Magic angle spinning NMR structure of human coflin-2 assembled on actin filaments reveals isoform-specific conformation and binding mode

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Actin polymerization dynamics regulated by actin-binding proteins are essential for various cellular functions. The coflin family of proteins are potent regulators of actin severing and filament disassembly. The structural basis for coflin isoform-specific severing activity is poorly understood as their high-resolution structures in complex with filamentous actin (F-actin) are lacking. Here, we present the atomic-resolution structure of the muscle-tissue-specific isoform, coflin-2 (CFL2), assembled on ADP-F-actin, determined by magic-angle-spinning (MAS) NMR spectroscopy and data-guided molecular dynamics (MD) simulations. We observe an isoform-specific conformation for CFL2. This conformation is the result of a unique network of hydrogen bonding interactions within the α2 helix containing the non-conserved residue, Q26. Our results indicate F-site interactions that are specific between CFL2 and ADP-F-actin, revealing mechanistic insights into isoform-dependent F-actin disassembly.
Monomeric (globular) actin (G-actin) undergoes dynamic cycles of polymerization into filamentous actin (F-actin) and depolymerization coupled to ATP hydrolysis, known as actin treadmilling (Fig. 1a)\(^1\). Actin treadmilling is the underlying mechanism for functions like cell migration and cell motility and is tightly regulated by different classes of actin-binding proteins (ABPs). These proteins work in concert to spatiotemporally carry out cellular functions\(^2\). The dynamics of F-actin is controlled by the actin nucleotide state, which acts as a so-called nucleotide clock. Nucleotide hydrolysis induces conformational changes at the intra-strand interface on the filament surface\(^3\), where they are sensed and amplified by several essential ABPs involved in the regulation of actin dynamics.

The cofilin family of proteins (referred to broadly as cofilin throughout the text) are responsible for actin filament severing and promote the turnover of G-actin monomers\(^4,5\). Cofilins are expressed in all eukaryotes, from yeast to humans. In humans, three separate genes encode for each cofilin isoform. Cofilin-1 (CFL1) is ubiquitously expressed; cofilin-2 (CFL2) is primarily expressed in muscle tissue; and destrin (DSTN, a.k.a. actin-depolymerizing factor) is expressed in neuronal and epithelial tissues. Cofilin is essential in mammals, and its different isoforms cannot fully compensate for each other in vivo due to

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**Fig. 1 MAS NMR structure of CFL2 bound to ADP-F-actin.** a Top: Structure of F-actin (left, PDB ID: 5ONV), composed of G-actin monomers (right, PDB ID: 1ATN). Separate strands are designated in pale cyan and dark teal. Bottom: TEM images of cofilinactin assemblies used for MAS NMR experiments. A representative image before (left) and after (right) MAS NMR experiments is shown (scale bars: 100 nm). TEM images were collected on three independent preparations with similar results. b 2D \(^1\)\(^3\)C-\(^1\)\(^3\)C CORD spectra acquired on CFL2 samples labeled with \(^1\)\(^6\)\(^1\)\(^3\)C-glucose (magenta) and \(^2\)\(^1\)\(^3\)C-glucose (teal) used for inter-residue distance restraints (CORD mixing time, 50 ms). Selected assignments are labeled on each spectrum. c Top: Ribbon representation of the lowest energy structure of CFL2 bound to ADP-F-actin (PDB ID: 7M0G). The number of unambiguous distance restraints are designated on the structure ranging from 0–5 restraints per residue (green) to greater than 20 restraints per residue (gray). Bottom: Sequence and secondary structure elements of CFL2. Residues constituting the canonical G- and F- binding patches are indicated with green and violet asterisks, respectively. d Top: Number of unambiguous distance restraints versus residue number. Bottom: Distance restraint networks and local alignment of structure ensemble for selected residues.
their specialized roles. For example, depletion of CFL1 in mice is embryonic lethal, and depletion of CFL2 causes abnormalities in α-skeletal muscle tissue development and fatal cardiomyopathies. Each isoform also exhibits distinct depolymerization and severing rates, thus finely tuning actin dynamics in different cellular compartments to achieve the desired phenotype. Consequently, aberrant co-assembly of CFL2 with rabbit MAS NMR structure of CFL2 bound to ADP-F-actin. 

Here, we report the atomic-resolution structure of CFL2 assembled with actin is shown in Fig. 1c. The structure of CFL2 assembled with actin is shown in Fig. 1c. Structure refinement and validation statistics are shown in Table 2. CFL2 exhibits an αβ domain fold typical of other cofilin isoforms. The core consists of five β-strands surrounded by six helices and loop regions. β1–β4 form anti-parallel β-sheets, and β5 is packed parallel to β4. Compared to yeast cofilin, vertebrate cofilins are slightly larger and have sequence insertions in loop regions corresponding to the nuclear localization sequence (NLS) (residues R21-K33), residues L56-D66, and a short β-hairpin at the C-terminus (residues G155-L166). The bundle backbone and heavy-atom RMSDs for CFL2 are 1.05 and 1.67 Å, respectively. With the multiple distance restraints, the side chain conformations are well defined for 117 residues, with most sidechains adopting a single conformation (Fig. 1d).

**RESULTS**

**MAS NMR structure of CFL2 bound to ADP-F-actin.** The assemblies of CFL2 with rabbit α-skeletal muscle actin (Fig. 1a) yield outstanding-resolution MAS NMR spectra, as shown in Fig. 1b and our previous work. Overall, we recorded nine two-dimensional (2D) and two three-dimensional (3D) spectra on three sets of samples (summarized in Supplementary Table 1). On the basis of these data sets, we completed 96% of 13C and 15N chemical shift assignments using 2D 13C-15N combined R2-driven (CORD) and 3D 13N-13C-15N (NCACX/NOCX) correlation spectra. Chemical shift assignments are listed in Supplementary Table 2. Long-range 13C-15N and/or 13C-13C distance restraints were determined using [1,6-13C-glucose,U-15N]-CFL2/actin and [2-13C-glucose,U-15N]-CFL2/actin samples, by recording proton-assisted insensitive nuclei cross-polarization (PAIN-CP) spectra and/or CORD spectra. In total, we assigned 1490 distance restraints corresponding to 1224 unambiguous restraints and 238 ambiguous restraints (fewer than 4 possibilities per assigned cross peak), as summarized in Table 1. For this degree of restraint completeness, the accuracy of a structure should be within 0.8 Å backbone RMSD of the true structure.

The structure of CFL2 assembled with actin is shown in Fig. 1c. Structure refinement and validation statistics are shown in Table 2. CFL2 exhibits an αβ domain fold typical of other cofilin isoforms. The core consists of five β-strands surrounded by six helices and loop regions. β1–β4 form anti-parallel β-sheets, and β5 is packed parallel to β4. Compared to yeast cofilin, vertebrate cofilins are slightly larger and have sequence insertions in loop regions corresponding to the nuclear localization sequence (NLS) (residues R21-K33), residues L56-D66, and a short β-hairpin at the C-terminus (residues G155-L166). The bundle backbone and heavy-atom RMSDs for CFL2 are 1.05 and 1.67 Å, respectively. With the multiple distance restraints, the side chain conformations are well defined for 117 residues, with most sidechains adopting a single conformation (Fig. 1d).

CFL2 exhibits an isoform-specific conformation distinct from other cofilin structures. To elucidate isoform-specific features of CFL2, we compared our atomic-resolution structure to cofilactin structures containing *Gallus gallus* cofilin-2 (designated hereafter as CFLGg) in a complex with chicken skeletal muscle F-actin (3.8 Å resolution, PDB ID: 5UY8) and human CFL1 in complex with mammalian α-skeletal F-actin (3.4 Å resolution, PDB ID: 6VAO)19,29. This comparison is shown in Fig. 2. Despite similarities in the protein core, we observe significant differences in surface regions of CFL2 containing the vertebrate-specific insertions (residues 18–34 and residues 57–67), as well as differences in other surface loops. These pronounced differences are surprising given that human CFL2 shares 98% sequence homology...
with CFLGg. The most significant conformational change is a rotation of the α₂ helix (α₂) by approximately 30°. This change corresponds to a local RMSD of 4.04 Å between CFL2 and CFLGg for residues 21–32, while the global RMSD for the full-length proteins is only 1.84 Å (Supplementary Fig. 1 and Supplementary Table 3). This region is part of the vertebrate-specific insertion containing the nuclear localization signal (NLS). In human CFL2, α₂ contains residue Q26, which is substituted by a conserved proline residue in other isoforms, including human DSTN and CFL1 as well as avian DSTN and CFLGg. The substitution of proline with glutamine at residue 26 modifies the local structure around α₂ and its preceding loop. Glutamine has a stronger helical propensity, and torsion angles derived from chemical shifts are consistent with a helix. This is also observed throughout the duration of the MD simulations on the entire cofilin assembly, as discussed below.

The P26Q substitution induces remodeling of the local hydrogen bond network in α₂ which reflects the differences in helical propensity between these two residues. Specifically, the Q26 backbone nitrogen atom forms a hydrogen bond with the backbone carbonyl atom of S23, and the mean hydrogen bond length is 3.1 Å. The Q26-S23 contacts are observed as cross peaks...
Interestingly, the conformation of CFL2 in this region is more the loop containing residues G155-S160 near the C-terminus. spectra as cross peaks between the corresponding residues, as P67 region is not surprising because it is structurally coupled to and the loop containing the second vertebrate-specific regions of CFL2, namely the loop containing residues G155-S160 – hydrogen-bonding length. E97 is in α to E97-Oe2 is ~13.0 Å. In our CFL2 structure, the distance between these residues is only 2.1 Å, well within hydrogen-bonding length. E97 is in β, which is one major structural component of the F-site binding interface with F-actin. We speculate that this is a functionally important interaction, as outlined below.

The rotation of α2 induces allosteric changes to other mobile regions of CFL2, namely the loop containing residues G155-S160 and the loop containing the second vertebrate-specific insertion, V57-P67. The conformational change associated with the V57-P67 region is not surprising because it is structurally coupled to the first vertebrate-specific insertion (residues 18–34) which contains α2. Interactions between these two insertions (residues 18–34 and residues 57–67) were directly detected in the NMR spectra as cross peaks between the corresponding residues, as illustrated in Fig. 2e. We also observed structural differences in the loop containing residues G155-S160 near the C-terminus. Interestingly, the conformation of CFL2 in this region is more similar to CFL1 than to CFLGg. In the structure of CFLGg, this region is more structured and contains a short helix, whereas this region of CFL2 and CFL1 exhibits mostly loop content with a folded β-hairpin (Fig. 2e). This region is likely dynamic on NMR timescales, as G155 is one of the two residues (along with M1) not present in the spectra.

We have compared the atomic-resolution CFL2 MAS NMR structure determined here to the 9 Å resolution cryo-EM map of CFL2 assembled with ADP-F-actin. Rigid body docking of the structure determined here to the 9 Å resolution cryo-EM map of CFLGg structures where S24 and E97 are conserved, the distance from S24-O of α2 is bent outwards, the distance between S23 and S24 is ~8 Å. In our CFL2 structure, the distance between these residues is only 2.1 Å, well within hydrogen-bonding length. E97 is in β, which is one major structural component of the F-site binding interface with F-actin. We speculate that this is a functionally important interaction, as outlined below.

Intermolecular interface reveals extended F-site on CFL2. Protein–protein interactions can be probed by MAS NMR spectroscopy through chemical shift perturbations (CSPs) that occur upon binding or by recording through-space correlations involving residues from each binding partner at the intermolecular interface, such as dREDOR experiments. In our previous study, we used CSPs in combination with dREDOR-based experiments to investigate the intermolecular interface between CFL2 and ADP-F-actin. Since we obtained a complete set of resonance assignments in the present study, the results here offer unique insights compared to our earlier study. To determine the CFL2 residues forming the interface with ADP-F-actin, we used dREDOR-CORD, to measure correlations arising exclusively from the residues of CFL2 involved in binding to F-actin. The spectra reveal multiple correlations involving CFL2 residues at the N-terminus (A2-T6), the NLS (M18-Q26), R45, β4 (E90-E97), a4 (L111-K127), T129, K132, the 310 helix (L140-D142), and α5 (E151-K152). While most of these regions are contained in the canonical interaction sites, to the best of our knowledge, this is the first report of the S24-Q26 region being part of the cofillin–actin binding site. These data are summarized in Supplementary Fig. 2.

Since the structure of CFLGg was solved using solution NMR, we also performed an analysis of CSPs between CFL2 and CFLGg to validate the isoform-specific differences we observed between these structures. As expected, large CSPs were mapped to the three non-conserved residues (CFL2-Q26, R45, and S70), residues in the canonical G-site and F-site as well as to multiple residues in both vertebrate-specific insertions. These CSPs span residues at the interface with ADP-F-actin as well as residues that are far from the interface (likely due to allosteric effects). This is summarized in Supplementary Fig. 3.

Atomic-resolution structure of cofilin filament. The results of our MAS NMR experiments indicate specific interactions between CFL2 and F-actin that cannot be deduced from prior cryo-EM structures using other coflin isoforms. To generate a complete atomic-resolution model of the entire CFL2/F-actin assembly (i.e., cofilactin), we employed data-driven all-atom molecular dynamics (MD) simulations using the distance restraint information from MAS NMR. The initial cofilactin model was prepared based on the 3.8 Å cryo-EM structure solved using CFLGg and F-actin. (Supplementary Fig. 4), the isoform with the closest homology (98%), highest resolution structural model, and lowest structural variability between subunits. The cofilactin model maintains its structural integrity within 200-ns simulations. As shown in Supplementary Fig. 4b, c, root mean square displacements (RMSDs) of Ca atoms of the entire complex and the individual CFL2 chains plateau after about 25 ns. In addition, secondary structure assignments (STRIDE) of both actin and CFL2 subunits in the complex suggest that no global unfolding occurs, and the model is stable throughout the MD simulations.

To evaluate the protein–protein interactions in cofilactin, we calculated and compared the pairwise interatomic distances in the cryo-EM CFLGg/F-actin structure, the initial cofilactin model built using our CFL2 structure, and the same cofilactin model at the end of 200-ns MD simulations (Supplementary Fig. 5). The results indicate that the coflin–actin and the actin–actin interactions observed in the cryo-EM structure are conserved in our model and are sustained during MD simulations. Additionally, in our model, the Ca-Ca interatomic distances between CFL2 α2 (containing residues S24-Q26) and the actin N-terminus region are shorter compared to the cryo-EM model (Supplementary Fig. 5a, e, i), suggesting potential CFL2–actin interactions in this region not seen by cryo-EM.

Contact analysis of MD trajectories identifies important CFL2–actin and actin–actin residue interactions. Fig 3 shows the CFL2–actin residue contacts with the highest contact occupancies >50% in seven regions, which fall into the canonical G- and F-site binding patches. These binding sites have been previously identified and correspond to coflin residues that can bind directly to bare F-actin (F-site) as well as residues that require actin conformational changes prior to coflin binding (G-site). In our model, residues in the G-site include CFL2 N-terminus interacting with actin residues 346–356, CFL2 α4 helix (residues 111 to 121) interacting with actin residues 140 to 149 and residues 341 to 349, CFL2 residue 45 interacting with actin residues 350 and 351, and CFL2 residues 129 and 132
interacting with actin residues 50, 56, 92, and 93. The F-site includes CFL2 residues 19 to 21 interacting with actin residues 91 to 96, CFL2 residues 93 to 97 interacting with actin residues 26 to 29 and 336 to 337, and CFL2 residues 147 and 148 interacting with actin residues 136 and 138. These interactions and their contact occupancies are summarized in Fig. 3 and Supplementary Table 4, respectively. Similarly, high-occupancy residue contacts in inter- and intra-strand actin–actin interaction interfaces are shown in Supplementary Fig. 6. We observed strong conservation of actin residues involved in both intra-strand interaction categories previously defined. These include inner domain (ID)—ID contacts between adjacent actin subunits as well as only a single outer domain (OD)—ID contact involving R62. Likewise, the inter-strand interactions evident in our structure are reduced compared to bare ADP-actin filaments, but consistent with previous coflinactin structures. Overall, both the actin–actin and CFL2–actin residue contacts identified in our MD simulations are similar to those reported in other coflin–actin structures and are consistent with our MAS NMR results.

In our MAS NMR structure of CFL2, we observe a unique conformation of α2 helix containing the non-conserved residue Q26. Q26 participates in hydrogen-bonding interactions with S23 and I29. Through dREDOR experiments, we also identified CFL2 residue Q26 as part of the coflin–actin interface. To investigate this further, we calculated the interatomic distances between Q26 and S23, I29, as well as D1 from actin’s N-terminus in the MD simulations. Gratifyingly, Q26 was found to strongly interact with S23 and I29. This is evidenced by the fact that most of the interatomic distances are shorter than 5.0 and 4.2 Å between the backbone nitrogen of Q26 and the carbonyl oxygen of S23 as well as the carbonyl oxygen of Q26 and the backbone nitrogen of I29, respectively (Fig. 3c). Interactions between CFL2-Q26 and actin-D1 were not stable throughout the simulations, although approximately 10% of the distributions have a distance shorter than 3.0 Å. It is worth noting that additional interactions between CFL2-Q26 with actin-E2, D3, and E4 were also observed during the simulations, but their contact occupancies were lower than those for the Q26–D1 interaction. The less frequent interactions...
between CFL2-Q26 and actin N-terminus are likely due to the inherent flexibility of the N-terminus of actin, as evidenced by the large root mean square fluctuations (RMSF) in this region (Supplementary Fig. 7). Therefore, it is possible that this is a low-population state under the sample conditions used here. Additionally, cross peaks corresponding to Q26 in the 13C-13C CORD spectra exhibit low peak intensities and are missing in our 3D NCACX dataset, which indicates some flexibility in this region of the protein. Indeed, the NLS region of CFL1 exhibits mobility based on 15N T1 and T2 relaxation rates from solution NMR. This interaction may still be important under severing conditions (i.e., at junctions between bare and decorated filaments) or in vivo during interactions with other ABPs.

Recent cryo-EM studies of bare actin filaments in all nucleotide states have provided insights into the actin structure. Specifically, the conformation of the DNase I-binding loop (D-loop) has been visualized, and its state is found to be coupled to the identity of the bound nucleotide. In the AMP-PNP- (a non-hydrolyzable ATP analog adenylyl-imidodiphosphate) bound state (mimicking the ATP-state), the D-loop exhibits an "open" conformation where it forms an extended hydrophobic network with F375 from the C-terminus of the adjacent actin protomer. Conversely, in the ADP-bound state, the D-loop assumes a "closed" conformation where this bridge with the C-terminus is broken, and the filament becomes more flexible.

Cofilin preferentially binds to aged ADP-actin filaments and induces disorder in the D-loop upon binding. However, any specific conformations of the D-loop present in cofilinactin are still unclear and have not been directly visualized in previous structures containing cofilin. In our structure, the D-loop samples a conformation similar to the closed state, and there are no extended interactions with the C-terminus of the neighboring subunit. We also observed frequent interactions of the D-loop with CFL2 residues D122 and K126 in the long α4 helix, which are part of the interface. However, due to the flexibility of the D-loop, these interactions are not stable. To investigate this further, we calculated the conformational dynamics of the D-loop from MD trajectories and identified several free energy minimum states and metastable states (Supplementary Fig. 8). In most states, D-loop exists as a coil, but also forms turn, and even a helix in certain states (Supplementary Fig. 8). Interestingly, the helical conformation has only been observed experimentally in the X-ray crystal structure of ADP-bound G-actin, and has not been seen in the recent cryo-EM structures. From our MD results, we observe that most of the barriers for conformational transitions between these states are smaller than 2 kT, confirming the flexibility of the D-loop, and its ability to easily transition between sub-states.

In vitro severing activity of F-actin by CFL1 and CFL2. Since Q26 is located in the α2 helix and is not conserved in CFL1 or CFL\textsubscript{Gg}, we hypothesized that this residue is important in actin disassembly. To test this assertion, we substituted Q26 in CFL2 with proline found at this position in CFL1 and CFL\textsubscript{Gg}. In addition, we prepared a CFL1 P26Q mutant, where P26 is substituted with glutamine, unique to CFL2. We then compared the F-actin severing activities of the generated cofilin constructs using single-filament total internal reflection fluorescence microscopy (TIRFM) and bulk pyrenyl-actin polymerization assays. These data were summarized in Fig. 4.

As reported previously, wild-type (WT) CFL2 showed much higher severing activity as compared to WT CFL1 in TIRFM experiments (Fig. 4a, b). In contrast, we observed only a marginal decrease in activity for CFL2 Q26P versus the WT. Similarly, we observed only a marginal enhancement in activity for CFL1 P26Q. In other words, the mutants behave similarly to their parent WT proteins. Therefore, the activity of each isoform is not determined by residue 26 alone. These results are consistent for both, single-filament TIRFM experiments (Fig. 4a, b) and bulk pyrenyl-actin polymerization assays (Fig. 4c, d).

Taken together, the results suggest that severing activity is modulated by cooperative interactions between multiple residues forming cofillin–actin interfaces, indicating a more complex regulation of severing activity. While at this time we do not have additional data to understand the structural basis for severing activity by different cofilin isoforms, we note that, for yeast cofillin, it is only the mutations in the F-site that are responsible for differences in severing activity. Within the F-site, there are multiple non-conserved residues between CFL1, CFL2, and DSTN that could synergistically tune the severing activity. This will be investigated in future work. It is also possible that under cellular conditions, there is a complex interplay between the combined effects from multiple non-conserved cofilin residues at the binding interfaces with actin and interactions with other ABPs that participate in severing.

**Discussion**

The atomic-resolution structure of CFL2 bound to ADP-F-actin determined herein revealed isoform-specific differences in the α2 helix: α2 is rotated 30° with respect to those of CFL1 and CFL\textsubscript{Gg} isoforms. The P26Q substitution specific to CFL2 remodels the local hydrogen-bonding network in this region of the protein through its increased helical propensity. Compared to P26, the polarity of the Q26 side chain is more optimal for interactions with the highly acidic N-terminus of α-skeletal F-actin, which would extend the F-site binding surface. We speculate that the combined effect from multiple non-conserved surface residues in the actin-binding site, potentially including Q26 in α2, contributes to isoform-specific differences observed in vitro and in vivo studies. This points to a severing mechanism that could be structurally distinct from that of other members of the cofilin family of proteins.

Various cofilin isoforms are often used to study actin filament disassembly since the early 1990s. However, it was only in the last decade that differences between the mammalian isoforms have been investigated systematically. Consistent with the results in this study, bulk severing assays showed that CFL2 is more efficient at severing both muscle actin and cytoplasmic actin compared to CFL1 and DSTN. More recent microfluidics-based experiments confirmed these findings and demonstrated that faster decoration of F-actin by CFL2 than by other isoforms could explain more efficient severing. Despite these interesting observations, the structural basis of isoform-specific severing activity of actin by cofilins is not yet understood. As demonstrated in our current study, resolving the interactive partners at atomic resolution is important because the rotation of α2 uniquely observed in CFL2 is a subtle feature that could not be seen in the 9 Å cryo-EM reconstruction. The conformational change of α2 most likely arises from the P26Q substitution as opposed to a conformational change induced by binding. This is supported by our MAS NMR data and by other structures of cofilactin containing CFL1, which exhibit very few differences with both the solution NMR and X-ray crystal structures of free CFL2. Additionally, we note that the MAS NMR structure reported in this work was determined using a large number of restraints (11 restraints per residue) in the region containing α2 (residues 20–34).

We hypothesize that this isoform-specific conformation has a role in actin binding. This hypothesis is supported by our current and previous findings, where a stretch of residues 24–26 was
detected in the dREDOR-CORD spectra, indicative of interactions with F-actin. Furthermore, in our data-driven all-atom MD simulations of cofailin, we observed shorter overall distances between the α2 helix and N-terminus of actin as compared to other structures. Many ABPs have interaction sites with the N-terminus of F-actin, which protrudes from the filament surface. Tropomyosin is one such protein, whose binding to F-actin excludes cofaillin binding. The actin sequence is 93% conserved between human and activity by ABPs.  

Finally, it is of note that F-actin possesses an additional degree of the regulation for actin binding other structures. 

**Methods**

**Materials.** $^{15}$NH$_4$Cl, U-$_{13}$C$_6$ glucose, 2-$_{13}$C-glucose, and 1,6-$_{13}$C-glucose were purchased from Cambridge Isotope Laboratories, Inc (Tewksbury, MA, USA). Common chemicals were purchased from Fisher Scientific (Hampton, NH, USA) or Sigma Aldrich (St. Louis, MO, USA).

**Expression and purification of sparse-labeled CFL2.** Cloning, expression, and purification of labeled tag-less human CFL2 was described previously²⁰. Tag-less full-length human CFL2 was expressed in *Escherichia coli* BL21-CodonPlus(DE3) cells. Transformed cells were grown at 37 °C in 4 L of nutrient-rich medium supplemented with 50 mg/mL ampicillin and 34 mg/mL chloramphenicol until OD 1–1.2 was reached. Bacterial cells were pelleted, washed in MJ medium²⁷ without
glucose and ammonium chloride, resuspended in 0.75 L of MJ medium, and incubated for 1 h at 25 °C. U-13C, glucose, 1.6-13C glucose, or 2-13C glucose was added with a total concentration of 0.75% (w/v) of 13NH4Cl (0.1 g/L total concentration) and expression was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were grown overnight at 25 °C and pelleted at 4 °C the following day using buffer A (10 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PPIPES), pH 6.8, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM mercaptoethanol, 0.5 mM p-phenylenediamine, and 0.2 mM ATP, 5 mM β-mercaptoethanol). G-actin was switched from Ca2+ to Mg2+ by incubating for 10 min with 0.1 mM MgCl2 and 0.4 mM EGTA. G-actin was polymerized as described previously by addition of buffer containing 20 mM PIPES pH 6.8, 25 mM KCl, 2 mM MgCl2, and 5 mM β-mercaptoethanol.

Preparation of f-actin. Skeletal muscle G-actin was prepared from acetonitrile powder of rabbit skeletal muscle (Pel-Freeze Biologicals) according to the published method48 and stored in G-buffer (5 mM Tris-HCl, pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 5 mM β-mercaptoethanol). Preparations of cofilin/actin assemblies were centrifuged at 18,000 rpm for 10 min. Preparations were packed, which contained ~3.5 mg of isotopically labeled CFL2.

Transmission electron microscopy. Sample morphologies for U-13C,15N-CFL2/ADP-F-actin, 2-13C,15N-CFL2/ADP-F-actin, and 1-13C,15N-CFL2/ADP-F-actin assemblies were confirmed using transmission electron microscopy (TEM). The preparations were stained with uranyl acetate (5% w/v), deposited on 400 mesh formvar/carbon-coated copper grids, and dried. The TEM images were acquired using a Tecnai F20 microscope operating at 120 kV. We acquired TEM images before and after MAS NMR experiments to confirm that no changes in filament properties occurred (Fig. 1a).

MAS NMR spectroscopy. Spectra were used for resonance assignments, distance restraints, and structure calculation. NMR spectra were acquired at 19.96 T using a Bruker AVIII spectrometer equipped with a 1.9 mm HCN probe. Larmor frequencies were 850.4 MHz (1H), 213.8 MHz (13C), and 86.2 MHz (15N). All experiments were performed at T = 273 K. Prior to MAS NMR experiments to confirm that no changes in filament properties occurred (Fig. 1a).

Structure calculation. Structure calculation was performed using XPLOR-NIH version 4.5.27 unambiguous 13C,15N distance restraints, 335 ambiguous 13C-13C distance restraints, 25 13C-15N distance restraints, and 291 TALOS-N torsion angle restraints were used as input in the structure calculation. Correlations from the CORD spectra were converted to a distance boundary of 1.5–6.5 Å for intra-residue correlations and 2.0–7.2 Å for inter-residue correlations28. The structure calculation of CFL2 was performed with similar input parameters used in our previous work22. In addition to potential energy terms that corresponded to distance restraints and torsion angle restraints, standard XPLOR-NIH energy terms were included. Standard terms for a bond, bond angle, and improper torsion angles were used to ensure proper covalent geometry. The gyration volume term was initialized excluding residues 1–5, and 166 to exclude disordered tails. A hydrogen bond database term, HBpot, was used to improve hydrogen bond geometries29. Backbone dihedral angle (ϕ, ψ) restraints were predicted using TALOS-N version 4.12 from experimental 13C and 15N chemical shifts. An extended structure was generated based on the primary structure, and 250 structures were generated using torsion angle dynamics and simulated annealing followed by a final gradient minimization in Cartesian space using a Powell energy minimization scheme. The initial simulated annealing was started at 500 K with a high-temperature run of 800 ps or until the temperature diminished first. Following the high-temperature run, the temperature was lowered to a final temperature of 20 K in steps of 20 K. At each temperature, a dynamics run was performed for 500 steps or 0.4 ps, whichever finished first. After this initial simulated annealing stage of refinement was performed using the 25 lowest energy structures. The refinement consist of a second simulated annealing step from 3000 to 20 K in steps of 4 K. Force constants for the distance restraints were ramped from 10 to 50 kcal/molÅ2 in the initial annealing step and from 2 to 30 kcal/molÅ2 in the refinement step. We generated 250 structures during the refinement step and chose a representative bundle of the 25 lowest energy structures for further analysis. The coordinates corresponding to the highest cross-correlation scores; in this specific case the score was 0.94 on a scale of 0–1.

Construction of atomic model for CFL2/F-actin. The initial coordinates of CFL2 were used directly from MAS NMR structure calculation. The initial structure of CFL2/F-actin was built using the cryo-EM structure of chicken cofilin (PDB ID: 5YUB). The sequence of rabbit actin used in this study is identical to chicken actin, and
Molecular dynamics simulations of CFL2/F-actin. Molecular dynamics simulations in this study were conducted using NAMD 2.146 and CHARMM36m67 protein and CHARMm TIP3P28 water force fields were employed. After the CFL2–actin filament was constructed, NaCl ions were added to neutralize the filament and solution of the whole system was performed using the TIP3P water model. Additional ions were added so that total bulk concentration of KCl was set to 25 mM and MgCl₂ to 2 mM. The resulting model contains 563,000 atoms including protein, ADP, the TIP3P water model, and ions.

The solvated system was then minimized for 10,000 steps using a conjugate gradient29 and line search algorithm30, with all backbone atoms of CFL2–actin filament fixed. The system was then heated from 50 to 310 K in 20 K increments for 1 ns while constraining the backbone atoms. Subsequently, the system was equilibrated for 10 ns. The equilibrated system was simulated in four independent NPT simulations and each one ran for 200 ns. In these simulations, the system temperature and pressure were maintained at 310 K at 1 atm using stochastic rescaling thermostat32.25 and Nose–Hoover Langevin piston pressure control, respectively. The backbone atoms in the helices of the first two and last two actin monomers were restrained with harmonic potentials (force constant of 0.5 kcal/mol Å²) during simulations, to maintain the actin filament conformation. The eight cofilin molecules were applied with flat-bottom harmonic potential distance restraints derived from the experimental NMR data. All bonds to hydrogen were constrained with the SHAKE and SETTLE algorithm for the solute and solvent, respectively. Long-range electrostatic force calculations used the particle mesh Ewald method, with a 1.2 nm cutoff. The rRESPA integrator and an integration time step of 2 fs were utilized, with the nonbonded interactions evaluated every 2 fs and electrostatics updated every 4 fs.

Analysis of MD simulation trajectories. Contact, secondary structure, RMSD, RMSF, and pairwise distance and analysis were performed in VMD.29 The contact is defined as the distance between sidechains of two residues are not greater than 3.4 Å. The contact occupancy was calculated by \( \frac{\sum_{i=1}^{n} C_{i}}{\sum_{i=1}^{n} C_{i} + b} \times 100\% \), where \( n \) is the number of MD simulations, \( m \) is the number of interaction interfaces, \( C_{i} \) is the number of frames in \( i \)th simulation and \( j \)th interface where residue \( a \) and residue \( b \) form contact, \( C_{total} \) is the total number of frames in \( i \)th simulation and at \( j \)th interface. The secondary structures of CFL2 and actin were assigned using the STRIDE algorithm. Homemade tcl scripts were written to compute the RMSDs, RMSF, and pairwise distances. Conformational dynamics of actin D-loop was calculated using time-lagged independent component analysis (TICA)63,64 implemented in pyEMMA 2.5.765. Pairwise backbone atom distances in the D-loop (904 distance pairs) were chosen as the feature to partition the conformational space of D-loop. D-loop trajectories from four independent simulations were used as input data to run TICA. Note that there are 10 D-loops in each simulation but four of them in the actin molecules at two ends of the filament were not included in the other TICA. TICA was computed with a selected lag time of 0.8 ns and the dimensionality of D-loop conformation was reduced to 10 independent components (ICs). The conformational dynamics of D-loop was projected onto the first two ICs.

Structure analysis and visualization. RMS deviation values were calculated using algorithms in Xplor-NIH (version 2.51)49,50. Restraint tailing and frame conversions were carried out with in-house Python 2.7 scripts. Structure ensembles were rendered for visualization in PyMOL 1.8.6.2 using in-house shell/bash scripts for batch rendering. Structures were classified according to TALOS-N predictions and manual inspection. All analyses of cofilin assembly were performed in VMD.

Cofilin mutagenesis and purification. For TIRFM and bulk pyrene-actin severing experiments, CFL2 and 2 were subcloned into a modified pCOut vector (Clontech) containing a 6xHis tag followed by a TEV protease cleavage site66,67 using NEB builder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA). Site-directed mutagenesis was carried out based on the Quick-change site-directed mutagenesis strategy (Agilent Technologies, Santa Clara, CA, USA) using Q5 DNA polymerase and DpnI restriction enzyme (New England Biolabs, Ipswich, MA, USA). The primer sequences used in this study are reported in Supplementary Table 5. WT and mutated cofilin constructs were expressed in E. coli BL21 CodonPlus (DE3) cells and purified using Talon metal affinity resin (Takara Bio USA, Inc., San Jose, CA, USA). 6xHis-tags were removed by treatment with recombinant TEV protease (1:20 w/w), which leaves a single glycine residue at the N-terminus. Following cleavage, 6xHis fragments, uncleaved 6xHis-constructs, and TEV protease were removed by passing through Talon metal affinity resin.

TIRFM analysis of Alexa488-F-actin severing by cofilin. TIRFM experiments were conducted as described previously48–50 using the protocathectic acid (PCA)/protocathectate-3,4-dioxoxygenase (PCD) O₂-scavenging system71. Skeletal actin (33% Alexa488-labeled, 1% biotinylated; 1.5 μM final concentration) was polymerized by the addition of an equal volume of 2x TIRF buffer in a TIRF flow chamber functionalized with 0.1 mg/mL streptavidin. Filaments were grown to 15–20 μm average length. Free actin monomers were then removed by washing in 1x TIRF buffer [final 1x buffer composition: 10 mM imidazole, pH 7.0, 50 mM KCl, 2.5 mM dithiothreitol (DTT), 1 mM MgCl₂, 0.4 mM ATP, 0.2 mM ethylene glycol-bis(β-aminoethoyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM acetic acid (neutralized to pH 7.0), 2.5 mM PCA (neutralized to pH 7.0), 0.1 μM PCD, 0.1% bovine serum albumin, and 0.5% methylcellulose-400K (Sigma Aldrich, St. Louis, MO, USA)]. Due to its notably higher severing potency, CFL2 was added at a 12-nM concentration as compared to 120-nM of CFL1 to obtain similar severing rates (Fig. 4a, b). Time-lapse images were collected every 15 s using a Nikon Eclipse Ti-E microscope equipped with a TIRF module, perfect focus system, CFI Plan Apochromat λ x100 oil objective (NA 1.45), and DS-Qi1MC camera (Nikon Instruments Inc., Melville, NY, USA). Data were quantified using Imagej software; number of severing events was counted in each time frame and normalized to the filament length measured prior to the addition of cofilin. Data were presented as the mean of three independent experiments with four fields of view analyzed within each experiment.

Bulk pyrene-actin polymerization assays. In a bulk mixture of 3.125 μM (5% pyrenyl-labeled) Ca²⁺-ATP G-actin with 3.44 μM human profilin-1 (PFN); purified as described previously69,70, Ca²⁺ in the nucleotide cleft of actin was switched to Mg²⁺ by adding 0.02 volumes of 50X switch buffer: 500 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.0, 5 mM EGTA, and 15 mM MgCl₂. Forty-microliter samples were promptly transferred to a 96-well plate and supplemented with 0.1 of the final volume (5 μL) of cofilins present at concentrations tenfold higher than the desired final concentrations. Time-based monitoring of pyrene fluorescence in an Infinite M1000 Pro plate reader (Tecan US Inc, Morrisville, NC) was initiated with λex = 407 nm at 25 °C. In 2 min, using a multichannel pipette actin polymerization was initiated by adding 0.1 volumes (5 μL) of the 10X initiation buffer, containing 10 mM MgCl₂ and 300 mM KCl. The samples were mixed promptly with a multichannel pipette set at 30 μL and the measurement was continued. The final concentrations of actin and PFN were 2.45 and 2.65 μM, respectively. Fluorescent intensity was normalized to the highest signal for each trace.

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

Data availability

The coordinates corresponding to the atomic-resolution structures in this work have been deposited in the Protein Data Bank under accession code PDB 7M0G for CFL2 and PDB 7U9K for cofilin. MAS NMR chemical shift, distance restraints, and dihedral parameters have been deposited in the Biological Magnetic Resonance Data Bank (BMRB) under accession code 30877. The coordinates corresponding to actin filaments decorated with CFL40 used in this study are available in the Protein Data Bank under accession code PDB 5YU8. Additional coordinates corresponding to other proteins analyzed in this study are available in the Protein Data Bank under the following accession codes. The accession code for the solution NMR structure of CFL40 is PDB 1IR7. The corresponding BMRB accession codes for actin filaments decorated with CFL40 is PDB 6VVO. Source data for in vitro TIRFM severing and bulk pyrene-actin polymerization assays are provided within this paper. Other data that support the findings of this study, such as structure calculation scripts, are available from the corresponding authors upon reasonable request. Source data are provided in this paper.

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**Author contributions**

T.P. and D.S.K. conceived and supervised the study. T.P. designed and supervised the MAS NMR experiments. J.K. processed MAS NMR spectra, analyzed the data, assigned distance restraints, performed structure calculations. E.K. and D.S.K. prepared samples for MAS NMR experiments and performed coﬁlin mutagenesis and the actin severing assays. J.R.P. designed and supervised the MD simulations. C.X. and N.K. performed the MD simulations. R.W.R. wrote scripts for structure calculation and restraint statistics and assisted with structural calculations. J.K., E.K., D.S.K., C.X., and N.K. prepared the manuscript ﬁgures. All authors discussed the results. J.K., E.K., D.S.K., and T.P. took the lead in writing the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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