved sites of positive charge and posttranslational modification disrupt cortactin–actin interactions and filament stabilization. A, sequence of cortactin repeats 3 and 4 showing sites where substitutions were generated with PCR-based mutagenesis in the context of CR16L, which fully recapitulates binding of full-length cortactin. Mutations targeted well-conserved clusters of positive charge that also are sites of posttranslational acetylation or phosphorylation in cells. B–D, cosedimentation assays measuring actin filament binding of CR16L constructs with substitutions at residues 181–184 (KTEK), 188–190 (QKD), or 207–209 (DKS) to alanine. See Fig. 1 for conditions. S, supernatant; P, pellet. E and F, densitometry values of CR16L mutants:actin in cosedimentation experiments were plotted versus the concentration of cortactin to determine a dissociation constant (Kd). All points are shown for three replicates. CR16L(DKS/AAA) reaches the same binding density as other mutants but has significantly reduced binding (p < 0.01, Student’s t test) compared with CR16L(KTEK/AAA) and CR16L(QKD/AAA), which bind with the same affinity as WT CR16L. G, DKS substitutions in CR16L disrupt actin stabilization. Actin depolymerization rates for three experiments with control, cortactin, and CR16L data are replicated from Figs. 3 and 4 for clarity. Analysis was performed grouping both by chamber and all filaments, plotting mean ± S.D. One-way ANOVA; *, relative to controls; #, relative to CR16L(DKS/AAA); N.S., not significant; ****, p < 0.0001; #, p < 0.05; ##, p < 0.01; ####, p < 0.0001.

Figure 7. Specific substitutions of serine 209 in CR16L disrupt actin binding. A and B, cosedimentation assay measuring binding of CR16L with S209A mutation to actin filaments. See Fig. 1 for conditions. Densitometry values of CR16L mutantactin in cosedimentation experiments were plotted versus the concentration of the CR16L construct to determine a lower limit for the dissociation constant (Kd) in the absence of the curve reaching saturation. S, supernatant; P, pellet. C and D, cosedimentation assay measuring binding of CR16L with S209D mutation to actin filaments, with accompanying quantification.
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Molecular cloning, purification, and labeling of recombinant proteins

Murine cortactin complementary DNAs were cloned with a Hics tag into the pFastBac1 vector (Invitrogen). Cortactin truncations of the NTA, proline-rich, and SH3 domains were generated using PCR-based amplification followed by cloning into pFastbac1. Recombinant baculoviruses expressing these constructs were generated using the Bac-to-Bac expression system (Thermo Fisher, Waltham, MA) in Sp9 insect cells as described previously (54). After expression in Hi5 insect cells for 48 h, cells were lysed in Hi5 lysis buffer (20 mM Tris (pH 8.0), 230 mM KCl, 1% NP-40, 5% glycerol, 20 mM imidazole, 1 mM DTT, and protease inhibitors (benzamidine, aprotinin, leupeptin, chymostatin, pepstatin A, and phenylmethylsulfonyl fluoride)). Cortactin proteins were affinity-purified on nickel-nitrilotriacetic acid resin (Qiagen, Hilden, Germany) and eluted with 250 mM imidazole followed by chromatography on a Resource Q column with a gradient of 100 mM to 400 mM KCl to elute. All cortactin proteins were dialyzed into cortactin buffer (20 mM Hepes (pH 7.25), 230 mM KCl, 0.01% NP-40, 5% glycerol, and 1 mM DTT) for biochemical assays.

Cortactin repeats and the adjacent linker (CR16L, residues 83–401), cortactin repeats alone (CR16, residues 83–324), and cortactin repeats 3–6 with the linker (CR36L, residues 154–401) were generated using PCR-based amplification and cloned into the pgex-6p1 vector (GE Healthcare, Little Chalfont, UK). CR16L mutants in repeats 3 and 4 were generated using PCR-based mutagenesis. After expression in BL21 *Escherichia coli* and induction overnight at 16 °C with isopropyl 1-thio-β-d-galactopyranoside, cells were lysed in PBS by sonication and affinity-purified with glutathione–agarose (Thermo Fisher), with elution in 10 mM GSH. GST tags were cleaved with PreScission Protease (GE Healthcare), and the untagged CR16L and CR16 were purified using cation exchange on a Resource S column with elution in 20 mM Hepes (pH 7.25), 0.01% NP-40, 5% glycerol, 1 mM DTT, and a gradient from 30 mM KCl to 200 mM KCl.

Cortactin repeat truncations (CR14, CR36) were generated using PCR-based amplification and cloned into the pmAL–TEV vector (New England Biolabs, Ipswich, MA). CR truncations were purified from *E. coli* as described above, with the maltose-binding protein (MBP) tag cleaved with TEV protease. Human HS1 complementary DNA was inserted into pFastBac1 with a Hisi tag using restriction digest–based cloning and purified in the same manner as cortactin above.

Actin was purified from an acetone powder of frozen chicken breast muscle (Trader Joe’s, Orange, CT) by one cycle of polymerization and depolymerization (55), followed by gel filtration over S300 resin (GE Healthcare) in G buffer (2 mM Tris-HCl (pH 8.0), 0.5 mM ATP, 0.5 mM DTT, 0.1 mM CaCl₂, and 1 mM NaN₃) and storage in G buffer at 4 °C.

Actin was labeled at Cys-374 with Oregon Green 488 or biotin as described previously (56). Unlabeled actin monomers
were clarified of actin polymers/aggregates by centrifugation at 135,000 × g for 2 h and polymerized in a 1:1 mixture with 2× labeling buffer (2× = 200 mM KCl, 4 mM MgCl$_2$, 50 mM imidazole (pH 7.0), 0.6 mM ATP, and 6 mM NaN$_3$) for 30 min. Actin was diluted to 24 μM in 1× labeling buffer. For Oregon Green labeling, a 30 mM solution of Oregon Green 488 iodoacetamide (Thermo Fisher) was prepared in dimethyl fluoride and added dropwise to polymerized actin in a 12.5× molar excess. For biotin labeling, a 20 mM solution of EZ-Link Maleimide-PEG2-anol and stored in ethanol up to 2 days before making flow filtered air before immediately forming flow chambers. Slides were sonicated for 10 min each in 2% Alconox, water, and ethanol and stored in ethanol up to 2 days before making flow chambers. Chambers were sealed by a metal block at 90 °C for a total of 10 s, followed by firm pressure with a gloved finger.

Prior to each depolymerization reaction, the chamber was incubated for 1 min with two 14-μl washes with each of the following solutions: 0.5% Tween 20 in HS-TBS buffer (50 mM Tris (pH 7.5) and 600 mM KCl), 5 mg/ml BSA (American Bio), 50 μg/ml streptavidin (Millipore Sigma) in HS-TBS, and 100 mg/ml BSA in HS-TBS. The chamber was washed twice with HS-TBS between each incubation step. Chambers were flushed with a 1:1 mixture of reaction buffer (20 mM Hepes (pH 7.25), 230 mM KCl, 5% glycerol, 0.01% NP-40, and 1 mM DTT) and 2× TIRF buffer (2× = 70 mM KCl, 1.4 mM MgCl$_2$, 1.4 mM EGTA, 14 mM imidazole (pH 7.0), 0.5% 4000 cPM methylcellulose, 30 mM glucose, 400 μM ATP, 100 mM DTT, 40 μg/ml catalase, and 200 μg/ml glucose oxidase) before actin was flowed into the chambers.

Depolymerization experiments were performed on a Nikon Ti-E microscope with a ×100 TIRF objective (numerical aperture = 1.49), an Andor Zyla 4.2 sCMOS camera, and Nikon Elements software. The microscope was equipped with a perfect focus system, automated TIRF angle motor, and 488-nm laser for these experiments. Final reaction conditions were 150 mM KCl, 0.7 mM MgCl$_2$, 0.7 mM EGTA, 7 mM imidazole (pH 7.0), 0.25% 4000 cPM methylcellulose, 15 mM glucose, 200 μM ATP, 50 mM DTT, 20 μg/ml catalase, 100 μg/ml glucose oxidase, 10 mM Hepes (pH 7.25), 2.5% glycerol, and 0.005% NP-40.

Actin monomers (0.1% biotin-labeled, 33% Oregon Green-labeled) were polymerized at a 1 μM in equal parts 2× TIRF buffer and reaction buffer and flowed into the chamber using Whatman paper. After 5 min of incubation, polymerization was imaged for 2 min to identify barbed and pointed ends. An actin mixture (0% biotin-labeled, 33% Oregon Green-labeled) at 0.24 μM was flowed into the chamber twice to allow nontethered barbed ends to grow for 15 min, the last 5 min of which also contained cortactin proteins of interest at varying concentrations. Finally, the chamber solution was replaced with cortactin buffer (with or without cortactin constructs) to observed depolymerization events. Exposure times were 100 ms at ~0.015-mW laser power, with frames collected 10 s apart during polymerization and depolymerization and 5 min apart during growth of nontethered ends.

Mg-ADP–actin monomers were prepared as described previously (57, 58). Ca-ATP–actin monomers (6 μM) were incubated with 10X ME buffer for 5 min (to a final concentration of 1× ME buffer). The resulting Mg-ATP–actin monomers were incubated with 2 mM glucose and 20 units/ml hexokinase (Millipore Sigma, St. Louis, MO) for 35 min, clarified at 100,000 × g for 10 min, and used for depolymerization experiments. TIRF buffers were identical to ATP–actin experiments with two exceptions: ADP was present in the TIRF buffer to replace ATP, and TIRF buffers contained 20 units/ml hexokinase. Tethered ADP–actin seeds (1 μM) were incubated in TIRF chambers for 2.5 min with little to no growth of seeds seen. Untethered ADP–actin filaments were polymerized from seeds in 2 μM ADP–actin monomers for 5 min with and without cortactin present. Filaments depolymerized in a 1:1 mixture of reaction buffer:2× TIRF buffer containing 0 μM or 1 μM cortactin.

**TIRF microscopy experiments**

Open-ended flow chambers of ~12 μl were made using a channel cut from Parafilm placed between a slide and coverslip arranged perpendicularly. Coverslips were plasma-cleaned for 2 min using H$_2$/O$_2$. Coverslips were coated overnight at 60 °C with 2 mg/ml methoxy-PEG5000-silane (Laysan Bio, Arab, AL) and 0.02 mg/ml biotin-PEG3400-silane in 95:4:1 (v/v) ethanol:water:acetic acid, sonicated for 2 min in ethanol, and dried with filtered air before immediately forming flow chambers. Slides were sonicated for 10 min each in 2% Alconox, water, and ethanol and stored in ethanol up to 2 days before making flow.
Changes in filament length were measured using ImageJ software with the JFilament plugin (59) for 8–12 filaments per experiment and at least three experiments per condition. Background correction was performed with a rolling ball radius of 7 pixels followed by drift correction with the TemplateMatching plugin (60). Depolymerization events were observed in non-tethered filaments with few pauses. If a pause occurred during depolymerization, depolymerization was quantified separately before and after the pause, and the rate for that filament was the weighted average of the two events over time. Events were seen as well in tethered filaments but not quantified because of much more pronounced pauses. For quantification, we assumed 370 actin subunits/μm of filament (45).

Statistical analysis

Differences in dissociation constant values were performed with unpaired, two tailed t tests as appropriate. Significance was defined as p < 0.05. Comparisons of depolymerization rates in TIRF microscopy experiments were performed with one-way ANOVA as appropriate. We quantify and analyze our depolymerization results using each chamber as an experimental replicate. Although each filament is analyzed individually, the variance of CR16L mutants in one experiment. Although this approach requires more data collection, it more accurately reflects small differences in buffers, concentrations, and conditions across multiple experiments compared with considering each filament as a replicate alone.

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