A structural model for microtubule minus-end recognition and protection by CAMSAP proteins

Joseph Atherton, Kai Jiang, Marcel M Stangier, Yanzhang Luo, Shasha Hua, Klaartje Houben, Jolien J E van Hooff, Agnel-Praveen Joseph, Guido Scarabelli, Barry J Grant, Anthony J Roberts, Maya Töpf, Michel O Steinmetz, Marc Baldus, Carolyn A Moores & Anna Akhmanova

CAMSAP and Patronin family members regulate microtubule minus-end stability and localization and thus organize noncentrosomal microtubule networks, which are essential for cell division, polarization and differentiation. Here, we found that the CAMSAP C-terminal CKK domain is widely present among eukaryotes and autonomously recognizes microtubule minus ends. Through a combination of structural approaches, we uncovered how mammalian CKK binds between two tubulin dimers at the interprotofilament interface on the outer microtubule surface. In vitro reconstitution assays combined with high-resolution fluorescence microscopy and cryo-electron tomography suggested that CKK preferentially associates with the transition zone between curved protofilaments and the regular microtubule lattice. We propose that minus-end-specific features of the interprotofilament interface at this site serve as the basis for CKK’s minus-end preference. The steric clash between microtubule-bound CKK and kinesin motors explains how CKK protects microtubule minus ends against kinesin-13-induced depolymerization and thus controls the stability of free microtubule minus ends.

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binds the transition zone between the regular lattice and the curved sheet-like structure of the minus-end extremity, which presents a subtly altered interprotofilament interface that is optimal for CKK binding. Finally, our structural and in vitro reconstitution data showed that CKK sterically hinders the interaction of kinesin-13 with MT minus ends, thereby explaining how CAMSAPs and Patronin protect MT minus ends against depolymerization by these factors.

RESULTS

End-recognition by CAMSAPs depends on the conserved CKK domain

We have previously shown that the CKK domain in mammalian CAMSAPs binds minus ends. To investigate whether other CAMSAP domains might also control minus-end recognition, we tested the ability of different purified GFP-tagged CAMSAP1 and CAMSAP3 domains to bind to MT minus ends and lattices in vitro.

None of the CAMSAP fragments that lacked the CKK domain showed any minus-end preference (Fig. 1a–f and Supplementary Fig. 1a–c). For CAMSAP1, we found that the predicted helical domain and the linker, which precede the CKK, had only very weak MT affinity of their own but increased MT binding of CAMSAP1 CKK, though not its minus-end selectivity (Fig. 1a–f). An additional negatively charged linker region of CAMSAP1 (Supplementary Fig. 1d), located upstream of its third coiled-coil domain, suppressed MT-lattice binding and enhanced minus-end selectivity when it was present together with the CKK domain (Fig. 1a–e; this construct is denoted CAMSAP1min). For CAMSAP3, we confirmed that the MT-binding domain located between coiled coils 2 and 3 specifically bound to GMPCPP but not GDP MTs, as we have previously demonstrated (Supplementary Fig. 1a–c). The predicted helical domain of CAMSAP3 together with the adjacent linker also specifically bound to GMPCPP MTs without showing end preference (Supplementary Fig. 1a–c). We concluded that the minus-end preference of CAMSAPs depends on the CKK domains, whereas additional domains modulate their end selectivity or ability to decorate MT lattices.

To establish whether CKK-mediated minus-end binding defines an evolutionarily conserved mechanism, we screened a set of nearly 100 eukaryotic genomes and found homologs of the CKK domain in all eukaryotic supergroups except for Amoebozoa, although homologs were absent in some well-studied lineages such as dikaryan fungi and land plants (Supplementary Fig. 2 and Supplementary Table 1).

Parasimoniously interpreting the occurrence of CKK across eukaryotes, we concluded that this domain was probably already present in the genome of the last eukaryotic common ancestor and did not emerge during early animal evolution, as suggested previously. In particular, our discovery of genes encoding CKK-containing proteins in the genomes of various members of the Excavata, a group that some consider to be sister to all other eukaryotes (discussed in ref. 22), strongly supports an ancient origin of CKK. Whereas in vertebrates, the array of CKK-containing proteins was expanded by gene duplications leading to the three CAMSAP paralogs in mammals, this outcome was not the case in many other genomes (Supplementary Data Set 1), thus suggesting that in most eukaryotes, a single copy of the domain fulfills CKK functions.

To test whether the sequence conservation of CKK translates into functional conservation, we next purified GFP-tagged CKK domains derived from flies (Drosophila melanogaster) and worms (Caenorhabditis elegans), as well as from four more evolutionarily distant eukaryotes (Trichomonas vaginalis, Tetrahymena thermophila, Naegleria gruberi and Phytophthora infestans). From these six CKK domains tested, four (D. melanogaster, C. elegans, T. vaginalis and T. thermophila) specifically tracked the growing minus ends of MTs assembled from pig-brain tubulin in in vitro MT-dynamics reconstitution experiments (Fig. 1g), whereas the two others (N. gruberi and P. infestans) showed strong binding along the entire MT lattice (data not shown). These data demonstrated that CKK is a protein module that recognizes MT minus ends, a property preserved over long evolutionary distances.

CKK domains bind an intradimer site between protofilaments

We determined the crystal structure of the mouse CAMSAP3 CKK-domain core (residues 1121–1239; denoted CKK3core) (Table 1). Similarly to a deposited but unpublished NMR structure (PDB 1UGI), the CKK3core has a compact, globular structure composed of two N-terminal α-helices, which are connected by a disordered loop (loop1) and pack against a central five-stranded β-sheet (Supplementary Fig. 3).

Although CKK shows a clear preference for MT minus ends, at high concentrations it can also decorate the entire MT lattice. We reasoned that a high-resolution structure of the lattice-bound CKK domain might shed light on the mechanism of its MT minus-end recognition. Therefore, we used cryo-EM to analyze extended CKK domains of CAMSAP1 and CAMSAP3 (residues 1474–1613 and 1112–1252, respectively) bound to 13-protofilament (pf) taxol-stabilized MTs. Filtered images of these MTs showed additional density corresponding to CKK domains spaced by 8 nm (Fig. 2a), a result indicative of binding every tubulin dimer. A striking feature of these CKK-decorated MTs is the presence of a right-handed skew in the protofilaments. This feature was evident in both the filtered images and the alignment parameters of the MT segments used for reconstruction and was specific to the CKK–MT data sets (Fig. 2a, b and Supplementary Fig. 4a). This result indicated that CKK binding modifies the MT lattice with which it interacts, in agreement with the idea that the regular MT lattice conformation is not the most favored substrate for CKK domains.

Asymmetric 3D reconstructions (~12-Å and ~9-Å resolution for CAMSAP1 and CAMSAP3, respectively) show the CKK domains binding the MT lattice every 8 nm between protofilaments except at the seam (Fig. 2c and Supplementary Fig. 4b). Using the pseudosymmetry of the MTs, we obtained averaged reconstructions with final resolutions that allowed α- and β-tubulin to be clearly distinguished (true Fourier shell correlation (FSCorr) 0.143 criterion of 5.3 Å (CAMSAP3) and 8 Å (CAMSAP1)) (Fig. 2d, e, Supplementary Fig. 4c–e and Table 2). From these data, we concluded that CKK domains bind at the tubulin intradimer B-lattice interface, where conserved differences between α- and β-tubulin explain this binding-site selection (Fig. 3a). The binding site of CKK is distinct from those of other proteins known to bind to protofilaments, end-binding proteins (EBs) and Doublecortin, which bind at the vertex of four tubulin dimers and not at the intradimer interface (Supplementary Fig. 4f–h). However, some overlap was found with the binding site of kinesin (Supplementary Fig. 4i).

For ease of description, the four tubulin subunits contacting a single CKK domain are referred to as β1-, β2-, α1- and α2-tubulin (Figs. 2d, e and 3a). In general, the CKK wedges snugly between the β-tubulins and forms more extensive contacts with the β-tubulins than with the α-tubulins (Fig. 3a). Contacts between the CKK domain and its MT-binding site are distributed across the domain and also involve the N and C termini (Figs. 2d and 3a, b). To confirm and extend the cryo-EM data, we also analyzed the structure of the CKK domain of CAMSAP3 bound to MTs, by using solid-state NMR (ssNMR), which provides a sensitive means to study ligand and protein binding to MTs at the atomic level. To do so, we compared the solution NMR results (PDB 1UGI) and our X-ray crystallographic results of free
CKK with 2D ssNMR data recorded on $^{13}$C-$^{15}$N-labeled CKK in complex with MTs. This procedure allowed us to identify CKK residues that underwent significant chemical-shift/intensity changes after MT binding (Fig. 3c and Supplementary Fig. 5a–d, labeled in red) and those that experienced no significant change (labeled in blue). For example, in our EM density, helix $\alpha$1 of the CKK domain sits across the $\beta$- and $\beta$2-tubulins, while beneath it, loop7 and adjacent regions of the $\beta$-barrel wedge in between the $\beta$-tubulin subunits (Fig. 3a–c and Supplementary Fig. 5e,f). Consistently with this configuration, residues from MT-bound CKK helix-$\alpha$1 and adjacent loop7 displayed ssNMR chemical shifts relative to free CKK. Similarly, our cryo-EM and ssNMR data corroborated interactions between CKK loop1 and $\beta$1-tubulin, and CKK loop7 and $\beta$2-tubulin, while CKK loop3 lies at the intradimer interface contacting both $\beta$1-tubulin and $\alpha$tubulin. CKK loop8 is well positioned to form contacts with $\alpha$2-tubulin, and our ssNMR data supported the involvement of this loop in the MT interaction. However, loop8 is visible only at less conservative cryo-EM density thresholds (Supplementary Fig. 5f), thus suggesting that this loop has greater flexibility than the rest of the CKK domain in this region, in agreement with its B factor in the crystal structure. Together, our results demonstrated that residues identified to undergo changes in ssNMR signals cluster on the CKK MT-facing surface. In contrast, residues displaying unaltered ssNMR signals are predominantly solvent exposed (Fig. 3c and Supplementary Fig. 5b–d). The ssNMR data thus supported the EM-derived binding mode at an atomic level.

The CKK N and C termini are not visible in the crystal structure, and both are flexible in solution (PDB 1UGJ). However, our cryo-EM and ssNMR data also supported a role of the CKK N and C termini in MT binding. Density corresponding to the final portion of the N terminus leading into helix-$\alpha$1 (starting at S1120) is visible in our cryo-EM reconstruction contacting $\alpha$2-tubulin (Figs. 2d and 3a,d), and the additional cryo-EM density suggests that more N-terminal regions make additional contacts with $\beta$2-tubulin (red asterisks in Supplementary Fig. S5f).

Figure 1 The CKK is a highly conserved domain for MT minus-end tracking. (a) Schematic of CAMSAP1 domain organization and the constructs used. (b,c) TIRFM images (b) and corresponding kymographs (c). Scale bars: horizontal, 2 µm; vertical, 2 min. (d-f) Quantification of localization of GFP-CAMSAP1 fragments to MT minus ends and lattice, based on data in b and c. The intensity was normalized to the average minus-end intensity of the C4 fragment. Data are mean ± s.d., n = 30 MTs. ND, not detectable; a.u., arbitrary units. (g) TIRFM images (top) and kymographs (bottom) of GFP-tagged CKK domains from humans (CAMSAP1), flies (D. melanogaster), worms (C. elegans), T. thermophila and T. vaginalis. Scale bars: horizontal, 1 µm; vertical, 30 s. Additional TIRFM images shown in Supplementary Figure 1, and source data for graphs are in Supplementary Table 2. Tub, tubulin.
Table 1 Data collection and refinement statistics

| Data collection | CKK3 (PDB 5LZN) |
|-----------------|-----------------|
| Cell dimensions | 94.6, 94.6, 63.3 |
| Resolution (Å)  | 68.1–1.4 (1.45–1.4) |
| Rmerge (%)      | 6.8 (475.0) |
| Completeness (%)| 100 (99.9) |
| Redundancy      | 25.5 (22.4) |

Data were collected from one crystal.

Fig. 3a; red dashed line in Fig. 3d). The CKK N terminus is therefore partially stabilized in complex with the MT, in agreement with the N-terminal truncation construct having lower MT affinity (Fig. 3c). Furthermore, although this region was not readily modeled, cryo-EM density corresponding to the conserved basic C terminus of the CKK domain and/or the β-tubulin C-terminal tail is visible (Fig. 3f). ssNMR measurements in the CAMSAP3 CKK–MT complex suggested that the CKK C terminus retains some conformational variability when interacting with MTs (Fig. 3c). The location of the CKK C terminus suggested that it interacts with the flexible acidic C-terminal tail of β-tubulin; this possibility was supported by the observation that the interaction of GFP-tagged CAMSAP3 CKK domain with MTs significantly decreased after the acidic C-terminal tails of tubulin had been cleaved (Fig. 3g).

We also used MD-based energy calculations, which can provide an estimate of the relative energetic contributions of individual residues to complex formation26, to analyze the CKK–MT interaction. This analysis also supported the involvement of the regions described above in the CKK–tubulin interface (Supplementary Fig. 6a). Overall analysis of the electrostatic potential of the contact surfaces showed complementary charges between the CKK domain and the MT (Supplementary Fig. 6b,c). This result was consistent with the large electrostatic contribution to binding energy from MD and the established sensitivity of CAMSAP–MT interaction to ionic strength18,19 (Supplementary Fig. 6a,d). CKK residues at the interface with the MT, especially basic ones, are particularly evolutionarily conserved in CKK domains (Supplementary Fig. 2b and 6c,e). In addition, these calculations also highlighted the greater energetic contribution of CKK binding to β-tubulin over α-tubulin (with a calculated ΔG of −25.1 ± 5.2 kcal/mol for β-tubulin positions compared with −3.6 ± 5.0 kcal/mol for α-tubulin). Together, our cryo-EM, ssNMR and MD data showed that CKK binds through multiple binding sites across two tubulin dimers at their intradimer interface between protofilaments and associates more closely with β-tubulin than α-tubulin.

Analysis of CKK mutants supports the identified CKK–MT interface

To further validate the identified CKK–MT binding mode, we made a series of CKK mutants in the context of a short version of CAMSAP1 (CAMSAP1 mini residues 1227–1613), which displayed more robust minus-end tracking activity than that of the CKK domain alone (Fig. 1a–e). Mutations of several conserved positively charged residues decreased the overall binding affinity of CAMSAP1 mini toward MTs and their minus ends (Fig. 4a–c), although they did not affect the integrity and folding of the CKK domain, on the basis of CD spectra and thermal unfolding profiles in the CAMSAP3 CKK context (Supplementary Fig. 7a). Truncation of the N-terminal extension or mutations of positively charged residues in this sequence had a similar effect, as did truncations of the positively charged C-terminal tail region (Fig. 4a). These data supported the validity of our CKK–MT lattice reconstruction and its relevance for CKK binding to the MT minus end.

Unexpectedly, a mutation of a conserved asparagine residue (N1492 of CAMSAP1 and N1130 in CAMSAP3) to alanine, which did not affect the integrity of the domain (Supplementary Fig. 7a), dramatically increased the ability of CKK to bind to the MT lattice and decreased, though did not abolish, its selectivity toward minus ends (Fig. 4a–c and Supplementary Fig. 7b–f). The importance of CAMSAP1’s N1492 residue was further emphasized by the observation that mutating it to bulky charged or uncharged amino acids decreased the affinity of CKK for both MT minus ends and lattices, whereas substituting it for either serine or threonine enhanced MT-lattice binding and decreased minus-end selectivity even further (Fig. 4d and Supplementary Fig. 7d–f). CAMSAP1 N1492 is expected to contact the C terminus of helix H4 of β-tubulin (cyan in Fig. 4b), and our data suggested that the formation of the CKK–MT complex is very sensitive to both the size and the chemical nature of the side chain in this position. For example, the potential hydrogen-bond formation between this residue and β-tubulin may affect the affinity of the CKK–MT interaction.

We also mutated several residues close to N1492 in the CKK structure to alanine; several of these substitutions had no effect (Supplementary Fig. 7g,h), whereas the D1572A mutation also enhanced CKK interaction with the MT lattice and decreased its minus-end selectivity (Fig. 4a). Overall, these experiments identified two main classes of mutations: (i) mutations of positively charged CKK residues, which weakened the interaction between CKK and the negatively charged surfaces of the MT ends and lattice, including less structurally ordered regions of both the CKK and MT, and (ii) mutations that specifically increased lattice binding. Remarkably, the effects of these latter mutations were rather subtle, thus suggesting that the mechanism of minus-end discrimination itself is also subtle.

We reasoned that understanding the mechanism underlying the higher affinity of the N1492A mutant for MTs would provide insight into CKK’s minus-end specificity. Therefore, we obtained a 3D reconstruction of the CAMSAP1 CKK N1492A mutant by using cryo-EM (Fig. 4e, Supplementary Fig. 8a–d and Table 2). As with the wild-type CKK, the N1492A mutant also binds to the tubulin intradimer B-lattice interface with the same overall orientation as the that of the wild-type CKK (Supplementary Fig. 8a,c,d). However, two distinct structural properties were observed for the mutant compared with...
wild type: (i) less protofilament skew in CKK N1492A–decorated MTs (Supplementary Fig. 8e) and (ii) a subtle shift of CKK N1492A itself toward the main MT body (Fig. 4e). These results suggested that very small changes in the CKK–MT interaction determine the binding selectivity of CKK between the lattice and the minus end. Moreover, the adjustment in the CKK–MT interaction by the N1492A mutant—movement of the CKK domain deeper between protofilaments—may reflect aspects of the mechanism of end selectivity by wild-type CKK via selection of a tubulin conformation present only at MT minus ends.

We also attempted to polymerize tubulin in the presence of a high concentration of CKK. In the presence of wild-type CKK, only very few short microtubules formed, and instead a variety of tubulin oligomers that formed clumps were generated. In contrast, in the presence of saturating concentrations of the N1492A mutant, many longer, predominantly 13-pf, microtubules formed (Supplementary Fig. 8f). These data supported the idea that wild-type CKK preferentially interacts with a polymeric conformation of tubulin that is distinct from the normal MT structure, whereas the binding of the N1492A mutant is more compatible with the regular MT lattice.

The high-affinity CKK-binding site at MT minus ends

Having defined the CKK–MT binding mode, we set out to determine whether the state of MT minus ends affects CKK binding. We found that the binding of CAMSAP1min to MT minus ends was insensitive to the nucleotide state of MTs, because the protein bound well to

Figure 2 The unique MT-binding site of the CAMSAP CKK domain. (a) Fourier-filtered images of 13-pf MTs. Left, filtering of a CAMSAP3-CKK-decorated 13-pf MT showing density corresponding to the CAMSAP3 CKK domain every tubulin dimer; center, filtering highlighting the MT moiré pattern and the presence of protofilament skew. Right, filtering of a kinesin-1-decorated 13-pf MT, highlighting a comparative lack of skew. (b) Schematic of three sets of three protofilaments depicting the skew detected in the CKK 13-pf MT data sets (left) compared with kinesin-bound 13-pf (middle) and 14-pf paclitaxel-stabilized MTs (right). The skew-angle size is exaggerated for ease of viewing. (c) The asymmetric reconstruction of the CAMSAP3-CKK-decorated 13-pf paclitaxel-stabilized MT low-pass filtered to 15-Å resolution, showing extra densities (green) every 8 nm corresponding to the CAMSAP3 CKK domains, which are absent at the seam (arrow). (d) The averaged reconstruction of the CAMSAP3 CKK domain viewed from the MT surface contacting two β-tubulins and two α-tubulins at the intradimer interprotofilament interface. The CKK is colored as in the X-ray structure (Supplementary Fig. 3), except for the N terminus (red) and loop1 (magenta), which are absent in our crystal structure but visible in our EM density. α-tubulin is shown in light gray, and β-tubulin is shown in dark gray. Term, terminus. (e) The averaged reconstruction of the MT-bound CAMSAP3 CKK domain viewed from the MT lumen, showing density corresponding to paclitaxel bound to β-tubulin (yellow). Along with the distinctive appearance of the H1–S2 and S9–S10 loops (arrowheads and arrows, respectively), this reconstruction differentiates between β- and α-tubulin, thus allowing for identification of the CKK-binding site at the intradimer interface. In d and e, schematic drawings appear on top. Ta, paclitaxel-binding site. Additional views are shown in Supplementary Figures 3 and 4.
the minus ends of GMPCPP-stabilized (GTP-like) or taxol-stabilized (GDP) MTs, as well as dynamic GTP or GTP-γS-bound (GDP-Pi-like) MTs (Fig. 5a, b). In contrast, we observed no accumulation of CAMSAP1mini at depolymerizing MT minus ends (Fig. 5c), thus suggesting that their structure does not support CKK binding.

To determine the precise localization of CKK on MT minus ends, we implemented the model-convolution method to determine the relative positions of the CKK and the MT minus end from their 1D intensity profiles along the MT axis (Fig. 5d, e). Our data showed that CKK accumulated ~16 nm, equivalent to a few tubulin dimers, behind the outermost MT minus end (estimated measurement error of ~8 nm; Online Methods). This result suggested that some structural features of MT minus ends at this specific location form a preferred CKK-binding site.

Cryo-ET shows preserved interprotofilament contacts at MT ends
To gain insight into the structure of this binding site, we analyzed individual ends of GMPCPP-stabilized (GTP-like) or taxol-stabilized (GDP) MTs by cryo-ET (Fig. 5f). Because no averaging was imposed, the tomographic 3D reconstructions contain information about overall polymer configuration and thus allow for direct visualization of the conformation of tubulin that CKK recognizes. The data quality readily allowed for discrimination of individual protofilaments and in many cases individual subunits (Fig. 5g), and provided critical 3D information about the MT ends.

Individual protofilament 3D trajectories within five minus and five plus ends (identified through previously established approaches (Fig. 5h) were analyzed. Overall, the individual MT ends are very heterogeneous, and there are no significant differences between minus and plus ends. However, their structures are not blunt but are also distinct from those of a MT-end model constructed on the basis of available high-resolution structures of curved tubulin (Supplementary Fig. 9c). First, plots of individual protofilament trajectories at both plus and minus ends show a range of curvatures and lengths of curved regions (example of a minus end in Fig. 5i). Second, although there is a wide variation of protofilament curvature, protofilaments at each end are on average less curved than in the model (Fig. 5j). The minus- and plus-end protofilaments are similar with respect to the length (18 ± 15 nm and 11 ± 8 nm at the minus and plus ends, respectively) and the extent of longitudinal curvature, results consistent with those of previous cryo-EM studies in 2D. Hence, CKK minus-end preference does not depend on longitudinal curvature alone.
Figure 3 The interaction with four tubulin monomers is distributed across the CKK domain. (a) CKK-interaction surface of the MT, with cryo-EM density colored according to CKK contacts (<6 Å distance; coloring as in Fig. 2d). Sequence alignments for contact regions in β-tubulin (top) and α-tubulin (bottom) indicate sequence differences between human α1a tubulin and β3 tubulins (asterisks) that may contribute to CKK binding to the inter- versus the interdimer site. Comparison between *H. sapiens* (Hs) β3 tubulin and α1a tubulin (most common isoforms in mammalian brain45); *C. elegans* (Ce) β1 tubulin and α3 tubulin; and *D. melanogaster* (Dm) β1 tubulin and α2 tubulin at 84B. Residues contacting the CKK are within green boundaries. (b) 180° rotations of the CKK domain, with loop coloring referring to MT contact sites in a. (c) CKK views as in b, showing ssNMR data on 13C-15N-labeled CKK–decorated MTs relative to free CKK. Red, residues showing significant chemical-shift or intensity changes; blue, residues showing no change; white, residues not analyzed. The unresolved N and C termini are represented as dashed lines with each dash depicting a single residue. (d) CAMSAP3 CKK–MT cryo-EM density at lower threshold, showing the CKK N terminus. (e) TIRFM experiments showing the importance of the CKK N-terminal extension in MT binding. Intensity is normalized to average CKK lattice intensity. Scale bar, 2 μm. Data are mean ± s.d.; CKK, n = 104 MTs; CKKΔN, n = 118 MTs. ***P < 0.001, two-tailed Mann–Whitney U test. (f) CAMSAP3 CKK–MT cryo-EM density probably corresponding to interaction between the CKK flexible C terminus (blue dotted line) and the β-tubulin C terminus (gray dotted line), not usually seen in MT reconstructions46. (g) X-rhodamine (rho)-labeled paclitaxel-stabilized MTs (red), either untreated or treated with subtilisin to remove their C-terminal tails, incubated with 200 nM GFP-tagged CAMSAP3 CKK and imaged with TIRFM. Scale bar, 4 μm. The intensity of MT labeling, normalized to that of wild type, is quantified. Data are mean ± s.d., n = 100 MTs. ***P < 0.001, two-tailed Mann–Whitney U test. Additional details of the CKK–MT binding site are shown in Supplementary Figure 5. Source data for graphs are in Supplementary Table 2.
Figure 4 Validation of CKK–MT contact sites with \textit{in vitro} assays and structure of a mutant CKK bound to MTs. (a) Left, TIRFM images of GFP-CAMSAP$_{1\text{mini}}$ wild type and mutants binding to the minus ends of dynamic MTs. Scale bar, 1 µm. The corresponding residues in CAMSAP3 and their locations are indicated. Right, quantification of GFP-CAMSAP$_{1\text{mini}}$ intensities at MT minus ends and on MT lattice. The intensity is normalized to the average minus-end intensity of wild type. Data are mean ± s.d.; \(n\) ranged from 17 to 87 MTs (individual data points in Supplementary Table 2). (b) View of the CKK-interaction surface of the MT cryo-EM density, with mutated CKK residues mapped (<8 Å distance) and colored according to the percentage change in the minus-end fluorescence signal in mutants relative to wild type (as in (a)). (c) Surface representation of the tubulin-interacting face of the CAMSAP1 CKK domain. Mutated CKK residues are colored according to the percentage change in the minus-end fluorescence signal of corresponding mutants relative to wild type in our TIRF assays (as in (a)). (d) Left, TIRFM images of GFP-CAMSAP$_{1\text{mini}}$N1492 mutants; scale bar, 1 µm. Right, quantification of GFP-CAMSAP$_{1\text{mini}}$ intensities at MT minus ends and on MT lattice. The intensity is normalized to the average minus-end intensity of wild type. Data are mean ± s.d., \(n = 30\) MTs. (e) The N1492A CAMSAP1 CKK binds at the intradimer, interprotofilament MT binding site but in an orientation subtly different from that of wild type. Ribbon representation comparing the position of N1492A CAMSAP1 CKK with wild-type CAMSAP1 CKK relative to the tubulin-binding surface. N1492A CAMSAP1 CKK (blue) is rotated 5° around the indicated axis and translated 1.9 Å into the interprotofilament-binding site, relative to wild-type CAMSAP1 CKK (green). Arrowhead depicts the position of N1492. Additional validation of CKK–MT contact sites by mutagenesis and analysis of the N1492A mutant is shown in Supplementary Figures 7 and 8. Source data for graphs are in Supplementary Table 2.
Intriguingly, adjacent protofilaments within an MT end retain connectivity with their neighbors (Fig. 5k and Supplementary Fig. 9d), even while the protofilaments spread away from the MT axis, and the total protofilament number is decreased. This behavior is in contrast to the MT-end model in which protofilament separation is an essential consequence of their curvature and is more consistent with the classical ‘rams horns’ of depolymerizing MTs\(^{31}\), to which CAMSAP do not bind (Fig. 5c). In other words, in nondepolymerizing MTs, protofilaments curving outward from the MT axis flatten from cylinders into gently curving sheet-like structures, which retain lateral connectivity\(^{30,32,33}\). Given that the CKK domain binds at the interprotofilament interface, the tomographic data suggest that potential CKK-binding sites are retained in the end structures characterized here. To determine whether CKK binding perturbs these curved sheet-like structures, we examined MT minus ends in the presence of CKK but detected no differences relative to the control MTs (Supplementary Fig. 9e–h). This analysis, when combined with our fluorescence-based localization of the CKK binding site, suggested that the CKK prefers neither the extreme end nor the MT lattice but a transition region from the straight lattice to a curved and flattened polymer.

**CKK blocks MCAK interaction with MT minus ends**

Fly Patronin and three mammalian CAMSAPs have been shown to protect MT minus ends from depolymerization by kinesin-13 (refs. 6,19), but the underlying mechanism is unknown. By superimposing the human (Hs) MCAK motor structure onto our CKK–MT cryo-EM reconstruction, we found that CKK and HsMCAK would strongly clash with each other at a number of positions (Fig. 6a). Hence, CKK and MCAK would be expected to compete for the binding sites at MT minus ends. To test this hypothesis, we measured in vitro disassembly of GMPCCP-stabilized MTs by 50 nM MCAK in the presence of different concentrations of CAMSAP\(_{\text{mini}}\) or the isolated CKK domain (Fig. 6b,c). CAMSAP\(_{\text{mini}}\) and CKK almost completely inhibited the minus-end binding and depolymerization of MCAK at 1.5 nM and 38 nM, respectively, whereas the activity of MCAK on plus ends was largely unaffected at those concentrations (Fig. 6d,e). Above 1 \(\mu\)M concentration, even though both CAMSAP\(_{\text{mini}}\) and CKK fully decorated the MT shaft with lattice intensities higher than those observed at the minus ends at low concentrations, this decoration did not lead to complete inhibition of MT plus-end accumulation and depolymerization by MCAK (Fig. 6d,e). This observation suggested that the CKK has the highest affinity for MT minus ends and then for the MT lattice, and has the lowest affinity for MT plus ends. The alternative mechanism—in which displacement of MCAK from MT minus ends is due to CKK-mediated alteration of the MT minus-end structure, which would be less favorable for MCAK binding—is not supported by our cryo-EM images even at a CKK concentration of >50 \(\mu\)M, which was much higher than the concentrations used in our in vitro assays (Supplementary Fig. 9e–h). This result indicated that the competition between CKK and MCAK on MT minus ends is primarily based on steric hindrance.
DISCUSSION

In this study, we demonstrated that the CKK is a highly conserved globular-protein module that interacts with an interprotofilament site between two tubulin dimers on the outer surfaces of MTs. Several lines of evidence suggest that an optimal site for CKK binding deviates from the interprotofilament interface within a regular, straight MT structure. First, the decoration by CKK distorts the normal MT lattice, thereby generating a right-handed protofilament skew. Second, tubulin copolymerization with CKK yields mainly non-MT, curved polymers. This binding site fits very well with the average position of the transition zone between the straight protofilaments in the MT shaft and the mildly curved tubulin sheets identified at MT ends by cryo-ET. Microscopy showed that the high-affinity CKK-binding region is located, on average, two tubulin dimers behind the outmost MT end. Microscopy showed that the high-affinity CKK-binding region is located, on average, two tubulin dimers behind the outmost MT end. Fifth, high-resolution microscopy showed that the high-affinity CKK-binding region is located, on average, two tubulin dimers behind the outmost MT end. This binding site fits very well with the average position of the transition zone between the straight protofilaments in the MT shaft and the mildly curved tubulin sheets identified at MT ends by cryo-ET.

At this transition zone, pairs of α-tubulins would be more laterally flattened and more flexible than the more constrained lattice-like β-tubulin pairs (Fig. 7a). In agreement with this scenario, our structures show that the lattice-bound CKK has tight shape complementarity and a large contact surface with the β-tubulin pairs, whereas the α-tubulin pairs surround the CKK more loosely. In contrast, at

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Figure 6 CAMSAP CKKs protect MT minus ends from MCAK-induced depolymerization via steric inhibition. (a) An MT tubulin dimer pair bound to CAMSAP3 CKK (green), shown with the expected position of an MD of HsMCAK (in complex with ADP; PDB 4UBF) by alignment with MT-bound kinesin-1 (ref. 47) in Chimera48. (b) Kymographs of MT-depolymerization assay with GMPCPP-stabilized MTs (blue) and GFP-MCAK (red). Scale bars: horizontal, 1 μm; vertical, 1 s (top) or 1 min (bottom). MT polarity was determined on the basis of the movement of the SNAP-Alexa647-tagged plus-end-directed motor kinesin-1 KIF5B (green, residues 1–560). (c) Kymographs of MT-depolymerization assays with GMPCPP-stabilized MTs (blue), GFP-MCAK (red) and different concentrations of SNAP-Alexa647-tagged CAMSAP1 mini or CKK (green). Scale bars: horizontal, 1 μm; vertical, 1 min. In b and c, MT minus (−) ends are shown on the left, and plus (+) ends are shown on the right. Data are mean ± s.d.; n ranged from 17 to 31 MTs (source data and individual data points in Supplementary Table 2).
the plus end, β-tubulin pairs are expected to acquire a more flattened and flexible arrangement than α-tubulins. This arrangement would disfavor the interaction of CKK with the plus end, thereby explaining its inability to effectively compete with MCAK at this location. In summary, flattening of β-tubulin pairs at the plus end may squeeze CKK out of the tubulin groove, thus avoiding steric clashes, whereas flattening of α-tubulin pairs at the minus end may tighten their multiple contacts around the CKK. This tightening would lead to a binding preference for the minus end, some capacity to bind the straight MT lattice and low affinity for MT plus end. The lack of CKK binding at rapidly depolymerizing MT minus ends, where the lateral contacts between protofilaments are expected to be lost, owing to their strong curling, is consistent with the requirement for maintenance of lateral protofilament connectivity and/or more gentle longitudinal curvature for CAMSAP binding.

Importantly, although our analysis showed that CKK was the only CAMSAP part able to autonomously recognize MT minus ends, some longer CAMSAP fragments, such as CAMSAP1\textsubscript{min}, had a higher affinity for the minus ends. These data indicated that CAMSAP regions outside of the CKK domain contribute to the affinity and selectivity of minus-end binding, and additional work will be needed to decipher the underlying mechanisms.

The MT minus-end-binding mode of the CKK domain is distinct from those of other known MT-tip-interacting proteins. The most conceptually simple mode of MT-end recognition relies on the presence of unique binding sites on α- and β-tubulin exposed on the outermost MT ends, as is the case for the γ-TuRC at the minus end\textsuperscript{5} and probably for the centriole protein CPAP at MT plus ends\textsuperscript{34}. However, the preference of most known MT regulators is based on recognition of certain MT-lattice features that are different at MT ends compared with the MT shaft. For example, EB proteins bind at the vertex of four tubulin dimers\textsuperscript{35,36} and sense a GTP-hydrolysis transition state of tubulin within the MT lattice at both plus and minus ends\textsuperscript{37,38}. Doublecortin, which also binds at the vertex of four tubulin dimers\textsuperscript{39} and acts as an MT-end-binding protein, is thought to be sensitive to the extent of protofilament longitudinal curvature\textsuperscript{40,41}. MT-depolymerizing
kinesin-13s can also accumulate at MT ends, probably because of a preference for the more curvy or flexed conformations of tubulin located outside the lattice\(^2,43\). Importantly, none of these mechanisms are specific for the plus or the minus end.

CAMSAPs are similar to most other MT-end-binding proteins in that they bind on the outer surfaces of MTs: in contrast to \(\gamma\)-TuRC binding, which caps MTs, CAMSAP binding is compatible with further MT polymerization at the minus end\(^18\). CAMSAPs and \(\gamma\)-TuRcs thus do not directly compete for binding to MT minus ends; however, the restricted lattice-like conformation of tubulin at \(\gamma\)-TuRC-capped minus ends probably prevents formation of CAMSAP-preferred binding regions. Importantly, in contrast to EB–MT association, CAMSAP–MT association is insensitive to the hydrolysis state of the tubulin-bound nucleotide. Whereas the tubulin interdimer interface to which EBs bind changes conformation in response to nucleotide, the intradimer interface to which CKks bind does not\(^36,44\). This difference in conformational changes accounts for the ability of CAMSAPs to recognize growing GTP- as well as GDP-bound minus ends generated by MT severing\(^18\).

CAMSAP's interaction with MT minus ends can be expected to have several consequences. First, by preferentially binding between laterally connected but curved protofilaments, the CKK, in the context of the full-length protein, might inhibit formation of a regular lattice, thus explaining why CAMSAPs slow down MT minus-end polymerization\(^18\). Second, CKK binding at intradimer-binding sites can also explain the protection of MT minus ends from kinesin-13-driven depolymerization by direct steric inhibition (Fig. 7b).

Together, our data show that the unique MT-binding mode of the CKK domains enables CAMSAPs to combine the ability to recognize MT minus ends with the preservation of the dynamic properties of these ends. Hence, CAMSAPs can be rapidly recruited to MT minus ends generated by release from the sites of MT nucleation, severing or breakage, and consequently protect the ends from disassembly by depolymerases and, by decorating polymerizing minus ends, form stretches of stabilized MT lattice. Future studies will show whether other proteins share the CKK's MT-binding mode or whether this mode is unique to this evolutionarily ancient and widespread protein module.

### METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

### ACKNOWLEDGMENTS

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### AUTHOR CONTRIBUTIONS

J.A., K.I., M.O.S., M.B., C.A.M. and A.A. designed experiments, analyzed data and wrote the paper; C.A.M. and A.A. coordinated the project. J.A. performed and analyzed cryo-EM and cryo-iFT experiments K.I. and S.H. performed in vitro reconstitution experiments; M.M.S. performed X-ray crystallography and biophysical experiments; Y.L. and K.H. performed and analyzed NMR experiments; J.J.E.V.H. performed bioinformatics analysis; A.-P.J. and M.T. performed cryo-EM-based molecular modeling; G.S. and B.J.G. performed molecular dynamics calculations; and A.J.R. performed subtilisin–MT TIRFM assays.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Protein expression and purification for crystallization. The DNA encoding the *Mus musculus* CAMSAP3 CKK core domain (denoted CKK3core residues 1112–1239; UniProt Q80VC9) was amplified from the *Mus musculus* CAMSAP3 CKK-domain vector (denoted CKK3) (residues 1112–1252) and cloned into the pET-based bacterial expression vector pSTCM2 (with an N-terminal hexahistidine tag) by positive-selection methods52. The CKK3 and the mutants used for biophysical characterization were cloned into the pET28a vector. Protein production was performed in *Escherichia coli* strain BL21(DE3) (Stratagene) in LB medium containing 50 µg/ml kanamycin. When the 1–1 cultures had reached an OD600 of 0.6 at 37 °C, the medium was cooled to 20 °C, and expression was induced with 1 mM IPTG. Expression was allowed to proceed for 16 h at 20 °C. After cells were harvested and washed with Dulbecco’s PBS (Millipore), the cells were sonicated in the presence of complete protease-inhibitor cocktail (Roche) in lysis buffer (50 mM HEPES, pH 8, supplemented with 500 mM NaCl, 10 mM imidazole, 2 mM β-mercaptoethanol and 0.1% bovine DNase I). Proteins were purified by immobilized metal-affinity chromatography (IMAC) on a HiTrap HP Ni²⁺–Sepharose column (GE Healthcare) at 4 °C, according to the manufacturer’s instructions. The column was equilibrated in IMAC A buffer (50 mM HEPES, pH 8, supplemented with 500 mM NaCl, 10 mM imidazole and 2 mM β-mercaptoethanol). Proteins were eluted with IMAC B containing buffer 400 mM imidazole after being washed with 5% IMAC buffer B. In the case of CKK3core, the N-terminal histidine tag was cleaved off by an in-house-produced HRV 3C protease in IMAC A buffer for 16 h at 4 °C. The cleaved sample was applied again to the IMAC column to separate cleaved from uncleaved protein. Proteins were concentrated and loaded on a SEC HiLoad Superdex 75 16/60 column (GE Healthcare) that had been equilibrated in 20 mM Tris-HCl, pH 7.5, supplemented with 150 mM NaCl and 1 mM DTT. The fractions of the main peak were pooled and concentrated to 10 ml protein. Protein quality and identity were assessed by SDS–PAGE and mass spectrometry, respectively.

Protein expression and purification for *in vitro* assays. All proteins used for *TIRFM* (human CAMSAP1 CKK (residues 1474–1613) and mouse CAMSAP3 CKK (residues 1112–1252)) were cloned into the pET28a vector. After purification with Ni–NTA resin (Qiagen), proteins were further purified on a MonoS ion-exchange column and a Superose 6 gel-filtration column (GE Healthcare). Purified proteins were concentrated to ~20 mg/ml in BRB20 buffer.

Protein expression and purification for crystallization were performed on a modified pTT5 expression vector (Addgene no. 44006 (ref. 54)) bearing Strept-GFP or Strept-Snap tags at either the N or the C terminus of the protein. The cDNA sequences encoding *T. thermophila* CKK, *T. vaginalis* and *N. gruberi* CKK were cloned into the pET51a vector (Addgene no. 44006 (ref. 54)) by positive-selection methods. The resulting full-length protein of commonly misidentified cell lines maintained by ICLAC and NCBI BioSample, was not authenticated and was negative for mycoplasma contamination.

**Total internal reflection fluorescence microscopy (TIRFM).** TIRFM was performed on an inverted research microscope Nikon Eclipse Ti-E (Nikon) with the perfect focus system (PFS) (Nikon), equipped with a Nikon CFI Apo TIRF 100×/1.49 NA oil objective (Nikon) and a Photometrics Evolve 512 EMCCD (Roper Scientific) camera, and controlled with MetaMorph 7.7 software (Molecular Devices). Images were projected onto the chip of an Evolve 512 camera with an intermediate 2.5× lens (Nikon C mount adapter 2.5×). To keep in vitro samples at 30 °C, we used an INUBG2E-ZILCS (Tokai Hit) stage-top incubator. For excitation, we used 491-nm/100-mW Stradus (Vortran), 561-nm/100-mW live (Cobolt) and 642-nm/110-mW Stradus (Vortran) lasers. We used an ET-mCherry 49008 filter set (Chroma) for imaging proteins tagged with GFP; an ET-mCherry 49008 filter set (Chroma) for imaging X-rhodamine-labeled tubulin mCherry-EB3; and an ET-405/488/561/647 filter set for imaging SNAP-Alexa467. For simultaneous imaging of green and red fluorescence, we used a triple-band TIRF polychrome filter (ZET405/488/561rps, Chroma) and triple-band laser emission filter (ZET405/488/561m, Chroma), mounted in the metal cube (91032, Chroma) together with an Optosplit III beam splitter (Cairn Research) equipped with a double-emission-filter cube configured with ET525/50m, ET630/75m and TS85PLXR (Chroma) filters. We used sequential acquisition for triple-color imaging experiments.

**In vitro MT assays.** The *in vitro* assays with dynamic MTs were performed under the same conditions as described previously18. Briefly, after coverslips were functionalized by sequential incubation with 0.2 mg/ml PLL–PEG–biontin (Susos) and 1 mg/ml neutravidin (Invitrogen) in MR800 buffer, GMPcPP-stabilized MT seeds were attached to the coverslips through biotin–neutravidin interactions. Flow chambers were further blocked with 1 mg/ml e-casein. The reaction mix with purified proteins (MR800 buffer supplemented with 20 µM porcine brain tubulin, 0.5 µM X-rhodamine–tubulin, 75 mM KCl, 1 mM GTP, 0.2 mg/ml κ-casein, 0.1% methyelulose and oxygen scavenger mix (50 mM glucose, 400 µg/ml glucose oxidase, 200 µg/ml catalase and 4 mM DTT)) were added to the flow chamber after centrifugation. The flow chamber was sealed with vacuum grease, and dynamic MTs were imaged immediately at 30 °C with a TIRF microscope. The conditions for the MT-depolymerization assay were essentially the same as in the assays with dynamic MTs, except that tubulin proteins were not included, and the reaction mix was optimized to image MT depolymerization (MR800 buffer supplemented with 100 mM KCl, 1 mM GTP, 1 mM ATP, 0.2 mg/ml κ-casein and oxygen scavenger mix). All tubulin products were from Cytoskeleton.

Quantification of the intensity of the wild-type and mutant CAMSAP1mini on dynamic MTs. To quantify the minus-end and lattice intensity of CAMSAP1mini in a time-lapse movie, kymographs were generated in ImageJ with the KymoResliceWide plug-in. The minus-end positions were marked by 5-pixel-wide linear ROIs corresponding to CAMSAP1mini signals. The maximum intensity within the 5-pixel region along the spatial axis of the kymograph was measured with a macro written in ImageJ.

**Determination of the CAMSAP1 position on MT ends.** To determine the position of CAMSAP1mini relative to the MT minus end, we simultaneously imaged CAMSAP1mini–GFP and X-rhodamine-labeled GMPCPP-stabilized MTs by using a beam splitter. Spatial registration between two channels was performed with a B-spline point-based transform (MathWorks File Exchange: 20057-b-spline-grid–image-and-point-based-registration), by using images of...
was incubated on ice for 5 min. MT polymerization was started by transfer to MgCl₂ and 1 mM DTT) to 1 mg/ml. After the addition of 0.5 mM GTP, the sample was pelleted using a Beckman SW28 1.4 rotor for 30 min at 25 °C, and the supernatant was removed. The pellet was resuspended in SDS sample buffer.

**Molecular dynamics simulations**. 4,000 cycles of energy minimization without any positional restraints were performed on the refined structural complex. Two consecutive molecular dynamics simulations runs of 10 ps and 200 ps were then used to increase the temperature from 100 K to 300 K and to equilibrate the systems at 300 K. Four replicate production runs of 40 ns each were then performed starting from the equilibrated system configurations. The simulations were run at constant temperature (300 K) and constant pressure (1 atm) with a 2-fs time step. Periodic boundary conditions and full particle-mesh Ewald electrostatics were used. A 12-Å cutoff value was applied to truncate the nonbonded interactions. The SHAKE algorithm was used to constrain the covalent bonds formed by hydrogen atoms. Each replicate simulation was run with different random starting velocities. All calculations were performed with the AMBER 12 package.

**MM/GBSA calculations**. Molecular mechanics with generalized Born and surface-area solvation (MM/GBSA) calculations were performed with the GB0EEG model in AMBER 12 (ref. 71). For each molecular dynamics simulation, pairwise electrostatic and solvation terms were scaled by the average number of CKK domain–tubulin dimers contacts (51 contacts) and averaged over four replica simulations.

**NMR sample preparation and experiments**. Uniformly 13C-15N-labeled variants of CKK were produced in E. coli strain Rosetta 2 in M9 minimum medium containing 25 µg/ml kanamycin and 35 µg/ml chloramphenicol. The cells were induced with 0.3 mM IPTG at 25 °C overnight after the OD₆₀₀ reached 0.6. Proteins were purified as described above with phosphate buffer instead of HEPES buffer. After purification, proteins were loaded onto a SEC HiLoad
Superdex 75 26/60 column (GE Healthcare), which was equilibrated in 40 mM phosphate buffer with 150 mM NaCl and 1 mM DTT, pH 7.0. Proteins were then concentrated and used for solution-state NMR measurements with 5% D₂O supplementation or for ssNMR sample preparation.

For ssNMR experiments, [¹⁵C,¹³N]CAMSAP1 CKK–MT complexes were prepared. 20 mg of lyophilized tubulin was first dissolved in BRB80 buffer to a final concentration of 2 mg/ml. Tubulin was then polymerized with the addition of 20 µM paclitaxel for 30 min at 37 °C. Paclitaxel-stabilized MTs were centrifuged at 55,000 r.p.m. (Beckman TLA-55 rotor) at 30 °C for 30 min. The pellet was resuspended in warm BRB80 buffer, and labeled CKK domain was added to a final concentration of 65.3 µM (4:1 CKK/tubulin). The mixture was incubated at 37 °C for 30 min and then centrifuged at 55,000 r.p.m. (Beckman TLA-55 rotor) at 30 °C for 30 min. The pellet was washed with phosphate buffer without disturbing the pellet. Finally, the pellet was transferred and packed into a 3.2-mm rotor.

Resonance assignments were obtained from previous results (PDB 1UGJ), and additional solution-state NMR experiments on free CKK were recorded on a 600-MHz spectrometer (Bruker Biospin) to assign missing residues (2D HSOQCs, 3D HNCA, HNCO, HN(C)CO, CBCA(CO)NH, HAHB(CO)NH and hCCH-DIPSI) ssNMR experiments involved 2D NCA and CC proton-driven spin–diffusion (PDSSD) experiments (temperature 260 K, MAS rate 14 kHz) as well as additional 2Q–1Q experiments (temperature 268 K, MAS 10 kHz). Mixing schemes used SPECIFIC–CP transfers and SPC5 as well as SPC5 (ref. 73) and spin diffusion under weak coupling conditions for longer PDSSD mixing times. Data were recorded on a 950-MHz standard-bore spectrometer (Bruker Biospin) equipped with a 3.2-mm triple-channel MAS HCN probe. ssNMR data were analyzed by ¹H–¹C CP over time.

Data availability. The structure of CKK3 core has been deposited in the Protein Data Bank (PDB) under accession code PDB 5ZNN. The CKK–MT models along with the side-chain and electron density maps have been deposited in the PDB and Electron Microscopy Data Bank, respectively: CAMSAP1 CKK–MT, PDB 5M54 and EMD–4156; CAMSAP1 N1492 CKK–MT, PDB 5SMC and EMD–3444; and CAMSAP3 CKK–MT, PDB 5SM0 and EMD–4154). NMR data have been deposited in the Biological Magnetic Resonance Bank under entry ID 27234. Source data for graphs in Figures 1 and 3–6 and Supplementary Figures 1.4 and 7–9 can be found in Supplementary Table 2. All data that support the conclusions are available from the authors on request, and/or available in the article itself. A Life Sciences Reporting Summary for this paper is available.
# Experimental design

1. **Sample size**
   
   Describe how sample size was determined.  
   - The sample size was chosen based on common practice in the field

2. **Data exclusions**
   
   Describe any data exclusions.  
   - Not applicable

3. **Replication**
   
   Describe whether the experimental findings were reliably reproduced.  
   - All replication attempts were successful

4. **Randomization**
   
   Describe how samples/organisms/participants were allocated into experimental groups.  
   - Not applicable

5. **Blinding**
   
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.  
   - Not applicable

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

### Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- **Confirmed**
  - The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
  - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
  - A statement indicating how many times each experiment was replicated

- **Not Confirmed**
  - The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
  - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
  - The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
  - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
  - Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

not applicable

Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

ATCC

b. Describe the method of cell line authentication used.

cell lines were not authenticated

c. Report whether the cell lines were tested for mycoplasma contamination.

yes

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

no

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

1. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

not applicable

Policy information about studies involving human research participants

2. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

not applicable