Structural basis of actin monomer re-charging by cyclase-associated protein

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Actin polymerization powers key cellular processes, including motility, morphogenesis, and endocytosis. The actin turnover cycle depends critically on “re-charging” of ADP-actin monomers with ATP, but whether this reaction requires dedicated proteins in cells, and the underlying mechanism, have remained elusive. Here we report that nucleotide exchange catalyzed by the ubiquitous cytoskeletal regulator cyclase-associated protein (CAP) is critical for actin-based processes in vivo. We determine the structure of the CAP-actin complex, which reveals that nucleotide exchange occurs in a compact, sandwich-like complex formed between the dimeric actin-binding domain of CAP and two ADP-actin monomers. In the crystal structure, the C-terminal tail of CAP associates with the nucleotide-sensing region of actin, and this interaction is required for rapid re-charging of actin by both yeast and mammalian CAPs. These data uncover the conserved structural basis and biological role of protein-catalyzed re-charging of actin monomers.
The actin cytoskeleton is critical for a wide range of cellular processes, including migration, morphogenesis, and endocytosis. Consequently, defects in the regulation of actin dynamics and actin network organization are linked to a number of diseases, including cancer metastasis, immune and neurological disorders. The rapid polymerization of actin filaments, that provides force for the above-mentioned cellular processes, must be balanced by the disassembly of “aged” actin filaments, and recycling of actin monomers for new rounds of filament assembly. This process, called “treadmilling”, consists of four phases: (1) incorporation of assembly-competent ATP-actin monomers to the rapidly growing actin filament barbed end; (2) ATP-hydrolysis, followed by Pi release, on actin subunits in the filament; (3) dissociation of ADP-actin monomers from the pointed end of filament; and (4) “re-charging” of ADP-actin monomers with ATP. In vitro, these four phases, as well as nucleation of new actin filaments, are relatively slow, and thus a large collection of actin-binding proteins such as the Arp2/3 complex, formins, and ADF/cofilin, evolved to enhance the rate of actin dynamics.

While previous studies have demonstrated the in vivo importance of these proteins that catalyze actin nucleation, polymerization, and disassembly, it has remained unclear whether additional protein machinery is also required to catalyze actin monomer “re-charging” (exchange of ADP for ATP) in cells. Two evolutionarily conserved proteins, profilin and cyclase-associated protein (CAP), can catalyze nucleotide exchange on actin monomers in vitro. However, CAP appears to be better suited for this function, because it binds the substrate (ADP-G-actin) with much higher affinity compared to profilin. Moreover, whereas all CAPs tested so far catalyze nucleotide exchange in vitro, only a subset of profilins accelerate nucleotide exchange in biochemical assays. Finally, only CAP has been shown in vitro to effectively catalyze nucleotide exchange on cofilin-bound ADP-actin monomers. These observations have called into question the popular view depicting profilin as the key driver of actin monomer recharging, and suggest instead that CAP may perform this conserved function. Until now, however, it has not been possible to rigorously test whether either or both proteins serve this function in vivo, due to an absence of mutants that disrupt nucleotide exchange activity without compromising actin binding.

CAPs are multi-domain, multifunctional proteins that oligomerize into hexamers and promote rapid actin filament turnover in vitro and in cells. Whereas yeasts and invertebrates have only one CAP protein, vertebrates express two CAP isoforms: ubiquitously expressed CAP1 and muscle-specific CAP2. The N-terminal half of CAPs binds ADF/cofilin-actin monomer complexes and actin filaments, and accelerates ADF/cofilin and twinfilin-mediated actin filament disassembly. The C-terminal half of CAPs harbors two proline-rich regions, PP1 and PP2, which bind to profilin and SH3 domain proteins, respectively, and a Wiscott Aldrich Syndrome protein homology domain.
2 (WH2) domain, which binds to both ADP-actin and ATP-actin monomers. At the C-terminus of CAPs is a homodimeric β-sheet domain, which displays structural similarity to other, functionally unrelated proteins, including X-linked retinitis pigmentosa 2 protein (RP2), and hence is referred to as a CAP and RP2 (CARP) domain. The CARP domain of CAPs binds specifically to ADP-G-actin, and together with the adjacent WH2 domain catalyzes nucleotide exchange on actin monomers.

Despite the fundamental requirement of CAPs for actin cytoskeleton organization and function across the eukaryotic kingdom, the underlying mechanism by which this protein regulates cytoskeletal dynamics in vivo has remained elusive. Moreover, the CARP domain does not display any structural homology to other known actin-binding domains, and despite extensive mutagenesis, the mechanism by which CAPs associate with actin monomers and catalyze nucleotide exchange has thus remained a mystery. Here, we determined the crystal structure of CAP1/ADP-G-actin complex. Combined with molecular dynamics (MD) simulations, biochemical experiments, and in vivo studies on budding yeast, we uncover the structural basis and biological role of CAP-catalyzed nucleotide exchange on actin monomers.

Results

Crystal structure of CAP1_{317–474}/ADP-G-actin complex. To reveal the principles of CAP–actin interactions, we crystallized the CARP domain of mouse non-muscle isoform, CAP1 (CAP1_{317–474}) in complex with unmodified muscle ADP-G-actin. Crystals diffracted anisotropically to 2.3 Å in the c direction, and to 3.2–3.3 Å in the a and b directions (Supplementary Table 1, see Methods). The obtained crystal structure of a symmetric complex containing a dimer of the CARP domain bound to two ADP-actin monomers reveals several new and unexpected features (Fig. 1a, 2).
Supplementary Fig. 1a, and Supplementary Table 1). First, each actin monomer in the complex contacts each of the two subunits in the CARP homodimer, and each CARP monomer (within the homodimer) binds to two actin monomers using two distinct interfaces. This results in a sandwich-like structure, with the CARP homodimer squeezed between two ADP-actin molecules. The intertwined CARP domain dimer has an S-shape organization, in which the two ADP-actin molecules fit perfectly on both sides (Fig. 1a, b). Second, CAP interacts with actin unlike any other actin monomer-binding motifs that have been structurally characterized. All of these other proteins bind to the "front side" or the barbed end interface of actin between subdomains 1 and 3, whereas the CARP domain binds to the "back side" on subdomains 1, 2, and 3 of actin (Fig. 1c). Third, the CARP domain binds G-actin through a much larger interface compared to other G-actin-binding proteins/domains. The large binding interface results from the two subdomains within the CARP domain dimer interacting with actin monomers through different interfaces. The "primary interface" of the CARP domain on subdomains 1 and 3 of actin overlaps with the binding site of profilin on actin. However, the "secondary interface," formed between the second CARP monomer and actin subdomain 2, is different from the interaction sites of other actin monomer-binding proteins characterized so far (Fig. 1a–c and Supplementary Fig. 1b). Analysis of the structures also revealed steric clashes between the CARP domain and profilin, as well as between the CARP domain and the twinfilin’s ADF-H domain (Supplementary Fig. 1g), providing a structural explanation for why these protein domains compete with each other for G-actin binding. Although interactions with actin do not alter significantly the structure of the CARP domain (Supplementary Fig. 1c), the CARP domain induces a conformational change in the actin monomers. In the crystal structure, the D-loop of actin subdomain 2 has a pulled-back conformation and associates with the secondary interface of the CARP domain (Fig. 1a, b). This orientation is drastically divergent from all other D-loop conformations of actin reported so far and (Fig. 2a, b, Supplementary Fig. 2a, and Supplementary Table 2). To confirm that the peculiar D-loop conformation does not result from crystal contacts (Supplementary Fig. 2b), we performed 1.2 μs all-atom MD simulations for the ADP-actin–CARP domain complex (System 1, Supplementary Table 3) and for the ADP-actin isolated from this complex (System 4, Supplementary Table 3). The simulations demonstrate that the CARP homodimer stabilizes the D-loop of actin subdomain 2 in the extended conformation, while in uncomplexed ADP-actin this loop is dynamic and adopts a variety of conformations (Fig. 2c, d and Supplementary Fig. 2d). Compared to the structure of uncomplexed ADP-actin, the CARP-bound ADP-actin monomer displayed also other small variations, which may be linked to the conformational change in the D-loop (Supplementary Fig. 1d–h). Together, the crystal structure and MD simulations reveal that the CARP homodimer binds to two ADP-actin monomers through a unique structural mechanism, and alters the conformation of the actin monomers.

### Table 1 Biochemical analysis of CAP1 mutants

| Mutant # | ATP-actin | ADP-actin | Nucleotide exchange |
|----------|-----------|-----------|---------------------|
| WH2 domain |           |           |                     |
| 1        | ++        | ++++      | ++++                |
| 2        | −         | ++++      | ++                  |
| 3        | −         | ++        | −                   |
| 4        | +         | ++++      | +                   |
| CARP domain primary interface |           |           |                     |
| 5        | ++        | +         | +                   |
| 6        | ++        | −         | −                   |
| CARP domain secondary interface |           |           |                     |
| 7        | ++        | +         | +                   |
| 8        | +         | ++        | +                   |
| 9        | +         | +         | +                   |
| 10       | +         | +         | +                   |

A summary of C-CAP mutant affinities for ADP-G-actin and ATP-G-actin, and their effects on nucleotide exchange on ADP-actin monomers (see Supplementary Figs. 3c, d). Symbols: $K_d$ for ATP-actin: ++ (0.5–2 μM), + (2–5 μM), − (unmeasurable); $K_d$ for ADP-actin: ++++ (0.005–0.025 μM), ++++ (0.025–0.1 μM), +++ (0.1–0.3 μM), ++ (0.3–1 μM), + (>1 μM), − (unmeasurable). Half-times of nucleotide exchange: ++++ (2–6s), ++++ (8–16s), ++++ (15–19s), ++ (19–24s), + (24–28s), − (>29s).

Interactions of CAP with ADP-G-actin and ATP-G-actin. We next performed mutagenesis and MD simulations experiments to test the roles of different CARP domain surfaces, and to reveal the mechanism by which the adjacent WH2 domain, which is unresolved in our structure (see Methods), contributes to actin monomer binding. We introduced four groups of mutations to the conserved clusters of residues in the WH2 domain of CAP1, and six groups of mutations in clusters of residues located at the primary and secondary actin-binding interfaces in our co-crystal structure (Table 1 and Fig. 3a–c). The mutant versions of the C-terminal fragment of mouse CAP1 (217–474) (C-CAP) were purified (Supplementary Fig. 3) and tested for ADP-G-actin and ATP-G-actin binding using a fluorometric NBD-actin assay (Supplementary Fig. 4). These experiments revealed that all four conserved clusters of residues in the WH2 domain are important for ATP-actin monomer binding, whereas none of the CARP domain mutations affected interactions with ATP-actin monomers. Consistent with the mutagenesis, MD simulations of the isolated ATP-actin–WH2 domain complex (System 3, Supplementary Table 3) revealed a stable association between the isolated WH2 domain (CAP1248–295) and an ATP-actin monomer (Supplementary Fig. 5a).

While the CARP domain was dispensable for ATP-G-actin binding, mutations in both WH2 and CARP domains affected the ability of C-CAP to bind ADP-G-actin. Whereas mutations in the WH2 domain modestly decreased ADP-G-actin binding, some mutations in the CARP domain (e.g., K365A, N367A, and D372A in the primary interface) resulted in a complete loss of ADP-G-actin binding (Table 1, Fig. 3c, and Supplementary Fig. 3c). Importantly, mutations in both the primary (mutant 5 and mutant 6) and secondary interfaces (mutant 7 and mutant 10) were defective in ADP-G-actin binding, demonstrating that both interfaces of the CARP domain are required for interactions with actin. We then generated a MD simulation model of CAP1248–474.
combining homology-modeled WH2 domain with the crystal structure of the CARP–actin complex (see Methods). Consistent with the mutagenesis results, both the CARP domain and the N-terminal helix of the WH2 domain displayed stable association with ADP-G-actin in the 1.2 μs simulations (Supplementary Fig. 5d). Collectively, these results reveal the structural mechanisms by which CAP interacts with ATP-actin using its WH2 domain (Fig. 3b), and with ADP-G-actin using a combination of its WH2 and CARP domains (Fig. 3c).

Mechanism of CAP-catalyzed nucleotide exchange. One fascinating feature of the CARP domain—ADP-actin co-crystal structure is the close proximity of the C-terminal tail of the CARP domain with the “nucleotide state sensing region” of actin. The occupancy of third phosphate alters the hydrogen bonding network in the nucleotide-binding loops P1 and P2 of actin. These differences are relayed to methylated His73 located in the “sensing loop” that changes conformation between the two nucleotide states of actin51. Our structure revealed that the C-terminal tail of the CARP domain forms a hydrogen bond between a backbone oxygen of CARP Ala473 and the imidazole ring of actin His73 (Fig. 4a, b). Moreover, two other possible contacts between the C-terminal tail of CAP and the nucleotide sensing region of actin were revealed in MD simulations of the ADP-actin–WH2 domain complex (System 1, Supplementary Table 3, Supplementary Fig. 6a), proposing that the C-terminal tail of CAP may contribute to nucleotide exchange on actin.

We generated mutant versions of mouse and budding yeast C-CAPs lacking the four C-terminal residues (∆4C). Importantly, this same mutation in both proteins caused severe defects in nucleotide exchange on actin without compromising ADP-G-actin binding. Instead, the ∆4C mutants bound ADP-G-actin...
with higher affinity compared to the wild-type proteins (Fig. 4c, d, f, g, Supplementary Fig. 6b). Moreover, gel filtration analysis demonstrated that also in the context of full-length protein, Δ4C mutant does not disrupt ADP-G-actin binding activity of yeast Srv2 (Supplementary Fig. 6c). Importantly, deletion of the four C-terminal residues converted the C-terminal halves of mouse and yeast CAPs into actin monomer sequestering proteins (Fig. 4h), and severely compromised the ability of mouse C-CAP to enhance actin filament turnover in the presence of coflin (Fig. 4e). These results suggest that deletion of the four C-terminal residues halts CAP's normal progression, leaving it bound to ADP-actin but unable to convert monomers to the ATP-bound state, and therefore severely impairing actin filament turnover.

Another peculiar detail of the nucleotide exchange activity was revealed by the CARP domain mutants that bind to ADP-G-actin with weaker affinity. Whereas mutants in the CARP domain that completely disrupted ADP-G-actin binding lead to severe defects in nucleotide exchange, the CARP domain mutants displaying compromised, but still detectable, affinity for ADP-G-actin were

**Fig. 4** The C-terminal tail of CAP is critical for nucleotide exchange. a C-terminal tail of CAP1 in the crystal structure displayed in 2Fo–Fc (σ = 1.0) electron density map. b Tail is positioned next to the nucleotide sensing region, loops P1 and P2 of actin that coordinate the nucleotide (σ = 1.0 in 2Fo–Fc electron density map). c The affinity of C-CAP and C-CAPΔ4C for ADP-G-actin was determined by a fluorometric competition assay with NBD-labeled actin (0.18 μM) and the C-terminal ADF-H domain of mouse twinfilin (0.44 μM) (see Supplementary Fig. 4a). d A representative example of rate of ADP-G-actin (0.5 μM) nucleotide exchange in the presence of different concentrations of wild-type C-CAP and C-CAPΔ4C. e A representative example of actin filament turnover as followed by Pi-release. F-actin (20 μM) was mixed with the indicated proteins (each 5 μM). f The affinity of C-Srv2 and C-Srv2Δ4C for ADP-G-actin (0.18 μM) was determined by fluorometric NBD assay. n = 3, error bars represent SD. g A representative example of rate of ADP-G-actin (0.5 μM) nucleotide exchange in the presence of C-Srv2 and C-Srv2Δ4C. h Monomer sequestering assay for C-CAP and C-Srv2 with different C-CAP/C-Srv2 concentrations using 2.5 μM actin. n = 3, error bars represent SD.
slightly more efficient in promoting nucleotide exchange compared to the wild-type protein (Table 1 and Supplementary Fig. 3d). Thus, stable association between CAP and ADP-G-actin is not necessary for nucleotide exchange, at least under the in vitro conditions used in this assay.

Physiological role of CAP-catalyzed nucleotide exchange. The Δ4C mutant described above enabled us to test the in vivo importance of CAP’s nucleotide exchange function, because the mutant does not disrupt actin binding. We integrated the srv2-Δ4C mutant at the SRV2 locus of budding yeast, and verified that it is expressed at levels similar to wild-type Srv2 (Fig. 5a and Supplementary Fig. 7). Strikingly, the srv2-Δ4C mutant impaired cell growth, cell morphology, and actin organization as severely as a full deletion of the SRV2 gene, despite missing only four residues located at its C-terminus (Fig. 5b–d). The srv2Δ and srv2-Δ4C mutants both caused a dramatic increase in the size of mother cells, a depolarization of cortical actin patches, and loss of normal actin cable staining. These results suggest that CAP’s nucleotide exchange activity plays a critical role in recharging actin monomers in vivo.

Discussion
A model for CAP-catalyzed re-charging of actin monomers is presented in Fig. 6. In cells, ADP-actin monomers either dissociate spontaneously from the filament pointed ends or their depolymerization is enhanced by the ADF-H domain proteins ADF/cofilin and twinfilin12,13,28. The C-terminal half of CAP efficiently catalyzes nucleotide exchange on both uncomplexed and ADF/cofilin-bound ADP-actin monomers22,23,34. We
and to promote the dissociation of ADF/cofilin-bound ADP-actin monomers, the WH2 domains of CAP, and then are released spontaneously, "re-charged" ATP-actin monomers remain transiently bound to the WH2 domain of CAP, and then are released spontaneously, or transferred to profilin, which binds to the adjacent PPII poly-proline region in CAP.

Our experiments provide evidence that three structural features of the CAP/ADP-G-actin complex are important for nucleotide exchange. First, we showed that the C-terminal tail of the CARP domain, which "penetrates" into the actin molecule and associates with its "nucleotide sensing region", is important for efficient "re-charging" of actin by CAP. Second, we found that the CARP domain induces a unique conformational change in the D-loop of actin. Importantly, subtilisin-cleavage of the D-loop of ADP-actin monomers results in ~3-fold increase in the rate of ADP-to-εATP nucleotide exchange on actin (Supplementary Fig. 2c), similar to what was previously reported for ATP-to-εATP exchange on subtilisin-cleaved actin,

Figure 6: A working model for how CAP catalyzes nucleotide exchange on actin monomers in cells. (1) CAP can interact with both free and cofilin-bound ADP-G-actin using CARP domain. This interaction puts the WH2 domain in position to competitively replace cofilin, leading to cofilin dissociation from the ADP-actin monomer, as previously observed biochemically. (2) The tight association of both CARP and WH2 domains with ADP-actin monomers, together with the penetration of the C-terminal tail of CAP into the nucleotide-binding pocket, catalyzes a change in conformation and dynamics of the ADP-actin monomer to enhance the rate of nucleotide exchange. (3) Nucleotide in actin is rapidly exchanged from ADP to ATP. (4) The CARP domain has little if any affinity for ATP-actin, leaving dimeric CAP molecules associated with ATP-G-actin solely through their WH2 domains. (5) Profilin has high affinity for ATP-actin monomers, and binds directly to the PPII domain of CAP, adjacent to the WH2 domain. Thus, as ATP-actin monomers dissociate from CAP, they are rapidly bound by profilin, replenishing the pool of ATP-actin monomers available for assembly. This leaves CAP primed for the next round of nucleotide exchange.

Our in vivo work using the yeast srv2Δ4C mutant provides the first direct evidence that CAP-catalyzed nucleotide exchange is critical for actin cytoskeleton organization and function. Earlier genetic evidence from yeasts suggested that nucleotide exchange catalyzed by profilin may be important in vivo. However, the role of the WH2 domain in nucleotide exchange varies between different species. Although malaria parasite CAP, which is entirely composed of a CARP domain, efficiently catalyzes nucleotide exchange on actin, mouse and budding yeast CAPs cannot efficiently promote nucleotide exchange without functional WH2 domain, especially in the presence of cofilin.

Our experiments demonstrate that the dimeric CARP domain associates with these newly depolymerized ADP-actin monomers through a unique structural mechanism that involves two separate CARP domain surface regions, which collectively interact with the "back side" of actin on subdomains 1, 2, and 3. The CARP domain specifically binds ADP-actin monomers, whereas the isolated WH2 domain of CAP displays no appreciable binding preference for ADP-actin vs. ATP-actin monomers.

Because ADP-actin and ATP-actin monomers do not display drastic structural differences, future work is required to reveal why CARP domain specifically associates with ADP-G-actin while the WH2 domain of CAP binds both ADP-actin and ATP-actin monomers. Nevertheless, our data suggest that CAP combines its WH2 and CARP domains to achieve high-affinity interactions with ADP-actin monomers, and to promote the dissociation of ADF/cofilin from ADP-actin monomers. Following the initial interaction of the CARP domain with ADP-actin monomers, the WH2 domains "embrace" the two ADP-actin monomers leading to a formation of a compact complex, where nucleotide exchange of actin occurs. The "re-charged" ATP-actin monomers remain transiently bound to the WH2 domains of CAP, and then are released spontaneously, or transferred to profilin, which binds to the adjacent PPII poly-proline region in CAP.

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mutants used in these studies all weakened actin-binding affinity, and thus the in vivo role of profilin in nucleotide exchange remains to be tested in the manner we have demonstrated here for CAP, with a mutation that disrupts nucleotide exchange without weakening actin affinity. Two other observations calling into question whether profilins catalyze nucleotide exchange in vivo are that profilins do not bind ADP-G-actin with high affinity\(^1\), and profilins are much less efficient than CAP in accelerating actin filament turnover in the presence of cofillin (Fig. 4e and ref. 23). Whether profilin has partially redundant roles with CAP in promoting nucleotide exchange in cells, or instead is required primarily to maintain homeostasis in distributing actin monomers between formin-dependent and Arp2/3 complex-dependent actin assembly pathways, remains to be determined\(^24,25\).

Collectively, our study uncovers the molecular mechanism by which the WH2 and CARP domains of CAP associate with actin monomers and accelerate nucleotide exchange on ADP-actin. However, it is important to note that most eukaryotic organisms, including yeast and mammalian cells, CAP oligomerizes into hexameric complexes that have additional functions in accelerating ADF/cofilin-dependent and twnfilin-dependent actin filament disassembly, which depend on the N-terminal helical folded domain\(^21,26-28\). Thus, in the future it will be important to reveal how the different activities of CAP are structurally and functionally coordinated between its N-terminal and C-terminal functional units.

**Methods**

**Proteins.** CAP1342-474 for crystallization experiments was expressed in BL21(DE3) E. coli (Sigma-Aldrich) as a 10XHis-3C fusion protein using pCodyf18 vector, a kind gift from Sabine Suppmann (Addgene plasmid #47937). After 20 h of culti-
vation at \(-22^\circ\text{C}\) in LB auto-induction media (AIML90210, Forremsd), cells were collected by centrifugation and suspended to lysis buffer (50 mM Tris-HCl 150 mM NaCl, 25 mM imidazole, pH 7.5) containing protease inhibitors (200 µg/ml PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin A, 20 µg/ml DNase I; all from Sigma-Aldrich). Cells were homogenized by EmulsiFlex C3 (Avestin Inc.) and the supernatants were clarified by centrifugation at \(15,000 \times g\) for 20 min.

Protein-containing fractions were pooled and dialyzed (50 mM Tris-HCl, 150 mM NaCl, 50 mM imidazole, pH 7.5) for 48 h against wash buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) and then concentrated to 10 mg/ml using Amicon Ultra-4 10 kDa centrifugal filter (Merck) and stored on ice for further use or were concentrated to 20 mg/ml PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin A, and stored as described above.

Crystal structure of CAP1375-474-ADP-actin complex. For complex formation with mouse CAP1342-474, actin was thawed and prepared by first exchanging Ca\(^{2+}\) metal to Mg\(^{2+}\) during ON dialysis (5 mM HEPES, 0.2 mM MgCl\(_2\), 0.2 mM EGTA, 0.2 mM ADP, 0.2 mM DTT, pH 8.0). Actin was washed with 5XCV of Buffer A and then 5XCV with Buffer B (100 mM Tris, 400 mM NaCl, pH 8.8). Finally, protein was eluted with a salt gradient, peak fractions were pooled and concentrated with Amicon Ultra-4 50 kDa centrifugal filter (Merck) and stored as described above.

**Biological experiments.** To determine binding of C-CAP proteins to actin with 4-chloro-7-nitrobenzofurazan (NBD) assay, NBD-actin was prepared as previously described\(^30,31\). Muscle α-actin was prepared from rabbit muscle acetone powder (Pel Freez) as previously described\(^32\) and stored at 2 mg/ml by snap-freezing with liquid N\(_2\) at \(-80^\circ\text{C}\). Full-length Src2 p proteins were expressed as GST-tagged fusion proteins in pGAT2 vector. Plasmids were transformed into BL21(DE3) E. coli and protein expression was performed at 37 °C in 2xLB media by IPTG induction at OD\(_{600}\) of 0.5–0.7. Expression was continued at 16 °C for 24 h. Cells were disrupted by sonication and supernatants clarified by centrifugation. Equilibrated glutathione agarose beads (Thermo Scientific) were added to the supernatants, incubated for 2 h at 4 °C. Beads were washed with wash buffer (50 mM Tris-HCl 150 mM NaCl, pH 8.0) for 20XCV and protein was eluted with 4XCV in a gravity column with elution buffer (100 mM Tris-HCl, 100 mM NaCl, 20 mM reduced glutathione, pH 8.8). Elution fractions were pooled and loaded to HiTrap Q HP (GE Healthcare) anion exchange column equilibrated in Buffer A (100 mM Tris, 100 mM NaCl, pH 8.8). Column was washed with 5XCV of Buffer A and then 5XCV with Buffer B (100 mM Tris, 400 mM NaCl, pH 8.8). Finally, protein was eluted with a salt gradient, peak fractions were pooled and concentrated with Amicon Ultra-4 50 kDa centrifugal filter (Merck) and stored as above.

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The structures of protein molecules (actin, CARP domain, WH2, and CAP1248-cr) were prepared using ProPka56 (PK_estimation based on the crystalized complex and determination of protonation states at pH = 6.8). Chimera72 (placing hydrogens), VMD73 (protein structure building and visualization), and PyMol (protein topology conversion from CHARMm to the GROMACS format). In all systems, E454 and H146 of the carp domain were mutated to N-terminus of actin (carp and cap) to neutralize the charges (parameters were obtained by analogy); and the crystal water molecules and the bound Mg ion were kept. In each system containing ATP-actin, ATP was docked into the binding pocket based on the ADP position. Each system containing the WH2 domain was initiated from the top scoring five Rosetta models as described above.

All simulations were carried out using GROMACS 5.172 employing the Charmm36 force field74 for the proteins and the TIP3P model75 for water. The equations of motion were integrated using a leap-frog algorithm with a 2 fs time step. All bonds involving hydrogens were constrained using the LINCS algorithm. Long-range electrostatic interactions were treated by the smooth particle mesh Ewald scheme75 with a real-space cutoff of 1.2 nm, a Fourier spacing of 0.12 nm, and a fourth-order interpolation. A Lennard–Jones potential with a force-switch between 1.0 and 1.2 nm was used for the van der Waals interactions.

Each protein complex was placed in a rhombic dodecahedral simulation box maintaining at least a distance of 15 Å to the box sides and solvated with 0.15 M NaCl solution, ensuring neutrality of the system. Before production runs, steepest descent minimization and successive equilibration simulations (in total ~500 ps) in the NVT and NPT ensembles using the Berendsen thermostat and barostat72 were performed. In these simulations, initially all protein heavy atoms, and later only the Ca atoms, were restrained with a time constant of 1.0 ps. The time step was then ramped up from 1 to 2 fs. The number of water molecules and the average box volume at the start of the production runs are given in Supplementary Table 3.

For production runs, each system was simulated in the NPT ensemble for ~1 μs. Reversing repeats were performed for each system (Supplementary Table 3). The total time scale covered in the simulations was 0.24 μs/atom. Protein–ADP/ATP-Mg complex, and solvent (water and NaCl) were coupled to separate temperature baths at 310 °C using the Nosé–Hoover thermostat5,6,7 with a time constant of 1.0 ps. Isotropic pressure coupling was performed using the Parrinello–Rahman barostat with a reference pressure of 1 atm, a time constant of 5 ps, and a compressibility of 4.5 × 10⁻¹⁰ bar⁻¹. Long-range electrostatic interactions were treated by Coulomb’s force-switch separately for each simulation repeat. The averages and standard deviations over independent repeats are reported.

In vivo experiments with yeast. All strains are in the s288c background from BGY331 (MATa, his3δ200, leu2-3,112, ura3-52, trp1-1, his3-200(oc)). Mutant yeast strains were svr2Δ:HSV3 (BGG3330) and svr2Δ:HSV3 (BGG3330M). Plasmid pPV16 was introduced into the strain svr2Δ:HSV3 (BGG3330) by using a single-crossover recombination event. Integration of pPV16 into the chromosomal Svr2 was confirmed by PCR analysis of isolated genomic DNA.

To measure Svr2/CAP protein levels in yeast cells, strains were grown in 10 ml cultures of YEPD at 25 °C to log phase, collected by centrifugation at 3000 g for 2 min, and resuspended in 20% TCA (trichloroacetic acid) at 25 °C. Cells were centrifuged at 16,000 g for 30 s, and the pellet was washed with 20% TCA, and vortexed with glass beads for 7 min. The mixture was diluted to final 5% TCA, and centrifuged at 3000 g for 10 min. Pellets were neutralized with 1 M Tris 8.0, resuspended in Laemli buffer (150 mM Tris-HCl (pH 6.8), 300 mM DTT, 6% SDS, 0.3% bromophenol blue, 30% glycerol, and 12% beta-mercaptoethanol), and immuno-blotted. Blots were incubated for 1 h with either 1:500 primary chicken anti-srv2Δ (Batch number 3439, Aves Labs, Inc., Tigard, OR) or 1:100 primary mouse anti-a-tubulin antibody (sc-32292; Santa Cruz Biotechnology), then washed and probed with secondary anti-chicken antibody (IRDye800CW, #603-131-126, LI-COR Biosciences) for Svr2 or anti-mouse antibody (IRDye-680, #926-32220, LI-COR Biosciences) for Tubulin. Blots were imaged using an Odyssey gel scanner (LI-COR Biosciences).

To visualize the actin cytoskeleton in cells, yeast strains were grown in YEPD to log phase and fixed with formaldehyde (4% final) for 30 min at 25 °C. Fixed cells were washed with 1x PBS and stained with AlexaFlour488 (Thermo Scientific) at a concentration of 1 μg/ml overnight at 4 °C. Cells were then washed with 1x PBS, and mounted on slides immediately before imaging. Cells were imaged on a Nikon A1R (Structured Illumination Microscopy) instrument (Nikon Instruments, Melville, NY) equipped with a SR APO TIRF AC 100x/1.49 NA oil immersion objective, a LU-N3-SIM laser unit, and an ORCA-flash4.0 CMOS camera (Hamamatsu, Japan). SIM image stacks were acquired with a Z-interval of 0.1 μm for a 0.9 μm section at the central plane of the cell. Images were captured with 500 ms exposure time. Fifteen raw images were acquired per Z-position, and reconstruction of images were performed using the reconstruction slice plugin from NI-Elements software (Nikon Instruments). Final images were then analyzed in ImageJ (NIH) to quantify actin patch number in mother cells and mother cell size, and the data were plotted in Graphpad Prism 6.
Quantification and statistical analysis. The statistical details, number of experiments and statistical analyses, are described in the figure legends or in the Methods section. Softwares for quantification and data analysis are indicated in experimental details in the Methods section.

Data availability. The WH2-PP2-CARP model(s) obtained from atomic simulations, together with the raw data, are available in Zenodo. The crystal structure has been deposited to the Protein Data Bank (PDB) under access code 6nm2. All relevant experimental data are available upon a reasonable request from the corresponding author.

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Author contributions

I.V., B.L.G., and P.L. designed the study. T.K. and K.K. conducted biochemical experiments, determined the crystal structure, and wrote the manuscript together with P.L. S.G. performed the in vivo experiments, and G.E. carried out the atomistic MD simulations. B.L.G. and P.L. edited the manuscript.

Additional information

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