A barbed end interference mechanism reveals how capping protein promotes nucleation in branched actin networks

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Heterodimeric capping protein (CP/CapZ) is an essential factor for the assembly of branched actin networks, which push against cellular membranes to drive a large variety of cellular processes. Aside from terminating filament growth, CP potentiates the nucleation of actin filaments by the Arp2/3 complex in branched actin networks through an unclear mechanism. Here, we combine structural biology with in vitro reconstitution to demonstrate that CP not only terminates filament elongation, but indirectly stimulates the activity of Arp2/3 activating nucleation promoting factors (NPFs) by preventing their association to filament barbed ends. Key to this function is one of CP’s C-terminal “tentacle” extensions, which sterically masks the main interaction site of the terminal actin protomer. Deletion of the β tentacle only modestly impairs capping. However, in the context of a growing branched actin network, its removal potently inhibits nucleation promoting factors by tethering them to capped filament ends. End tethering of NPFs prevents their loading with actin monomers required for activation of the Arp2/3 complex and thus strongly inhibits branched network assembly both in cells and reconstituted motility assays. Our results mechanistically explain how CP couples two opposed processes—capping and nucleation—in branched actin network assembly.
Heterodimeric capping protein (CP/CapZ) is an evolutionarily ancient actin regulator found in nearly all eukaryotic organisms and cell types\(^1\) that controls the fate and interactions of actin filament ends in a variety of cellular contexts. Comprised of two closely related α and β subunits, it forms a constitutive heterodimer that tightly binds to the barbed end of actin filaments to terminate their growth. Within sarcomeres, it caps the barbed ends of filaments in the z-disk. In non-muscle cells, CP collaborates with a core set of proteins—profilin, the Arp2/3 complex and a membrane-localized nucleation promoting factor (NPF) of the WASP protein family—to create dense, polarized networks of short and branched filaments\(^2\). These branched actin networks generate pushing forces to move cellular membranes\(^8\) in a large number of cellular processes ranging from cell motility\(^1\), endocytosis\(^12\), phagocytosis\(^13\), autophagy\(^14\) to cell-cell adhesion\(^5\). CP’s central role in branched network assembly is commonly thought to simply result from its eponymous biochemical activity— to cap actin filament barbed ends and inhibit the addition of further subunits. However, in vitro reconstructions of branched network assembly suggest a more complex, multi-functional role\(^6\). Capping protein is known to contribute to branched actin network assembly in several ways. First, it prevents the elongation of filaments beyond a critical length at which they would easily buckle under load\(^6\). Second, it rapidly stops the non-productive elongation of filaments distant from the cell membrane. This generates a requirement for continuous nucleation of new filament branches via the Arp2/3 complex, which is in turn activated by a membrane-bound NPF. Confinement of Arp2/3 complex activation to the membrane is therefore responsible for polarized network growth.\(^5\) In addition to these established functions directly related to the termination of filament growth, CP has been shown to stimulate nucleation of new filaments by the Arp2/3 complex. Perturbing CP activity either in vivo or in reconstituted actin motility results in comparable effects that are consistent with it acting as a positive regulator of Arp2/3-dependent branching nucleation.\(^5\) Yet, the responsible biochemical mechanism has remained enigmatic.

Branching nucleation itself is a multi-step process that requires the transient formation of a higher-order complex between the Arp2/3 complex bound to the side of an actin mother filament and a NPF that is loaded with an actin monomer bound to its WH2 domain.\(^25\) It is presently unclear, which steps in this process control the rate of filament nucleation within growing actin networks. The activity of CP seems to be a key factor,\(^5\) but we currently do not understand how CP might either directly or indirectly influence the Arp2/3 complex, its upstream NPF or any intermediates formed between the two during nucleation. Prior structural and biochemical work on CP provides little insight into this crucial question.\(^2\) One potential hint comes from the observation that each CP subunit possesses a C-terminal helical extension that protrudes from the body of the heterodimer known as tentacle.\(^26\) Both tentacles share sequence similarity with the actin-binding WH2 domain of NPFs. Based on a low-resolution structure of capped skeletal muscle actin filaments,\(^27\) it has been proposed that most of the interaction occurs between the body of CP, which, together with its α tentacle, contacts the penultimate actin protomer. In addition, the β tentacle constitutes a potential secondary actin-binding region that has been speculated to bind to the last actin subunit.\(^27\) While this is indeed the case in the homologous complex found within the dynactin complex, in which CP caps a short Arp1 filament,\(^29\) the structural details of capped actin filaments are still unknown. As a consequence, we understand very little how CP might influence Arp2/3 complex activity.

One conceptual model of why CP stimulates nucleation in branched networks is offered by the so-called monomer gating hypothesis.\(^5\) Key to monomer gating is a presumed kinetic competition between growing filament ends and the Arp2/3 complex for a limiting flux of actin monomers already in complex with membrane-bound NPFs. As polymerizing filament ends become more numerous through branching, they should consume larger amounts of NPF-bound monomers, which might reduce their partitioning into nucleation via the Arp2/3 complex. While this model offers a conceptual explanation for the nucleation stimulating function of CP, it lacks direct experimental evidence. Indeed, alternative mechanisms based on the local depletion of soluble actin monomers have also been proposed.\(^31\)

We are currently lacking a comprehensive mechanism that explains and unifies the multiple roles of CP based on its interactions with the other core components of branched actin networks. Here, we combine electron cryo-microscopy (Cryo-EM) with in vitro reconstitution and cell biology to establish that CP not only prevents the elongation of actin filaments as commonly thought. Instead, we show that it selectively masks the actin filament end to control interactions with other key proteins via a conserved actin-binding motif—the β tentacle. Its removal does not prevent capping, but dramatically inhibits the assembly of Arp2/3-generated actin networks in vitro. In line with this, we find that the CP β tentacle is selectively required for efficient lamellipodial protrusion of mammalian cells and localization of CP to the leading edge. We uncover the responsible molecular mechanism by demonstrating that NPFs activating the Arp2/3 complex are tethered and inactivated by capped filament ends when this binding motif is lost on capping protein. Our results reveal an unanticipated mechanism at the core of the branched actin network engine that couples the creation of polymerizing filaments by Arp2/3 complexes to their elimination through capping protein.

**Results**

**The structure of CP-bound actin filament barbed ends.** The ends of actin filaments have emerged as important regulatory sites, to which a diverse set of regulators bind to control filament dynamics. Despite their obvious importance, there are— with a single exception— no high-resolution structures of filament ends available. Because filament elongation is kinetically far more favorable than nucleation, the exceedingly sparse density of ends in common actin filament preparations has prevented single particle analysis until now. To overcome this fundamental limitation, we established a general procedure to generate short actin filaments amenable to single-particle Cryo-EM analysis (Fig. 1A). To this end, we premixed CP with actin in buffers of low ionic strength containing Ca\(^2+\), which keep it from polymerizing. We then triggered rapid filament nucleation by salt addition. Under these conditions, and in the absence of monomer-binding proteins such as profilin, CP potently nucleates filaments that slowly elongate from the pointed end.\(^33\) We stabilized these capped filament stubs using phallolidin shortly after the initiation of polymerization. To separate stabilized filaments from residual soluble capping protein and actin, we subjected these reactions to size exclusion chromatography (Fig. 1B). SDS PAGE revealed the presence of both CP and actin at roughly 1:2 stoichiometry in the high molecular weight fractions (Fig. 1B, Supplementary Figure 1).

Cryo-EM images of samples from these fractions revealed the presence of densely packed filaments of near-uniform lengths of 80–120 nm (Fig. 1C, Supplementary Figure 1). Using these samples and an iterative classification strategy, we determined a
Cryo-EM structure of CP-bound cytoplasmic actin barbed ends to an overall resolution of 3.8 Å (Fig. 2A, Supplementary Fig. 2, Supplementary Table 1). Local resolution ranges from 3.5–7.5 Å, with most actin monomers resolved better than 4 Å and CP to 6–7 Å (Supplementary Fig. 2). We attribute this large variation in map quality to two factors. (i) CP and individual actin monomers are similar in size, making it extremely challenging to differentiate between capped and uncapped filaments during classification. (ii) The directional FSC analysis shows preferential orientation of the particles (Supplementary Fig. 2). Combined with the observation that not all ends are capped (Supplementary Fig. 1), this suggests that ends might become uncapped when CP interacts with the grids in a certain orientation.

As expected, the structure of cytoplasmic actin filaments closely resembles the previously determined structures of skeletal actin34,35. We observed clear density for phalloidin in all the expected binding sites36, with the exception of the cleft between the ultimate and penultimate actin subunits (Supplementary Fig. 2). This is likely due to the absence of the missing third actin protomer required to form the full binding site. In agreement with our previous work34,36, we find the D-loop in a closed state. Due to the comparably high resolution within the nucleotide-binding site of actin, we could directly determine the identity of the nucleotide bound to each subunit. Consistent with the early addition of phalloidin during the polymerization reaction, most subunits show clear density for inorganic phosphate (Pi) (Supplementary Fig. 2). Interestingly, the two terminal barbed end subunits lack this density, suggesting that they are in an ADP-bound state. Whether this was due to the direct interaction with CP favoring Pi release or simply the result of our filament preparation procedure, in which these two subunits are the “oldest” in the filament as they form the nucleus for polymerization with CP is unclear.

The primary interaction between CP and the filament occurs at the barbed end face of the penultimate and the inner filament side of the terminal actin subunit (Fig. 2B). The overall interaction between CP and the actin barbed end is very similar to what has been observed in the dynactin complex30,37 (Supplementary Fig. 3). In spite of the sequence differences between Arp1 and actin, their complexes with CP superimpose almost perfectly (Supplementary Fig. 3). The core of CP together with the α tentacle block all of the exposed barbed end surface of the penultimate actin protomer (Fig. 2, Supplementary Fig. 3), with the α tentacle...
deeply wedged in a hydrophobic cleft at the barbed end of this subunit. In contrast, CP makes few contacts with the barbed end face of the ultimate protomer (Supplementary Fig. 3). Contacts between CP and the terminal subunit are dominantly formed between CP’s core and the inner side of the ultimate protomer. However, a clear density occupies the hydrophobic cleft at the barbed end of this subunit, which can be unequivocally assigned to the \( \beta \)-tentacle (Fig. 2B, C). The density in this region was slightly weaker, but comparable to the body of the terminal actin protomer (Fig. 2A), suggesting that the \( \beta \)-tentacle remains mostly docked to actin but retains some flexibility. The placement of the \( \beta \)-tentacle has only been speculated from a previously reported low-resolution structure of capped skeletal actin\(^27\) and agrees with prior computational models and mutational analysis\(^28,29\). As predicted previously\(^28\), this secondary barbed end binding site conspicuously overlaps with and mirrors the interaction between WH2 domain-containing proteins of the WASP family and actin monomers (Fig. 2C). The strong conservation of the \( \beta \)-tentacle among even evolutionarily distant CP family members suggests that this specific type of interaction serves an important function.

Deletion of the CP \( \beta \)-tentacle modestly affects barbed end capping kinetics. To understand the role of this conserved secondary actin-binding site in CP, we removed the last 23 amino acids of the beta subunit (CP\(\alpha\beta\Delta23\)) comprising the full \( \beta \)-tentacle
and studied the effect on filament barbed end association and dissociation kinetics by TIRFM assays in vitro (Fig. 3).

First, we measured the rate of CP dissociation from growing, surface-tethered actin filaments at the single filament level (Fig. 3B, C). Due to the extraordinarily long lifetime of CP at the barbed end (>10 min), we could not visualize single CP molecules directly, but inferred dissociation indirectly from the moment of filament regrowth (Fig. 3B, C). Mono-exponential fits to the dwell time courses yielded the dissociation rate constants (koff, Fig. 3D).

In a second line of experiments, we probed CP association to the barbed ends of surface-tethered filaments by dual-color single molecule assays (Fig. 3E, F). Observed capping rates directly, but inferred dissociation indirectly from the moment of filament regrowth (Fig. 3B, C). Mono-exponential fits to the dwell time courses yielded the dissociation rate constants (koff, Fig. 3D). In a second line of experiments, we probed CP association to the barbed ends of surface-tethered filaments by dual-color single molecule assays (Fig. 3E, F). Observed capping rates
Fig. 3 The CP β tentacle is dispensable for capping of single actin filaments. A Overview of the location of CP mutations. Colors are as in Fig. 2. Insets highlight the region of the β tentacle deleted (green), and the color scale on the actin surface depicts increasing hydrophobicity from gray to white to yellow. B Scheme of actin filament barbed end re-growth after dissociation of CP via TIRFM (top). Single capped filaments were visualized by Cy5-UTRN261N (10 nM, green) in the presence of profilin-actin (2 μM) and myotrophin/V1 (20 nM) after CP washout. CP dissociation leads to growth of the filament visualized in the kymograph (bottom). Over 50 such kymographs were analyzed per experiment, which was repeated independently three times with similar results. C TIRFM time lapse images of filaments following the washout of either wt CP or mutants, as indicated. Conditions are as in B. Filled red arrowheads indicate the positions of barbed ends at the moment of CP dissociation in each case, and empty red arrowheads follow the positions of (re-) growing barbed ends. The experiment was repeated three times with similar results. D Time traces of the fraction of un capped barbed ends after washout of either wt CP or mutants, as indicated, at t = 0. Dissociation rate constants (k_off) were derived from mon-exponential fits (see Methods). Source data are provided as a Source Data file. E Scheme of single molecule TIRFM assays (top). Individual surface-attached filaments visualized by Cy5-UTRN261N (10 nM, green) grow from profilin-actin (2 μM) in the presence of TMR-CP (4 nM, magenta). CP association results in the termination of filament growth as visualized in the kymograph (bottom). Over 50 such kymographs were analyzed per experiment, which was repeated independently three times with similar results. F TIRFM time lapse images of filament elongation (green) in presence of wt or mutant capping protein (magenta) as indicated under conditions as in E. Empty yellow arrowheads indicate initial positions of barbed ends and filled yellow arrowheads the positions at the moment of CP association. The experiment was repeated three times for each CP concentration with similar results. G Observed reaction rates (k_on) for capping protein wt and mutants as a function of CP concentration (Supplementary Fig. 4). The observed reaction rates (k_on) were derived from the mean of N = 3 independent experiments for each CP concentration. Plotted data also contains the independently measured dissociation rates as y-intercepts. Association rate constants (k_on) are calculated from linear fits to the data with the y-intercepts fixed to the measured off-rates. Error bars are SD. Source data are provided as a Source Data file. H Summary table of dissociation (k_off), association rate constants (k_on) and equilibrium dissociation constants (K_D, calculated from the former rate constants) for the interaction of CP (wt or mutants as indicated) with actin filament barbed ends. Errors are SD of the mean values from 3 independent experiments (k_on) or the SEM of the linear fit (k_off).

( Supplementary Fig. 4) were plotted against the total CP concentration, and linear fits to the data, constrained by the independently measured off-rate, yielded association rate constants (k_on, Fig. 3G). In overall agreement with prior bulk measurements, we found that the deletion of the entire CP β tentacle reduced the affinity for the barbed end only by less than one order of magnitude. This was the result of moderate changes in the association (1.8-fold) and dissociation (8-fold) rate constants. The moderate drop in affinity suggests that engagement of this secondary binding site is not essential for filament capping.

The dwell time of CP at filament ends in vitro exceeds its lifetime in cellular actin networks by two orders of magnitude. This is in part the result of its active removal from filaments ends via twinfilin and potentially other allosteric regulators. It is reasonable to assume therefore that its association rate should be functionally more relevant compared to its exceedingly slow in vitro dissociation rate. Even in the absence of its β tentacle, CP still dwells at filament ends far longer than it should be necessary to carry out its capping function at the leading edge of motile cells. To control for the moderate contribution of the β tentacle to barbed end capping kinetics and to uncouple this effect from a potential unrelated secondary function, we designed weakening mutants distant from β tentacle within the primary barbed end binding site of CP (Fig. 3A, see Methods). We identified two single site mutants within the CPα subunit (αW271Rβ and αY277Vβ) that altered the thermodynamics and kinetics of barbed end binding to a similar degree as β tentacle removal (Fig. 3D, G, H). We therefore refer to these two variants as “kinetics-mimicking” mutants that cause effects on single filament capping rates closely resembling those seen after β tentacle deletion.

Deletion of the CP β tentacle drastically inhibits reconstituted branched actin network assembly. Because the deletion of the CP β tentacle results in only mild changes in the kinetics of capping, we wondered how this relates to its potentially more complex role in regulating branched actin network dynamics. To address this question, we assembled branched actin networks from polystyrene beads that were coated with a minimal Arp2/3-activating fragment of the NPF protein WAVE1 (WAVEAN). We initiated network assembly by the addition of Arp2/3 complex (100 nM), CP (wildtype or mutants, 100 nM), and 1:1 complexes of profilin–actin prepared by size exclusion chromatography (5 μM, see Methods); conditions previously shown to promote rapid and sustained actin network growth. The tight affinity of cytoplasmic actin together with isolation of stoichiometric profilin-actin complexes reduces the free actin monomer concentrations in our assays sufficiently (to about 290 nM) to prevent significant nucleation of filaments in solution. To visualize the kinetics of all biochemical processes contributing to the assembly of the actin network—actin polymerization, Arp2/3-driven nucleation and CP-mediated capping—we included a fluorescently-labeled filament binding probe (Alexa488-lifect) and used fractions of Arp2/3 complex and CP labeled with matching fluorescent dyes (Fig. 4A). We kinetically arrested network assembly very soon (4 min) after the initiation of growth by adding a 3-fold molar excess of Latrunculin B and phalloidin, which stabilizes existing actin filaments and prevents the nucleation of new ones. Upon kinetic arrest, we immediately proceeded to multi-color imaging of formed actin networks (Fig. 4B). We observed that reactions with CP lacking the β tentacle generated actin networks with drastically altered assembly dynamics compared to reactions with wildtype CP (Fig. 4B, top). Averaged intensity profiles showed that networks grown in the presence of CPαβA23 contained less polymerized actin and drastically reduced Arp2/3 complex and CP amounts (Fig. 4B, bottom). Relative to the wildtype CP control, these networks grew with about 3-fold reduced average velocities (Fig. 4C). To directly quantify the rates at which actin, CP, and the Arp2/3 complex join the growing network at the NPF-coated surface, we integrated the fluorescence intensity of each component in individual actin networks and divided these values by the reaction time (Fig. 4D). This analysis revealed that the deletion of the CP β tentacle dramatically (20-fold, Supplementary Table 2) reduced network incorporation rate of CP much more than predicted by its modest defect in capping at the single molecule level (Fig. 3H). Conversely, we also observed a drastic reduction in the rate of Arp2/3 complex network incorporation (Fig. 4D). To test whether this reduced filament nucleation rate at the network level was simply a consequence of its modestly reduced capping rate, we turned to the CP mutants kinetically mimicking the β tentacle deletion (αW271Rβ and αY277Vβ). Decisively, we observed that both mutants were capable of generating branched actin networks...
with assembly kinetics nearly indistinguishable from wildtype CP (Fig. 4B–D).

Because we reconstituted branched network assembly at near-saturating levels of profilin, which reduces the availability of free actin monomers, we were wondering whether our observations depended on the exact composition of the soluble actin pool. We therefore repeated our bead motility experiments by adding an excess of either profilin (500 nM) or thymosin-β4 (4.5 μM) to 5 μM profilin-actin. According to mass action, either addition will reduce the free actin monomer concentration by about 50% (from 290 to 140 nM). Importantly, we observed that the selective defects in branched network assembly resulting from the CP beta tentacle deletion were preserved in either condition (Supplementary Fig. 5). We therefore deduced that these defects are robust towards changes in the actin monomer pool and can be similarly observed at various levels of free actin monomers. In conclusion, we found that the strongly diminished rates of network incorporation of both CP and Arp2/3 complex resulting from the CP β tentacle deletion were not caused by its modestly altered capping rates observed at the single filament level. Instead, the data indicates that the CP β tentacle serves an important secondary function in branched network assembly not directly related to the termination of actin filament growth.

**Deletion of the CP β tentacle inhibits Arp2/3-dependent branching by tethering NPF proteins to capped filament ends through their WH2 domain.** The CP β tentacle interacts with the central binding cleft of the terminal actin protomer at the barbed end in a manner that sterically prevents binding of other WH2 domain-containing proteins such as NPFs (Fig. 2C). We therefore hypothesized that capping protein controls the rate of Arp2/3-mediated branching at the level of the NPF as part of a feedback mechanism (Fig. 5A): CP occupies both WH2 binding sites at the penultimate and terminal barbed end subunits, effectively masking the capped filament end from NPF binding. Loss of the β tentacle liberates one of these binding sites, which might drive the sequestration of proximal NPF WH2 domains. This type of tethering should therefore reduce the amount of NPF molecules loaded with actin monomers ready to activate the Arp2/3 complex.

To test this hypothesis, we employed a recently developed Förster resonance energy transfer (FRET) method to directly measure partitioning of WH2 domains between actin monomers and filament ends [44] (Fig. 5A, Supplementary Fig. 6). To this end, we conjugated a fluorescent donor (Alexa488) to a site immediately upstream of the WH2 domain of WAVE1 and

**Fig. 4 The CP β tentacle is essential for reconstituted branched actin network assembly.** A Scheme describing bead-motility-assay. Dendritic actin networks are assembled from polystyrene microspheres (Ø = 3 μm) coated with WAVEΔN via biotin-streptavidin linkages. Dense branched actin networks assemble from these beads and incorporation of all constituting components can be visualized via matching fluorescent labels as indicated. B Top: Representative epifluorescence images of dendritic actin networks grown from WAVEΔN-coated microspheres using 5 μM profilin-actin complexes (see Methods), 100 nM CP (either wt or mutants as indicated, 20% TMR-labeled), 100 nM Arp2/3 (20%-Alexa647 labeled). Reactions were kinetically arrested after 4 min using phalloidin (15 μM), Latrunculin-B (15 μM) and Alexa488-lifeact (15 nM) to visualize filamentous actin. The experiment was repeated three times with similar results. Bottom: Quantification of the averaged intensity profiles for the indicated dendritic network components. Source data are provided as a Source Data file. C Plots of network growth velocities of dendritic actin networks grown in presence of either wt or mutant CP. Source data are provided as a Source Data file. D Plots of polymerization, capping and nucleation rates of dendritic actin networks grown in presence of either wt or mutant CP (see Methods). Source data are provided as a Source Data file. Quantifications in C and D were done for n = 25 actin networks from N = 3 independent experiments, error indicator = SD of the mean. P-values were derived from one-way ANOVA Tukey tests.
conjugated a non-fluorescent quencher (Atto540Q) to monomeric actin in a manner that does not affect their binding affinities (see Methods). We then immobilized this labeled WAVE1 fragment on polystyrene beads and assembled branched networks in the presence of either wildtype CP or mutants. We added quencher-labeled and Latrunculin B-stabilized actin monomers at the moment of kinetic network arrest through Latrunculin B, phalloidin and myotrophin/V-1. The latter inhibitor preserves free barbed ends. To minimize the impact of CP dissociation from capped ends after network arrest, we immediately proceeded to imaging and did not analyze NPF-coated beads longer than 5 minutes after inhibitor and...
Fig. 5 Loss of the CP β tentacle tethers NPFs to capped filament ends via their WH2 actin binding site. A Scheme of the FRET setup. Surface-bound WAVEΔN molecules are donor-(Alexa488-) labeled upstream of their WH2 site. NPF WH2 can either interact with quencher- (Atto540Q-) labeled actin monomers resulting in a decrease of donor fluorescence or unlabeled terminal protomers of filament barred ends, resulting in no change in fluorescence. Terminal protomers are unlabeled, since quencher-labeled monomers are introduced only upon network arrest. Loss of the β tentacle vacates the WH2 binding site at the terminal protomer of capped ends, tipping the balance in favor of end-binding. B Left: Representative epifluorescence images of Alexa488-WAVEΔN-coated microspheres (green) and dendritic actin networks (visualized by Cy5-UTRN, blue) 3 min after arrest before (top) and after (bottom) quenching with Atto540Q-labeled actin monomers. Protein concentrations were as in Fig. 4 in the presence of wt or mutant capping proteins, as indicated. Right: Average intensities (continuous lines at the center of the error band, dashed lines error bars) of the NPF-fluorescent signal intensity on the microsphere surface in the presence or absence of Atto540Q-labeled actin monomers (n = 25 beads per condition). Source data are provided as a Source Data file.

quencher addition. Alexa488-WAVEΔN-coated beads in physical contact with actin networks assembled from either wildtype or kinetics-mimicking (αW271Rβ and αY277Vβ) CP mutants displayed similarly strong (by 59–69%) quenching of NPF donor fluorescence, indicating that a majority of NPF molecules were able to bind quencher-labeled actin monomers from solution (Fig. 5A, B). Strikingly, quenching was substantially diminished (to 14%) for Alexa488-WAVEΔN beads tethered to actin networks grown in the presence of CP lacking its β tentacle (Fig. 5B, Supplementary Fig. 6). These results verify that the loss of the β tentacle locks the majority of NPF molecules in an inactive state tethered to capped filament ends. In summary, this strongly suggests that the conservation of this secondary end binding site in CP arises from the necessity to mask the terminal actin subunit at the barbed end from WH2 binding.

Inspired by this result, we wondered whether CP exerts a similar function in blocking the NPF binding site also at the penultimate actin subunit through its α tentacle. If this were the case, removing the α tentacle could vacate this second NPF binding site, which might cause defects similar to β tentacle deletion. Previous work established that truncation of the entire α tentacle dramatically inhibits barbed end binding and therefore cannot be used to test this hypothesis29. Our structure, however, suggested that removing the very end of the α tentacle by only a few amino acids might liberate the NPF WH2 binding site at the penultimate subunit (Supplementary Fig. 7). We therefore created two additional CP variants that removed the last 8 or 9 residues of the α tentacle and studied the effect of either deletion in reconstituted branched network assembly (Supplementary Fig. 7). Decisively, both CP variants behaved like wildtype capping protein, indicating that they were active in barbed end binding as anticipated, but did not inhibit nucleation like the β tentacle deletion (Supplementary Fig. 7). We believe the latter finding reflects either of two possibilities: (i) The short deletions do not liberate the WH2 site at the penultimate subunit entirely. Indeed, the NPF binding site of this subunit comes very close to the CP body (Supplementary Fig. 7). Moreover, the WH2 domain represent only a fraction of the NPF, suggesting possible clashes with CP. (ii) Ultimate and penultimate actin subunits are differentially conducive to NPF WH2 binding. While we do not observe obvious structural differences between these subunits, the penultimate subunit will be more distant (by about 2.5 nm) from the surface-immobilized NPF on average. This in itself might lead to preferential NPF binding to the last subunit, explaining why the α and β tentacles of CP are not functionally equivalent.

The β tentacle is essential for CP localization to lamellipodial actin networks and efficient leading edge protrusion. To determine how our in vitro results translate to the function of CP in regulating branched actin network dynamics in mammalian cells, we generated engineered murine B16-F1 cell lines, in which expression of either one of the two CP subunits was genetically disrupted by CRISPR/Cas9-mediated genome editing. We first stably knocked out the single gene (CapZβ) from which all three known CPβ isoforms are derived47. This successfully resulted in a clonal cell line devoid of detectable CPβ expression (CapZβ KO/+10, Fig. 6A). In parallel, we attempted to inactivate both genes (CapZα1 and −2) responsible for the expression of the two ubiquitous CPα isoforms1,48. The murine genome contains a third CPα gene (CapZα3), however the resulting protein is more divergent and only expressed in the male germ cell lineage1,49. Simultaneous, CRISPR/Cas9-mediated targeting of CapZα1 and −2 did not yield clones entirely devoid of CPα, indicating that the CPα subunit might be essential for cell viability. Nonetheless, we were able to select a clone resulting from synchronous targeting of CapZα1 and -2 that showed markedly reduced total CPα expression levels, with the CPα isoform being virtually absent (Fig. 6A).

In agreement with prior knockdown/knockout studies22,23,39, functional interference with either CP subunit caused consistent defects in leading edge dynamics reflected by significantly altered morphologies at the peripheries of these cells compared to wild type (Fig. 6B, C). These phenotypes coincided with strongly compromised protrusion velocities in each genotype (by app. 70%), which could be largely rescued by EGFP-tagged wildtype variants of respective, disrupted gene (Fig. 6C, D). Inefficient protrusion observed in CRISPR/Cas9-treated clones derived from their leading edge membranes undergoing rapid fluctuations, strongly contrasted by the smooth, homogeneous protrusion seen upon rescue with wildtype CPβ and –α as well as the kinetics-mimicking mutants of the latter (αW271Rβ or αY277Vβ, Fig. 6D bottom right) when expressed at similar levels (Supplementary Fig. 8). Furthermore, the restoration of protrusion velocity was similarly efficient with the latter CPα-mutants as seen with wild type CPα (Fig. 6D, right panel). As only exception, expression of CPβ lacking its tentacle region in the CPβ-deficient clone was significantly less potent in its rescue efficiency compared to wildtype CPβ when expressed at comparable levels (Supplementary Fig. 8), with a protrusion efficiency found in the latter condition statistically indistinguishable from untransfected KO cells (Fig. 6D, left panel). The functional difference between CP lacking its β tentacle (αβΔ23) and the corresponding kinetics-mimicking mutants (αW271Rβ or αY277Vβ) also correlated with their accumulation in restored protrusions, since all EGFP-tagged constructs accumulated robustly at protruding cell edges except for αβΔ23 (Fig. 6C). Importantly, we confirmed that the levels of both, lamellipodial F-actin and Arp2/3 complex were substantially reduced in the absence of the beta tentacle, as expected (for representative images see Supplementary Fig. 8), albeit not abolished. These effects concur with our model and closely resemble the observations obtained with our reconstitutions, in which we saw a similarly strong reduction of both factors in branched networks in vitro. We therefore concluded that the strongly diminished accumulation of β tentacle-truncated CP (αβΔ23) in lamellipodial networks at least partly coincides with...
reduced Arp2/3 complex-mediated branching activity in those cells. Taken together, these data show that the CP β tentacle serves an important function in regulating lamellipodial actin dynamics secondary to simply contributing to high-affinity barbed end binding of actin filaments.

Discussion
Combining structural and cell biology with in vitro reconstitution, we have elucidated a mechanism that links the two central antagonistic biochemical reactions –capping and nucleation- in the assembly of branched actin networks. Our results identify the WH2 domain of the NPF as the central coupling element that can...
assume distinct states (Fig. 7): (1) Bound to an actin monomer ready to activate the Arp2/3 complex and (2) tethered to a free filament barbed end in a nucleation-inactive configuration. We propose that the latter interaction provides negative feedback to Arp2/3 complex activity and refer to this mechanism as barbed end interference. Importantly, both free, polymerizing barbed ends and capped ends unmasked by the CP β tentacle deletion are capable of sequestering WH2 domains. In the absence of the CP β tentacle, WH2 domains cannot discriminate between capped and free barbed filament ends, effectively creating an additional third state that tethers and inactivates the NPF (Fig. 7). We propose that this results in excessive NPF WH2 sequestration and strong inhibition of Arp2/3-dependent branching in the context of the motile network.

Barbed end interference clearly differs from the previously proposed monomer gating hypothesis5. Monomer gating assumes a kinetic competition between free barbed ends and the Arp2/3 complex for a limiting "flux" of monomeric actin transiently

**Fig. 6 The β tentacle is essential for CP localization to lamellipodial actin networks and efficient leading edge protrusion.** A Lysates of B16-F1 control cells, cells with CapZβ KO (clone #10, left) and CapZα1/2 CRISPR/Cas9-treated cells (clone #1, right) subjected to western blotting using CapZβ (left) or CapZα1 and CapZα1/2 (right) antibodies. The experiment was performed once. B Cell morphologies of B16-F1 versus CapZβ KO and CapZα1/2 CRISPR cells stained for the actin cytoskeleton with phalloidin. The experiment was performed once. C Representative phase contrast and fluorescence live cell imaging frames of B16-F1 control cells, CapZβ KO and CapZα1/2 CRISPR/Cas9-treated B16-F1 cells with or without expression of EGFP-tagged CapZβ2 (left) or CapZα1 (right) variants, as indicated. Boxed regions (orange) highlight insets at the bottom to reveal localization patterns of respective, EGFP-tagged rescue constructs. The experiment was repeated three times with similar results. D Quantification of protrusion velocity (with representative kymographs at the bottom) of B16-F1 control cells, CapZβ KO (clone 10, left) or CapZα1/2 CRISPR clone 1 (right) cells and the latter expressing CapZβ2 or CapZα1 variants as indicated. Quantifications were done for n = 32 cells from N = 3 independent experiments, error indicator = SD of the mean. P-values were derived from one-way ANOVA Tukey tests. Source data are provided as a Source Data file.
bound to the nucleation promoting factor. Barbed end interference on the other hand simply reflects a direct interaction between NPFs and filament barbed ends. Our results strongly argue for the latter, because a significant fraction of NPFs bind to actin filament ends even when network assembly is kinetically arrested i.e. in the total absence of monomer flux30 (Fig. 5).

WH2 domains of WAVE-family proteins bind actin monomers more tightly ($K_D < 1 \mu M$) than filament barbed ends ($K_D > 10 \mu M$).41,52 How can barbed ends—either free and polymerizing or capped and "unmasked" by the loss of the CP $\beta$ tentacle—compete with monomers for NPF binding given this large difference in affinity? Several factors might strongly tip the balance towards end binding. First, the density of polymerizing barbed ends at the active front of lamellipodial networks is extremely high (500–2000/µm²) (refs. 5,8,19)). Second, in the context of a growing actin network, free ends are confined to the immediate proximity of the NPF, which is attached to the boundary they push against5,19. This type of dimensionality reduction might greatly favor association. Third, bare actin monomers unlikely exist at levels significantly above their critical concentration under physiological conditions ($c_c < 1 \mu M$). Indeed, the majority of soluble actin is bound to profilin in living cell45,53, which itself competes with and reduces the monomer occupancy of NPF WH2 domains44. The exact contributions of each of these mechanisms to barbed end interference remains to be tested. Importantly, both profilin44,54 and filament barbed ends can indirectly reduce NPF activity through competition with WH2 for monomer binding. While we presently do not fully understand how these two distinct effects combine in the cellular environment, our results show that barbed end interference must constitute an important layer in the control of NPF activity in vivo. In the absence of such an indirect negative feedback, capping and nucleation within branched networks are not tightly coupled, as observed both in cells and in reconstitutions.

In addition to resolving the stimulating effect of CP on Arp2/3-dependent nucleation, barbed end interference explains a multitude of observations otherwise hard to rationalize. Most importantly, it explains why Arp2/3 complex activation by WASP-family proteins requires the transfer of an actin monomer bound to the NPF WH2 domain. The recent discovery of a second class of Arp2/3 complex activators devoid of an actin monomer binding site42,43 demonstrates that monomer delivery is not a mandatory step in nucleation32. Instead, we propose that the NPF WH2 domain illustrates the necessity to study them not only individually as done previously appreciated and have evolved as a functional unit. This structural approach established here provides a powerful strategy to study how all of these and other unrelated end-binding proteins target actin filament ends.

**Methods**

**Protein biochemistry—purification and labeling**

Native (β, γ)-actin. Native bovine (β, γ)—actin was purified from bovine thymus tissue according to the methods described previously45. Briefly, (β, γ)—actin was purified from fresh bovine thymus tissue. After lysis of the thymus tissue, the solution was hard spun and filtered to generate a cleared supernatant that was incubated with His$_6$-gelsolinG4-6 fragment to promote the formation of actin-gelsolin G4-6 complexes. Next, the lysate was circulated over a Ni$_2$-agarose superfactor column. Actin monomers were eluted and polymerized into filaments. After ultracentrifugation, actin filaments were resuspended in F-buffer (1×KMEI, 1×KMPA, 0.5×ATP) and stored in continuous dialysis at 1°C. F-buffer containing fresh ATP and TCEP was continuously exchanged every 4 weeks. Actin was depolymerized through dialysis into BufferA (2 mM Tris, 0.2 mM ATP, 0.5 mM CaCl$_2$, 0.5 mM TCEP) for 6 days and gelfiltered over a Superdex 200 16/600 column prior to all experiments.

Profilin and profilin-actin complexes. Human profilin1 and stoichiometric complexes of profilin and β, γ—actin were purified as described45,44. Briefly, human profilin1 was expressed as untagged protein in E. coli Rosetta cells at 30°C for 4.5 hr. Profilin1 was purified by ammonium sulfate precipitation, followed by ion-exchange (DEAE) and hydroxyapatite (HA) chromatography steps, followed by size exclusion chromatography (Superdex 200 16/600) into storage buffer (20 mM Tris–Cl pH 7.5, 50 mM NaCl, 0.5 mM TCEP). Proteins were snap-frozen in liquid nitrogen upon addition of 20% glycerol to the storage buffer, and stored at −80°C. To generate profilin-actin complexes, freshly gelfiltered actin monomers were incubated with 1.5x molar excess of profilin1 at 4°C overnight. Next, profilin-actin complex was separated from free profilin by gelfiltration over a Superdex 200 10/ 300 GL into Buffer A. The complex was concentrated to working concentrations between 100 and 200 µM and stored at 4°C up to 2 weeks without inducing nucleation.

**Atto540Q-channel labeled actin.** For the labeling of actin monomers with Atto540Q-NHS, the complex of human profilin1 and Acanthamoeba castellanii actin59 was generated in Buffer A using a 2× molar excess of profilin1. Following gelfiltration into labeling buffer (2 mM Hepes pH 8.0, 0.1 mM CaCl$_2$, 0.2 mM ATP, 0.5 mM TCEP), the profilin-actin complex was labeled at its reactive lysine residues by incubating with a 3-fold excess of Atto540Q-NHS for 40 min at 23°C. The reaction was terminated by adding Tris–Cl (pH 8.0) to a final concentration of 2 mM to the reaction mix. After 10 min incubation, soluble dye and protein were separated from solid particles by ultracentrifugation for 10 min at 300,000 g. Next, polymerization of actin was initiated by adding KMEI-buffer (10 mM imidazole pH 7.3, 50 mM KCl, 1.5 mM MgCl$_2$, 1 mM EGTA) and 1% of pre-polymerized, freshly-sheared actin filaments to the labeling mix. After 1 hr of polymerization at 23°C, filaments were separated from excess soluble dye, non-polymerizable actin and profilin by ultracentrifugation for 10 min at 300,000 xg. Actin filaments were stored in F-buffer dialysis at 4°C and diaлизed into Buffer A (for 6 days) prior to experiment. The degree of labeling (73–87%) was determined by absorbance at 280 nm and 543 nm.

**MyosinV/VI.** Human myosin was expressed in E. coli BL21 Rosetta cells from a pETM11 vector containing an N-terminal 10xhis tag followed by a
TEV-cleavage site. After protein expression for 16 hrs at 18 °C, cells were lysed (50 mM KPi, pH 7.3, 400 mM NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF, DNaseI). The lysate was hard spun and clarified by IMAC over a 5 mL HitTrap Chelating column. After gradient elution (50 mM KPi, pH 7.3, 400 mM NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol), the His₆-tag was cleaved by TEV protease overnight. After cleavage, the protein was desalted into lysis buffer and filtered over a HitTrap Chelating column. The flow-through was gel-filtered over a Superdex 200 column (20 mM Hepes pH 8.0, 50 mM KCl, 0.5 mM TCEP) and snap-frozen in liquid nitrogen supplemented with 20% glycerol. For long-term storage, the protein was stored at −80 °C.

Capping protein. Capping protein mutants were either generated via PCR amplification and Gibson cloning40,44,83 or via site-directed mutagenesis (W271K, a277V, EGFP-CapZβ2). Primers sequences are listed in Supplementary Table 3. Murine heterodimeric capping protein (α in pETM20, β in pETM3344) were co-expressed in E. coli BL21 Rosetta cells. Following protein expression, cells were lysed (50 mM KPO₄ pH 7.3, 400 mM NaCl, 5 mM imidazole, 1 mM EDTA, 2 mM PMFS, 0.75 mM β-mercaptoethanol, 15 μg/mL benzamidine, DNasel) and purified by IMAC over a 5 mL HitTrap Chelating column. After gradient elution (50 mM KPO₄ pH 7.3, 400 mM NaCl, 5 mM imidazole, 1 mM EDTA, 2 mM PMFS, 0.75 mM β-mercaptoethanol), the His₆-tag was cleaved by TEV/Prezcision proteases overnight. Following cleavage, the protein was desalted into low salt MonoQ buffer (50 mM KCl, 0.5 mM TCEP) and snap-frozen in liquid nitrogen supplemented with 20% glycerol. For long-term storage, the protein was stored at −80 °C.

Arp2/3 complex. Native bovine Arp2/3 complex was purified from fresh bovine thymus extracts using a combination of exchange chromatography (DEAE, Source Q and Source S) followed by gel filtration over a Superdex 75 column as described106,107. The protein was either directly snap frozen with 20% glycerol and stored at −80 °C or transferred into a labeling reaction.

Arp2/3 complex was fluorescently labeled by the addition of 5-fold molar excess of maleimide-Alexa647 dye conjugate. After incubation on ice for 2 hrs, the reaction was terminated by adding 2 mM DTT for 30 min. Next, the complex was loaded onto an N-WASP-VCA column (5 mM Tris-Cl pH 8.0, 50 mM NaCl, 0.5 mM MgCl₂, 0.1 mM ATP, 2 mM DTT) and gradient-eluted over 100 volume column (5 mM Tris-Cl pH 8.0, 1 M NaCl, 0.5 mM MgCl₂, 0.1 mM ATP, 2 mM DTT). Fractions containing the full complex were gel-filtered (5 mM Hepes pH 7.5, 400 mM NaCl, 0.5 mM MgCl₂, 0.1 mM ATP, 0.2 mM TCEP) over a Superose 6 column, concentrated, snap-frozen with 20% glycerol and stored at −80 °C.

Preparation of an N-WASP-loaded column for Arp2/3 complex labeling. Before immobilization onto a TIRF-labeled coverslip (22 × 22 mm, 1.5 H, Marienfeld-Superior) functionalized as described105, reaction flow chambers were blocked with a Pluronic solution (0.1 mg/mL k-casein, 1% Pluronic F-127, 1 mM TCEP, 1xKMEI) followed by 2 washing steps with 40 μL each (0.5 mM ATP, 1 mM TCEP, 1xKMEI, 0.1 mg/mL β-casein). Next, the channels were incubated with 75 mM streptavidin for 4 min following a washing step and incubation of 90 mM biotin-phalloidin29,59 for 4 min.

Actin filament elongation assays using TIRF-M were performed as described59,61. Briefly, 9 μL of a 4.4 μM profilin-actin solution was incubated with 1 μL of 10x ME (0.5 mM MgCl₂, 2 mM EGTA) for 1 min at RT following the addition of 4 μL oxygen scavenging mix (1.25 mg/mL glucose-oxidase, 0.2 mg/mL catalase, 400 mM glucose)85,86. 400 mM glucose ATP with 26 μL reaction buffer containing TIRF- buffer (20 mM Hepes pH 7.0, 100 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 20 mM β-mercaptoethanol, 0.1 mg/mL β-casein, 1 mM ATP). This buffer has a molar ionic strength of 0.133 M, which is close to the physiological ionic strength found in the literature (between 0.1 and 0.2 M).

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Biochemical assays

Buffers. All experiments were carried out in a common final assay buffer of the following composition if not stated otherwise: 20 mM Hepes pH 7.0, 100 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 20 mM β-mercaptoethanol, 0.1 mg/mL β-casein, 1 mM ATP. This buffer has a molar ionic strength of 0.133 M, which is close to the physiological ionic strength found in the literature (between 0.1 and 0.2 M).

Functionnalization of glass coverslip surfaces and protein immobilization. For TIRF-M experiments, microscopy counter slides were passivated with PLL-PEG and maleimide-functionalized glass coverslip. The glass coverslips were functionalized as described105. Reaction flow chambers were blocked with a Pluronic solution (0.1 mg/mL k-casein, 1% Pluronic F-127, 1 mM TCEP, 1xKMEI) followed by 2 washing steps with 40 μL each (0.5 mM ATP, 1 mM TCEP, 1xKMEI, 0.1 mg/mL β-casein). Next, the channels were incubated with 75 mM streptavidin for 4 min following a washing step and incubation of 90 mM biotin-phalloidin29,59 for 4 min.

Measuring capping protein association and dissociation kinetics for the filament barbed end. Experiments were performed as described in the previous section with the following modifications: kₐ, measurements of capping protein for filament barbed ends by TIRF-microscopy. To determine the association rate constant for capping protein (wt and affinity mutants) for the actin filament barbed end, experiments were conducted similarly to protocols outlined in59,63 with modifications. Briefly, small pre-polymerized phallloidin-stabilized actin seeds were immobilized to the glass surface of a TIRF chamber. Increasing capping protein (TIRF-labeled or non-labeled CP) concentrations (0.5–15 ng/mL) were used with 2 μM Mg-ATP actin monomers and 2 μM profilin1 in TIRF reaction buffer and flowed into the chamber. Elapsed time between mixing and imaging was ~40 s. The time courses of capping protein binding to the filament barbed end (termination of barbed end polymerization) were recorded in single molecule or single filament TIRF time-lapse experiments. Only filaments that were visualized in the first frame were followed throughout the experiment. Filaments that were immobilized from solution during the experiment or new filaments that originated from spontaneous nucleation were excluded from the analysis. Therefore, only filament barbed ends that were initially free were scored. The experiment was recorded over a time period of 5 min in 1 s intervals. ~80 filaments were measured for each capping protein concentration from ≥3 experiments.

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Integration of protein intensity on SDS gel after SEC. After SDS-gel run, actin and capping coomassie signal intensities were analyzed using ImageJ. After selecting protein signal areas for each protein, intensities were plotted in a plot representing the intensity signal across the selected area.

Cell culture of B16-F1 cells. B16-F1 cells and derived CapZa1/2 CRISPR#1, and CapZb KO#10 were cultured in DMEM (4.5 g/L glucose; Invitrogen, Germany), supplemented with 10% FCS (Gibco), 2 mL glutaamine (Thermo Fisher Scientific), and penicillin (50 Units/mL) streptomycin (50 µg/mL) (Thermo Fisher Scientific) and 1% CO2. Cells were routinely transferred in 35 mm dishes or 6-well plates, using 500 ng DNA in total and 1 µL JetPrime. After overnight transfection, cells were either plated onto acid-washed, laminin-coated (25 µg/mL) coverslips for phallolidin or ArpCA5 staining or in a µSlide 4 Well µ (Ibidi) chamber slides, coated with 25 µg/mL laminin, for live cell fluorescence microscopy.

Generation of CapZa1/2 and CapZb CRISPR/Cas9 clones. Disruption of Cpa or β expression in B16-F1 cells was achieved by targeting CapZb or CapZa1 and CapZb2 simultaneously using CRISPR/Cas9-mediated genome editing, using targeting-guide sequences CCTCAGCGATCTGTCGACC (for CapZb exon 2), GAGTTTAATGAGATTTACCAA (for CapZa1 exon 2), and CAGAAGGAAAGTGGCGGATGC (for CapZa2 exon 1), cloned into plasmid psPc9/BB)-2A-Puro (Addgene, ID 40139). Plasmids were transfected into B16-F1 cells, followed by selection of transfected cells for three days using 2.5 µg/mL puromycin (Sigma-Aldrich, Tautkirchen, Germany). Puromycin-resistant cells were then extensively diluted and single cell-derived colonies grown for 5 to 7 days. After colony isolation, cells were expanded and cloned screened by Western Blotting using CapZb or CapZa1 and CapZb2-specific antibodies, allowing selection of CRISPR-clones #10 (for CapZb) and #1 (for CapZa2). The genotypes of these clones were examined using TIDE sequencing. In case of CapZb KO#10, all functional alleles could be disrupted. Instead, for CapZa1/2 CRISPR clone #1, sequencing revealed that all CapZa1 alleles were disrupted, whereas expression from CapZa2 alleles appeared partially possible from alleles harboring a 9 bp deletion. Additional, subsequent targeting of CapZa2 alleles on exon 2 failed to eliminate expression from those 9 bp-deletion alleles.

Western blotting. For preparation of whole cell lysates, cells were washed with PBS, lysed using Laemmli-buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.05% bromophenol blue, 63 mM Tris-HCl pH 6.8), sonicated to shear genomic DNA and boiled for 5 min at 95°C. Western blotting was carried out using standard techniques, and with antibodies as listed in the Key Resources Table (Supplementary Table 4). Chemiluminescence signals were obtained upon incubation with ECL Prime Western Blotting Detection Reagent (GE Healthcare), and recorded with ECL Chemocam imager (Intas, Goettingen, Germany). Original, uncorrected versions of all blots used for figures in the paper can be found in Supplementary Fig. 9.

Phalloidin and immune-stainings. For solely stainings of the actin cytoskeleton of untransfected B16-F1, CapZa1/2 CRISPR#1 and CapZb KO#10 cells, respective cells were fixed with pre-warmed 4% paraformaldehyde in PBS, supplemented with 0.25% glutaraldehyde for 20 min, and permeabilized with 0.05% Triton X-100 in PBS for 30 min. The actin cytoskeleton was subsequently stained using Alexa 488-conjugated phallolidin (dilution 1:200 in PBS). For stainings upon rescue experiments of CapZb KO#10 cells with EGFP-tagged CapZb2 variants, B16-F1 wildtype and rescued CapZb2 KO#10 cells were fixed as described above with the exception that glutaraldehyde was omitted from the fixation mixture. Subsequently, cells were either stained with ATTO-S94-conjugated phallolidin (dilution 1:200 in PBS) or, after blocking with 5% horse serum / 1% BSA in PBS with anti-ArpCA5 antibody (undiluted hybridoma supernatant; see Key Resources Table (Supplementary Table 4)). The primary antibody was then visualized with Alexa-S94-conjugated anti-mouse IgG (1:100 dilution, see Key Resources Table (Supplementary Table 4)). Samples were mounted using ProLong Gold or VectaShield Vibrance antifade reagents and imaged using a ×63/1.4NA Plan apochromatic oil objective.

Fluorescence microscopy data acquisition TIRF-Microscopy data acquisition. All in vitro experiments were performed at 23°C, unless otherwise specified. Images were acquired using a customized Nikon Ti2 TIRF-Microscope with Nikon perfect focus system. TIRF-images were acquired by a dual EM CCD Andor iXon camera system (Cairn) controlled by NIS-Elements software. Dual color imaging was performed through an AXITIRF6000-0.05 objective using a custom multilayer launch system (AcralFlIc) at 488 nm and 560 nm and a Quad-Notch filter (400-410/488/561/631640). Images were acquired at intervals of 1-30 s.

Wide-field epifluorescence-microscopy data acquisition. All in vitro experiments were performed at 23°C. Bead motility assays were carried out using a Olympus IX81 wide field epifluorescence microscope with LED illumination (E4000, CoolLED). Image acquisition was performed by a Hamamatsu c9100-13 EMCCD.
camera controlled by Micromanipulator 1.4 software. Multi-color images were taken through an UPlanS APO 40x or 60x oil objective.

**Live cell fluorescence microscopy and quantification of protrusion velocity.** B16-F1, CapZa1/2 CRISPR/F1 and CapZb KO10 cells, untransfected or transfected with indicated EGFP-tagged CapZ constructs, were plated in 14-Well Ph + (Ibidi) chamber slides overnight. Thereafter, the medium was replaced with microscopy medium (F12 HAM HEPEs-buffered medium, Sigma), including 10% FCS (Gibco), 2 mM glutamine (Thermo Fisher Scientific) and penicillin (50 Units/mL)/streptomycin (50 µg/mL) (Thermo Fisher Scientific). The slide was mounted onto an Axiovert 100 (Carl Zeiss Microscopy) equipped with a 37 °C incubator, and with an HXP 120 lamp for epifluorescence illumination, a halogen lamp for phase-contrast imaging, a CoolSnap-HQ2 camera (Photometrics) and electronic shutters driven by MetaMorph software (Molecular Devices). Unpolar cells were chosen for analysis. Phase contrast images, and fluorescence images in case of cells transfected with EGFP-tagged CapZ constructs, were acquired every 5 seconds using a 63x/1.4NA Plan apochromatic oil objective for a period of 5 min. Protrusion velocities were determined based on phase contrast-derived kymographs using MetaMorph 7.8. For every cell analyzed, kymographs at three different positions of the leading edge were taken. For EGFP fluorescence intensity measurements of transfected CapZb or CapZa1/2 cells shown in Supplementary Figure 8, average fluorescence intensities of large cytosolic regions were measured, and corrected for background by subtracting average fluorescence intensities of respective, adjacent extracellular regions. Obtained data were displayed as counts/pixel.

**Electron microscopy**

Negative-stain microscopy. Immediately after SEC, the quality of the short actin filament preparations was assessed with negative stain EM. Briefly, 4 µL of sample were deposited onto a glow-discharged-carbon-coated copper grid and incubated for 30 s. After blotting the excess sample, the grid was washed twice with KMEI buffer and stained with 0.75% (w/v) uranyl formate. The grids were subsequently imaged at 120 kV in a Tecnai Spirit microscope equipped with a LaB6 cathode and 4k × 4k CMOS detector F416 (TVIPS). Most importantly, we used these images to decide on the concentration used for preparing the cryo-EM grids.

**Cryo grid preparation.** Cryo-EM samples were prepared using graphene-oxide-coated Quantifoil 1.2/1.3 300 mesh grids (GO-grids). We produce our GO-grids as Cryo grid preparation. We then exported the particles to RELION where we performed Bayesian particle cleaning followed by local re-optimization within SPHIRE. Supplementary Fig. 1 shows a schematic summary of the procedure.

**Resolution estimation and maps postprocessing.** The final map was obtained from particles with some preferential orientation. To estimate the directional FSC of the map, we used the 3D-FSC package. Local resolution was estimated using the false-discovery rate FSC method implemented in SPROX. The final map was locally filtered using deepEMHancer. Statistics of the Cryo-EM model of capped actin filaments are summarized in Supplementary Table 1.

**Atomic modeling.** We used MODELLER to build a homology model of the complex between CP and the barbed end of human β-actin. As template, we used the capped end of the Arp1 filaments within the dynactin complex (PDBID: 5AXD). We run this model through the Rosetta alanaine scan to identify side chains whose deletion would provide a similar decrease in binding affinity as the removal of the β-tentacle.

To build an atomic model of the complex, we fitted the initial homology model into the density using a combination of ISOLDE and Coot. In order to deal with the strong quality gradient of our density, we used the locally filtered map produced by deepEMHancer for modeling. High-resolution refinement is possible in the area close to the center of the filament, where the resolution is highest. To deal with the least resolved areas, we used adaptive distance restraints during our ISOLDE runs. In this way, CP could be flexibly fitted into our density, but the high-resolution details were largely inherited from the initial model. Likewise, we manually included several distance restraints aimed at maintaining proper conformation of the Mg2+ complex at actin’s active site. A final round of refinement in Phenix using positional restraints was used to remove some geometric violation and estimate the atomic B-factors, while keeping the overall structure produced by ISOLDE.

**Quantification and statistical data analysis.** All analyzed data were plotted and fitted in Origin9.0 G. All fluorescence microscopy experiments were analyzed in ImageJ manually via kymograph analysis and plotting of intensity profiles unless described otherwise.

**Determination of the association and dissociation rate constants of capping protein for actin filament ends.** Images were analyzed by manual filament tracking using the segmental line tool from ImageJ and further analyzed by the kymograph plugin. The parameters were measured to determine the polymerization rate of individual actin filaments. The pixel size/length was converted into microns/s. One actin monomer contributes to 2.7 nm of the actin filament length. For each experimental condition, the filament polymerization velocity was measured from ≥50 filaments per condition, and reported as mean value with error bars representing SD. The time courses of the fraction of re-growing filament ends were fitted with mono-exponential functions to yield the observed pseudo-first order reaction rates (kon) as a function of capping protein concentration. The association rate constants (koff) were determined from linear regression fits of the kon values (error = SD) as a function of the total capping protein concentration [nM]. The time courses of the fraction of re-growing filament ends were fitted with single exponential functions to yield the dissociation rate constants (koff). The equilibrium dissociation constants (Kd) of capping protein for actin filament barbed ends were calculated from the measured dissociation rate constants (koff) and association rate constants (koff) using the following equation:

\[
K_d = \frac{k_{off}}{k_{on}}
\]

**Errors for the equilibrium dissociation constants were calculated using error propagation.**

**Quantification of fluorescence intensities in actin dendritic networks.** The mean fluorescence intensities of all network components (actin, CP and Arp2/3 complex) from multicolor fluorescence images were measured with ImageJ (ROI Manager) and exported to Microsoft Excel for comparison to image intensity from line regions of interest (ROIs) matching the network area. The background fluorescence intensities for all components was determined from identified lines drawn adjacent to each network. The background signal was subtracted from the network signal. The integrated mean fluorescence intensities (representing polymerization, capping and nucleation rates) for each component was plotted as a function of distance from the bead surface [µm]. The rates for actin polymerization, capping and nucleation were calculated from integrated fluorescence intensities divided by reaction time.
Quantification of the NPF-WH2-occupancy on actin filament ends. The fluorescence signal intensity of the Alexa488-labeled NPF was measured over the entire bead surface and after addition of quencher-labeled Atto480Q actin monomers. The intensity data was plotted over the bead distance/diameter and represents the amount of WH2 domains occupied by actin filament ends. For each condition, ≥25 dendritic actin networks from ≥3 experiments were quantified.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
All quantitative data presented in the manuscript are contained in the source data file. Additional data are available from the corresponding authors upon request. A reporting summary for this Article is available as a Supplementary Information file. Our structure of capped barbed end is deposited in Protein Data Bank PDB, 6B2D, 7PDZ and Electron Microscopy Data Bank, EMDB-13343. The previously published structure of the dynactin complex69 is available in the Protein Data Bank PDB, 6AXD. The previously published structure of monomeric actin in complex with the WH2 domain of WASP49 is available in the Protein Data Bank PDB, 6AZX. Source data are provided with this paper.

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**References**

1. Cooper, J. A. & Sept, D. New insights into mechanism and regulation of actin capping protein. *Int. Rev. Cell Mol. Biol.* **267**, 183–206 (2008).
2. Edwards, M. et al. Capping protein regulates fine-tune actin assembly dynamics. *Nat. Rev. Mol. Cell Biol.* **15**, 677–689 (2014).
3. Prost, J. M., Robinson, K. A., Titus, M. A. & Fritz-Laylin, L. K. The actin networks of chytrid fungal cells reveal evolutionary loss of cytoskeletal complexity in the fungal kingdom. [http://bioreport.org/lookupdoi/10.1101/2020.06.12.142943](http://bioreport.org/lookupdoi/10.1101/2020.06.12.142943) (2020). [https://doi.org/10.1101/2020.06.12.142943](https://doi.org/10.1101/2020.06.12.142943).
4. Rivero, F. & Cvrcková, F. Origins and evolution of the actin cytoskeleton. *Adv. Exp. Med. Biol.* **607**, 97–110 (2017).
5. Akin, O. & Mullins, R. D. Capping protein increases the rate of actin-based motility by promoting filament nucleation by the Arp2/3 complex. *Cell 133*, 841–851 (2008).
6. Cameron, L. A., Svitkina, T. M., Vignjevic, D., Theriot, J. A. & Borisy, G. G. Endocytic organization of actin comet tails. *Curr. Biol. 11*, 130–135 (2001).
7. Leissl, T. P., Boujemaa, R., Pantaloni, D. & Carlier, M. F. Reconstitution of the actin cytoskeleton-autophagy connection. *Curr. Biol. 28*, R318–R326 (2017).
8. Efimova, N. & Svitkina, T. M. Branched actin networks push against each other at adherens junctions to maintain cell-cell adhesion. *J. Cell Biol.* **217**, 1827–1845 (2018).
9. Blanchin, L., Boujemaa-Paterski, R., Sykes, C. & Plastino, J. Actin dynamics, architecture, and mechanics in cell motility. *Physiol. Rev. 94*, 235–263 (2014).
10. Isenberg, G., Aebi, U. & Pollard, T. D. An actin-binding protein from Acanthamoeba regulates actin filament polymerization and interactions. *Nature 288*, 455–459 (1980).
11. Schaffer, D. A., Jennings, P. B. & Cooper, J. A. Dynamics of capping protein and actin assembly in vitro: uncapping barbed ends by polyphosphoinositides. *J. Cell Biol. 135*, 169–179 (1996).
12. Vinzenz, M. et al. Actin branching in the initiation and maintenance of lamellipodia. *J. Cell Sci. 125*, 2775–2782 (2012).
13. Yelden, C. A., Smith, B. A., Breitsprecher, D., Gelles, J. & Goode, B. L. Cease-fire at the leading edge: new perspectives on actin filament branching, depolymerization, and capping. *Curr. Cytoskeleton 68*, 596–602 (2011).
14. Achard, V. et al. A ‘primer’-based mechanism underlies branched actin filament network formation and motility. *Curr. Biol. 20*, 423–428 (2010).
15. Hug, C. et al. Capping protein levels influence actin assembly and cell motility in dicytostelium. *Cell 81*, 591–600 (1995).
16. Mejillano, M. R. et al. Lamellipodial versus filopodial mode of the actin nanomachinery: pivotal role of the filament barbed end. *Cell 118*, 363–373 (2004).
17. Mullins, R. D., Bieling, P. & Fletcher, D. A. From solution to surface to filament: actin flux into branched networks. *Biophys. Rev. 10*, 1537–1551 (2018).
18. Rotty, J. D., Wu, C. & Bear, E. J. New insights into the regulation and cellular functions of the Arp2/3 complex. *Nat. Rev. Mol. Cell Biol. 14*, 7–12 (2013).
19. Yamashita, A., Maeda, K. & Maéda, Y. Crystal structure of CapZ: structural basis for actin filament barbed end capping. *EMBO J. 22*, 1529–1538 (2003).
20. Narita, A., Takeda, S., Yamashita, A. & Maeda, Y. Structural basis of actin filament capping at the barbed-end: a cryo-electron microscopy study. *EMBO J. 25*, 5626–5636 (2006).
21. Kim, T., Cooper, J. A. & Sept, D. The interaction of capping protein with the barbed end of the actin filament. *J. Mol. Biol. 404*, 794–802 (2010).
22. Wear, M. A., Yamashita, A., Kim, K., Maeda, Y. & Cooper, J. A. How capping protein binds the barbed end of the actin filament. *Curr. Biol. 13*, 1531–1537 (2003).
23. Unruh, L. et al. The structure of the dynactin complex and its interaction with dynemin. *Science 347*, 1441–1446 (2015).
24. Wang, R. & Carlsson, A. E. How capping protein enhances actin filament growth and nucleation on biomimetic beads. *Phys. Biol. 12*, 066008 (2015).
25. Akin, O. & Mullins, R. D. Capping protein binding to actin in yeast: biochemical mechanism and physiological relevance. *J. Cell Biol. 164*, 857–860 (2004).
26. Miura, K. & Aoe, M. Twin-linkin and formin constitute a pacemaker system for robust capping of actin in dendritic nucleation actin network: evidence of frequent capping and uncapping activity. *Curr. Biol. 17*, 395–406 (2007).
27. Lai, F. P. L. et al. Arp2/3 complex interactions and actin network turnover in lamellipodia. *EMBO J. 27*, 982–992 (2008).
28. Miyoshi, T. et al. Actin turnover-dependent fast dissociation of capping protein in the dendritic nucleation actin network: evidence of frequent filament severing. *J. Cell Biol. 175*, 947–955 (2006).
29. Hakala, M. et al. Twinfilin uncaps filament barbed ends to promote turnover of lamellipodial actin networks. *Nat. Cell Biol. 23*, 147–159 (2021).
30. Mwangangi, D. M., Manber, E. & Robinson, R. C. The structure of the actin filament uncapping complex mediated by twinfilin. *Sci. Adv. 7*, eabd5271 (2021).
31. Bieling, P. et al. WH2 and proline-rich domains of WASP-family proteins collaborate to accelerate actin filament elongation. *EMBO J. 37*, 102–121 (2018).
32. Funk, J. et al. Profilin and formin constitute a pacemaker system for robust actin filament growth. *Elife 8*, e05063 (2019).
33. Reidl, I. et al. Lifact: a versatile marker to visualize F-actin. *Nat. Methods 5*, 605–607 (2008).
34. Schaffer, D. A., Korshunova, Y. O., Schroer, T. A. & Cooper, J. A. Differential localization and sequence analysis of capping protein beta-subunit isoforms of vertebrates. *J. Cell Biol. 127*, 435–463 (1994).
35. Hart, M. C., Korshunova, Y. O. & Cooper, J. A. Vertebrates have conserved capping protein isoforms with specific expression patterns. *Cell Motil. Cytoskeleton 38*, 120–132 (1997).
36. Hurst, S., Howes, E. A., Coadwell, J. & Jones, R. Expression of a testis-specific putative actin-capping protein associated with the developing acrosome during rat spermiogenesis. *Mol. Reprod. Dev. 49*, 81–91 (1998).
37. Cao, W., Wang, D. T., Gierke, S., Chang, V. & Taunton, J. Mechanism of actin network attachment to moving membrane barbed end capture by N-WASP WH2 domains. *Cell 128*, 901–913 (2007).
51. Chereau, D. et al. Actin-bound structures of Wiskott-Aldrich syndrome protein (WASP)-homology domain 2 and the implications for filament assembly. *Proc. Natl Acad. Sci. USA* 102, 16644–16649 (2005).

52. Marchand, J. B., Kaiser, D. A., Pollard, T. D. & Higgins, H. N. Interaction of WASP/Scar proteins with actin and vertebrate Arp2/3 complex. *Nat. Cell Biol.* 3, 76–82 (2001).

53. Kaiser, D. A., Vinson, V. K., Murphy, D. B. & Pollard, T. D. Profilin is predominantly associated with monomeric actin in Acanthamoeba. *J. Cell Sci.* 112, 3779–3790 (1999). Pt 21.

54. Rotty, J. D. et al. Profilin-1 serves as a gatekeeper for actin assembly by Arp2/3-dependent and -independent pathways. *Dev. Cell* 32, 54–67 (2015).

55. Balzer, C. J., Wagner, A. R., Helgesen, L. A. & Nolen, B. J. Dp1 co-opts features of branching nucleation to create linear actin filaments that activate WASP-bound Arp2/3 complex. *Curr. Biol.* 28, 3886–3891 (2018). ed.

56. Helgesen, L. A. & Nolen, B. J. Mechanism of synergistic activation of Arp2/3 complex by cortactin and N-WASP. *Elife* 2, e00884 (2013).

57. Bombardier, J. P. et al. Single-molecule visualization of a formin-capping protein ‘decision complex’ at the actin filament barbed end. *Nat. Commun.* 6, 8707 (2015).

58. Schekkars, S. et al. Formin and capping protein together embrace the actin filament in a ménage à trois. *Nat. Commun.* 6, 8730 (2015).

59. Hansen, S. D., Zucher, J. B. & Mullins, R. D. Cytoplasmic actin: purification and single molecule assembly assays. *Methods Mol. Biol.* 1046, 145–170 (2013).

60. Gibson, D. G. Enzymatic assembly of overlapping DNA fragments. *Methods Enzymol.* 498, 349–361 (2011).

61. Keppler, A. et al. A general method for the covalent labeling of fusion proteins with small molecules in vivo. *Nat. Biotechnol.* 21, 86–89 (2003).

62. Doolittle, L. K., Rosen, M. K. & Padrick, S. B. Purification of native Arp2/3 complex from bovine thymus. *Methods Mol. Biol.* 1046, 231–250 (2013).

63. Antos, J. M. et al. Site-specific protein labeling via sortase-mediated transpeptidation. *Curr. Protoc. Protein Sci.* 89, 15.3.1–15.3.19 (2017).

64. Kuhn, J. R. & Pollard, T. D. Real-time measurements of actin filament polymerization by total internal reflection fluorescence microscopy. *Biophys. J.* 88, 1387–1402 (2005).

65. Atkin, C. E., Marshall, R. A. & Puglisi, J. D. An oxygen scavenging system for improved dye stability in single-molecule fluorescence experiments. *Biophys. J.* 94, 1826–1835 (2008).

66. Rasnik, L., McKinney, S. A. & Ha, T. Nonblinking and long-lasting single-molecule fluorescence imaging. *Nat. Methods* 3, 891–893 (2006).

67. Hansen, S. D. & Mullins, R. D. VASP is a processive actin polymerase that requires monomeric actin for barbed end association. *J. Cell Biol.* 191, 571–584 (2010).

68. Kuhn, J. R. & Pollard, T. D. Single molecule kinetic analysis of actin filament capping. Polyphosphoinositides do not dissociate capping proteins. *J. Biol. Chem.* 282, 28014–28024 (2007).

69. Brinkman, E. K., Chen, T., Amendola, M. & van Steensel, B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res.* 42, e168 (2014).

70. Edelstein, A. D. et al. Advanced methods of microscope control using μManager software. *J. Biol. Methods* 1, e10 (2014).

71. Zheng, S. Q. et al. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat. Methods* 14, 331–332 (2017).

72. Rohou, A. & Grigorieff, N. CFIFFIND: Fast and accurate defocus estimation from electron micrographs. *J. Struct. Biol.* 192, 216–221 (2015).

73. Stabrin, M. et al. TranSPHIRE: automated and feedback-optimized-on-the-fly processing for cryo-EM. *Nat. Commun.* 11, 5716 (2020).

74. Moriya, T. et al. High-resolution single particle analysis from electron cryomicroscopy images using SPHIRE. *J. Vis. Exp.* (2017). https://doi.org/10.3791/55448.

75. Wagner, T. et al. SPHIRE-cryoYOLO is a fast and accurately fully automated particle picker for cryo-EM. *Commun. Biol.* 2, 218 (2019).

76. Tang, G. et al. EMA2N: an extendible image processing suite for electron microscopy. *J. Struct. Biol.* 157, 38–46 (2007).

77. Yang, Z., Fang, J., Chittiluru, J., Astarias, F. I. & Penczek, P. A. Iterative stable alignment and clustering of 2D transmission electron microscope images. *Structure* 20, 237–247 (2012).

78. Scheres, S. H. W. RECLION: implementation of a Bayesian approach to cryo-EM structure determination. *J. Struct. Biol.* 180, 519–530 (2012).

79. Zivanov, J., Nakane, T. & Scheres, S. H. W. A Bayesian approach to beam-induced motion correction in cryo-EM. *Nat. Struct. Mol. Biol.* 6, 5–17 (2019).

80. Tan, Y. Z. et al. Addressing preferred specimen orientation in single-particle cryo-EM through tilting. *Nat. Methods* 14, 793–796 (2017).

81. Beckers, M. & Sachse, C. Permutation testing of Fourier shell correlation for resolution estimation of cryo-EM maps. *J. Struct. Biol.* 212, 107579 (2020).

82. Sanchez-Garcia, R. et al. DeepEMhunter—a deep learning solution for cryo-EM volume post-processing. http://bioxrv.org/lookup/doi/10.1101/2020.06.12.148296 (2020). https://doi.org/10.1101/2020.06.12.148296.

83. Eswar, N., Eramian, D., Webb, B., Shen, M.-Y. & Sali, A. Protein structure modeling with MODELLER. *Methods Mol. Biol.* 426, 145–159 (2008).

84. Kortemme, T., Kim, D. E. & Baker, D. Computational alanine scanning of protein-protein interfaces. *Sci. STKE* 2004, pl2 (2004).

85. Croll, T. I. ISOLDE: a physically realistic environment for model building into low-resolution electron-density maps. *Acta Crystallogr. D. Struct. Biol.* 74, 519–530 (2018).

86. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. *Acta Crystallogr. D. Biol. Crystallogr.* 66, 486–501 (2010).

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