Kinesin-8-specific loop-2 controls the dual activities of the motor domain according to tubulin protofilament shape

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Kinesin-8s are dual-activity motor proteins that can move processively on microtubules and depolymerize microtubule plus-ends, but their mechanism of combining these distinct activities remains unclear. We addressed this by obtaining cryo-EM structures (2.6–3.9 Å) of Candida albicans Kip3 in different catalytic states on the microtubule lattice and on a curved microtubule end mimic. We also determined a crystal structure of microtubule-unbound CaKip3-ADP (2.0 Å) and analyzed the biochemical activity of CaKip3 and kinesin-1 mutants. These data reveal that the microtubule depolymerization activity of kinesin-8 originates from conformational changes of its motor core that are amplified by dynamic contacts between its extended loop-2 and tubulin. On curved microtubule ends, loop-1 inserts into preceding motor domains, forming head-to-tail arrays of kinesin-8s that complement loop-2 contacts with curved tubulin and assist depolymerization. On straight tubulin protofilaments in the microtubule lattice, loop-2-tubulin contacts inhibit conformational changes in the motor core, but in the ADP-Pi state these contacts are relaxed, allowing neck-linker docking for motility. We propose that these tubulin shape-induced alternations between pro-microtubule-depolymerization and pro-motility kinesin states, regulated by loop-2, are the key to the dual activity of kinesin-8 motors.

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Members of the kinesin-8 family are unique, as they often integrate the two enzymatic activities of pro- cessive microtubule plus-end-directed motility and microtubule depolymerization into a single ~40 kDa motor domain. These activities allow kinesin-8s to localize to the ends of cytoplasmic or spindle microtubules, where they can regulate mitotic spindle size, spindle position, and chromosomal congression. In eukaryotes in which these motor proteins have been studied, the motile and depolymerization activities are produced by their N-terminal motor domain, while their C-terminal tail has an additional microtubule-binding site that helps regulate the functions of the motor domain. A lack of high-resolution structures capturing a kinesin-8 motor domain at each major step of its motile and microtubule depolymerization cycles has limited our understanding of the relationship between these two catalytic cycles and the structural elements of the motor domain that control them.

At this time, our knowledge of the catalytic cycles of kinesins is limited to motile kinesins that are incapable of microtubule depolymerization, and to microtubule-depolymerizing kinesins that are not motile. In motile kinesins, such as kinesin-1 and kinesin-3, microtubule lattice binding by one of their two motor domains opens the nucleotide-binding pocket, allowing ADP release. Subsequent entry of ATP initiates closing of the nucleotide-binding pocket and docking of a short peptide, known as the neck-linker, along the side of the motor domain. Neck-linker docking in the microtubule-bound motor domain propels the second, microtubule-unbound, motor domain towards the microtubule plus-end, enabling a single stepping event. In the non-motile microtubule-depolymerizing kinesin-13s, which move on microtubules by diffusion, ATP binding does not induce closure of the nucleotide-binding pocket on the microtubule lattice. Only when kinesin-13 is bound to curved αβ-tubulin prototifilaments found at microtubule ends does the nucleotide-binding pocket close in the presence of ATP. In this case, closing the nucleotide-binding pocket allows kinesin-13-specific structural elements to promote further tubulin bending, leading to microtubule depolymerization. The motility and depolymerization cycles in purely motile and purely depolymerizing kinesins are thus very different. The features of the kinesin-8 cycle that allow their motors to combine both activities remain unknown.

An important gap in our understanding of the kinesin-8 depolymerization mechanism is the lack of a kinesin-8 structure bound to curved tubulin. Functional studies of Saccharomyces cerevisiae kinesin-8 Kip3 have proposed that upon reaching the curved conformation of tubulin at the plus-end, the motor domain experiences a conformational switch that suppresses ATP hydrolysis. As a result, prolonged, tight interactions between kinesin-8 and the microtubule plus-end stabilize prototifilament curvature and promote microtubule depolymerization. The suggested mechanism for this tubulin shape-dependent ATPase switch involves formation of an interaction between loop-11 of the motor domain and an aspartate residue on helix-3 of α-tubulin (Asp118—yeast tubulin, Asp116—bovine or porcine tubulin). Subsequent molecular dynamics studies identified an arginine within loop-11 (Arg351–ScKip3) as a potential candidate residue that interacts with α-tubulin Asp118/116 in the curved conformation. Studies on the human kinesin-8s KIF18A and KIF19A have also implicated separate regions of the microtubule-binding interface, namely loop-2 and loop-8, as elements that are likely involved in stabilizing curved tubulin prototifilaments. Ultimately, a structure of a kinesin-8 motor bound to curved tubulin is needed to elucidate how discrete parts of the motor domain contribute to kinesin-8-mediated microtubule depolymerization.

Mechanistically, it is well-established that some kinesin-8s operate cooperatively to induce microtubule depolymerization. Both in vitro and in vivo, a build-up of motors at the plus-end is required for effective disassembly of microtubule polymers. As long microtubules accumulate more motors than shorter microtubules, longer microtubules are depolymerized faster—an effect termed length-dependent depolymerization. The structural basis underlying this cooperative length-dependent microtubule depolymerization is not understood. It is also not clear if and how kinesin-8s physically interact to collectively produce forces needed to dissociate tubulin dimers.

To understand the mechanistic basis for the dual-functionality of kinesin-8, we obtained seven cryo-EM structures of Candida albicans Kip3 bound to either the microtubule lattice or curved microtubule end mimics, and a microtubule-unbound CaKip3 X-ray structure. We also performed biochemical experiments on CaKip3 and human kinesin-1 constructs with their loop-2 region swapped or loop-1 region truncated. On straight prototifilaments within the central microtubule lattice, we observed that loop-2 forms extensive contacts with α-tubulin that temporarily restrict the ATP-bound motor in a non-motile, open conformation. Only in the post-ATP-hydrolysis state of microtubule-bound CaKip3 are the loop-2-tubulin interactions relaxed enough to allow nucleotide-binding pocket closure and neck-linker docking, producing a pro-motility state. On curved prototifilaments, we observe that loop-2 retracts and promotes closing of the nucleotide-binding pocket around ATP, while loop-1 inserts into the preceding motor domain, forming a pro-depolymerization state. When we replaced CaKip3’s loop-2 with the short loop-2 of kinesin-1, and truncated loop-1, the microtubule depolymerization activity of CaKip3 decreased substantially. Alternatively, swapping loop-2 from kinesin-1 into CaKip3 increased the microtubule-stimulated ATPase and microtubule-gliding speed of GaKip3. These findings show that the kinesin-8-specific extended loop-2 is a central element that coordinates the motility and depolymerase activities of kinesin-8 in accord with the shape of tubulin it binds and that GaKip3’s microtubule depolymerization activity involves loop-2-amplified conformational changes of its motor domain and inter-motor domain contacts through loop-1. We also show that the unconventional conformations of CaKip3’s ATPase cycle on microtubules, modulated by loop-2, bear several similarities to kinesin-13s.

Results

Structures of CaKip3 reveal a unique ATPase cycle. The pathogenic yeast Candida albicans expresses a single kinesin-8 homolog, dubbed CaKip3, whose motor domain shares 60% sequence identity with that of S. cerevisiae Kip3 (Supplementary Fig. 1a). C. albicans cells lacking this protein exhibit striking growth defects that are indicative of cell cycle arrest and are unable to form hyphae under conditions that induce filamentous growth (Supplementary Fig. 2a–d). Similar to observations in other eukaryotes, loss of kinesin-8 activity in C. albicans produces unusually long and more numerous astral microtubules, long mitotic spindles with a “fishhook” morphology, and delays in the timing of anaphase entry (Supplementary Fig. 2e–j).

These effects of CaKip3 loss are consistent with a functional role in nuclear positioning and chromosome segregation by regulating microtubule length. We therefore used CaKip3 as a model to understand the molecular mechanisms of motility and microtubule depolymerization by kinesin-8s.

We solved high-resolution structures of the CaKip3 motor domain at key intermediates in its motile cycle, as well as its structure on curved αβ-tubulin rings that mimic prototifilaments at a depolymerizing microtubule plus-end (Fig. 1). To visualize the
Fig. 1 Structures of microtubule-unbound, microtubule-bound, and curved tubulin-bound CaKip3. 

a. Cartoon representation of catalytic intermediates of CaKip3’s motility and microtubule depolymerization cycles. Bottom—Example of a CaKip3-decorated microtubule (MT-CaKip3-MDC-ANP) cryo-EM map and the full CaKip3-decorated dolastatin-tubulin-ring cryo-EM map. 

b. X-ray crystallographic density of CaKip3-MDN in the ADP state. 

c-e Cryo-EM maps of microtubule-bound CaKip3-MDC in the APO, AMP-PNP, and ADP-AlF₃ nucleotide states. 

f. Cryo-EM maps of curved tubulin-bound CaKip3-MDC in the AMP-PNP state. Map surfaces are colored regionally according to the segment of the fitted protein model they enclose: α-tubulin (cornflower blue), β-tubulin (sky blue), kinesin motor core (orange), Switch I loop (forest green), loop-11 of Switch II (magenta), neck-linker (red), nucleotide (tomato), loop-1 (yellow), loop-2 (lime green), helix-0 (dark blue). The figure was prepared with UCSF ChimeraX. 

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microtubule-unbound ADP state of CaKip3, we determined a 2.0 Å X-ray crystal structure of a construct containing the motor domain and a slightly truncated neck-linker (CaKip3-MDN, residues 1–436) (Fig. 1b and Supplementary Fig. 1b). Using a longer CaKip3 construct containing the motor domain, neck-linker, and the first predicted coiled-coil-forming domain (CaKip3-MDC, residues 1–482) (Supplementary Fig. 1b), we obtained 2.6–3.3 Å (Supplementary Fig. 3) cryo-electron microscopy (cryo-EM) structures of microtubule-bound CaKip3 in: (1) a nucleotide-free state (Fig. 1c), (2) an ATP-like state using the non-hydrolysable ATP analog AMP-PNP (Fig. 1d), and (3) an ADP-Pi-like transition state using ADP-AlF₄⁻ (Fig. 1e). To view CaKip3’s interactions with curved αβ-tubulin protifilaments, we obtained a 3.9 Å cryo-EM structure of CaKip3-MDC bound to dolastatin-stabilized curved tubulin rings and AMP-PNP (Fig. 1f and Supplementary Fig. 4)⁴³. The data processing and model refinement statistics for the X-ray crystal structure and all cryo-EM structures are reported in Tables 1 and 2, respectively.

Comparing the different CaKip3 structures reveals that the conformational changes experienced by the kinesin-8 motor domain core during microtubule binding in relation to its ATP hydrolysis cycle are different from purely motile kinesins. The CaKip3-MDN-ADP crystal structure has a partially open nucleotide pocket and an undocked neck-linker (Fig. 1b and Supplementary Figs. 5 and 6). The Switch I region (loop-9) is well-defined but angled away from ADP, while the Switch II region (loop-11) that coordinates microtubule sensing and nucleotide binding is mostly disordered. When CaKip3-MDC binds the microtubule (MT-CaKip3-MDC-APO), theminus subdomain rotates relative to the plus subdomain, opening the nucleotide-binding pocket further (Figs. 1c and 2a; Supplementary Movie 1). Here, the full length of loop-11 is visible and interacts with helices 3’, 3, 11’, and 12 of α-tubulin (Supplementary Fig. 12a). Unexpectedly, binding of ATP to the MT-CaKip3-MDC complex, as mimicked by AMP-PNP, fails to induce a “pro-motility” conformational change (i.e., nucleotide-binding pocket closure and neck-linker docking on the microtubule lattice) even though the CaKip3-MDC construct includes the full neck-linker (Figs. 1d and 2b; Supplementary Figs. 6 and 7, Supplementary Movie 1). The cryo-EM map of the MT-CaKip3-MDC-ANP complex clearly shows that the nucleotide-binding pocket is open, and the neck-linker is undocked, similar to MT-CaKip3-MDC-APO (compare Fig. 1c, d). This is striking because all microtubule-bound structures of motile kinesins with a full neck-linker that is not pulled backward by a trailing head exhibit a closed nucleotide-binding pocket in the ATP-bound state (as mimicked by AMP-PNP) (compare Supplementary Fig. 7b, c, and j)③１４４－４６. In this regard, CaKip3 is even more akin to depolymerizing kinesins than to motile kinesins. Only in the post-ATP-hydrolysis state (MT-CaKip3-MDC-AAF) does the microtubule-bound CaKip3 motor domain adopt the pro-motility conformation with a closed nucleotide-binding pocket and docked neck-linker, allowing CaKip3 to complete its motile cycle (Figs. 1e and 2c; Supplementary Fig. 3b, Supplementary Movie 1).

Our library of structures defines the subdomain motions associated with nucleotide-binding pocket closure in CaKip3. When the nucleotide-binding pocket closes in the MT-CaKip3-MDC-AAF complex, the minus subdomain has a strong rotational component in the plane perpendicular to the microtubule axis (Fig. 2c and Supplementary Movie 1). We observe these same conformational changes in CaKip3-MDC when it is bound to curved tubulin rings and AMP-PNP (CT-CaKip3-MDC-ANP) (Fig. 2d and Supplementary Movie 1). When viewed from the microtubule minus end, it is evident that closure of the nucleotide-binding pocket is associated with a counter-clockwise rotation of the minus subdomain and a clockwise rotation of the Switch I and II regions (Fig. 2c, d; left panels, Supplementary Movie 1). Movement of these subdomains in the plane parallel to the microtubule axis results in a relative displacement of the tubulin-interacting regions to better fit curved tubulin, which is similar overall to what was reported in kinesin-13 (Supplementary Fig. 7h)②４. In the MT-CaKip3-MDC-AAF and CT-CaKip3-MDC-ANP complexes, the displacement of tubulin-interacting regions relative to β-tubulin is greatest at the kinesin-8-specific elongated loop-2 that extends the kinesin-tubulin interface (Fig. 2c, d; right panels, Supplementary Movie 1). Prominent as well is movement of the loop-8 lobe toward helix-12 of β-tubulin. Unexpectedly, we also observe a small rotation of α-tubulin relative to β-tubulin in the MT-CaKip3-MDC-AAF complex, giving a slight outward curvature to each CaKip3-bound αβ-tubulin subunit in the microtubule lattice when the nucleotide-binding pocket of CaKip3 is closed (Fig. 2c; right panels—blue arrows on α-tubulin, Supplementary Movie 1).

### Table 1 X-ray diffraction data collection, refinement, and validation statistics.

| Data collection                  | CaKip3-MDN-ADP (PDB 7LFF) |
|---------------------------------|----------------------------|
| Wavelength                      | 0.9795                     |
| Resolution range (Å)            | 45.91-2.01 (2.08-2.01)     |
| Space group                     | P1                         |
| Unit-cell                       | a, b, c (Å)                 |
| a, b, c (Å)                     | 43.99, 52.68, 86.75         |
| a, b, c (°)                     | 86.9, 79.96, 89.924         |
| Unique reflections              | 49660 (4913)                |
| Multiplicity                    | 2.7 (2.69)                  |
| Completeness (%)                | 97.09 (96.7)                |
| Average I(0)                    | 4.6 (0.3)                   |
| Average C1/2                    | 0.995 (0.149)               |
| Wilson B-factor (Å²)            | 46.5                       |
| Rmerge (%)                      | 11.4                       |
| Rp (%)                          | 8.2                        |
| Rfree (%)                       | 14.1                       |
| Number of reflections           | 49471 (4807)                |
| Completeness (%)                | 97.1 (92.79)                |
| Rwork (%)                       | 23.23 (48.59)               |
| Rfree (%)                       | 26.76 (51.13)               |
| RMSD bond lengths (Å)           | 0.0049                     |
| RMSD bond angles (°)            | 1.0467                     |
| Biso (Å²)/protein atoms         | 60.07/5273                 |
| Biso (Å²)/ligand atoms          | 46.37/56                   |
| Biso (Å²)/water molecules       | 53.09/142                  |
| Ramachandran favored (%)        | 95.05                      |
| Ramachandran allowed (%)        | 3.86                       |
| Ramachandran outliers (%)       | 1.08                       |

Statistics for the highest-resolution shell are shown in parentheses.
Table 2 Cryo-EM data collection, refinement, and validation statistics (1/2).

|                      | MT-CoKip3-MDC AMP-PNP (EMDB-26074) (PDB 7TQX) | MT-CoKip3-MDC ADP-AIF₁ (EMDB-26075) (PDB 7TQY) | MT-CoKip3-MDC APO (EMDB-26076) (PDB 7TQZ) | MT-CoKip3-MDN AMP-PNP (EMDB-26077) (PDB 7TR0) |
|----------------------|-----------------------------------------------|-----------------------------------------------|--------------------------------------------|-----------------------------------------------|
| **Data collection and processing** |                                               |                                               |                                            |                                               |
| Magnification (actual) | 60,606                                        | 58,893                                        | 60,606                                     | 60,241                                        |
| Voltage (kV)          | 300                                           | 300                                           | 300                                        | 300                                           |
| Electron exposure (e⁻/Å²) | 70.4                                          | 63.3                                          | 71.1                                       | 69.6                                          |
| Defocus range (µm)    | −0.8; −2.1                                    | −0.8; −1.7                                    | −0.9; −1.8                                 | −0.9; −1.7                                    |
| Pixel size (Å)        | 0.825                                         | 0.849                                         | 0.825                                      | 0.830                                         |
| Symmetry imposed      | Helical                                       | Helical                                       | Helical                                    | Helical                                       |
| Rise (Å)              | 5.56                                          | 5.44                                          | 5.55                                       | 5.56                                          |
| Twist (deg)           | 168.09                                        | 168.07                                        | 168.09                                     | 168.09                                        |
| **Particle images identified as 15R symmetry (no.)** | 12,120                                        | 23,783                                        | 16,061                                     | 77,728                                        |
| **Particle images in helical reconstruction (no.)** | 12,120                                        | 23,783                                        | 16,061                                     | 77,728                                        |
| **Overall resolution (Å)** | 2.8                                           | 2.6                                           | 2.7                                        | 2.7                                           |
| **FSC threshold**     | 0.143                                         | 0.143                                         | 0.143                                      | 0.143                                         |
| **Kinesin resolution (Å)** | 3.1                                           | 2.9                                           | 3.0                                        | 2.9                                           |
| **Tubulin resolution (Å)** | 2.8                                           | 2.6                                           | 2.7                                        | 2.7                                           |
| **Re**                |                                               |                                               |                                            |                                               |
| **Model composition** |                                               |                                               |                                            |                                               |
| Non-hydrogen atoms    | 9824                                          | 9892                                          | 9791                                       | 9836                                          |
| Protein residues      | 1229                                          | 1235                                          | 1229                                       | 1229                                          |
| Ligands               | 6                                             | 7                                             | 4                                          | 5                                             |
| **R.m.s. deviations** |                                               |                                               |                                            |                                               |
| Bond lengths (Å)      | 0.0054                                        | 0.0054                                        | 0.0061                                     | 0.0058                                        |
| Bond angles (°)       | 1.14                                          | 1.17                                          | 1.19                                       | 1.17                                          |
| **Validation**        |                                               |                                               |                                            |                                               |
| MolProbity score      | 1.48                                          | 1.36                                          | 1.48                                       | 1.36                                          |
| Clashscore            | 4.28                                          | 3.23                                          | 5.01                                       | 4.27                                          |
| Poor rotamers (%)     | 0.47                                          | 1.12                                          | 0.94                                       | 0.66                                          |
| **Ramachandran plot** |                                               |                                               |                                            |                                               |
| Favored (%)           | 96.07                                         | 96.66                                         | 96.64                                      | 97.13                                         |
| Allowed (%)           | 3.93                                          | 3.34                                          | 3.36                                       | 2.87                                          |
| Disallowed (%)        | 0.00                                          | 0.00                                          | 0.00                                       | 0.00                                          |
| **Refinement**        |                                               |                                               |                                            |                                               |
| **Model composition** |                                               |                                               |                                            |                                               |
| Non-hydrogen atoms    | 9708                                          | 9588                                          | 9588                                       | 10,224                                        |
| Protein residues      | 1216                                          | 1207                                          | 1207                                       | 1289                                          |
| Ligands               | 6                                             | 4                                             | 4                                          | 4                                             |

Table 2 Cryo-EM data collection, refinement, and validation statistics (2/2).

|                      | MT-CoKip3-MDN₁₂-HKHC AMP-PNP (EMDB-26078) (PDB 7TR1) | MT-CoKip3-MDN₁₂-HKHC APO (EMDB-26079) (PDB 7TR2) | CT-CoKip3-MDC AMP-PNP (EMDB-26080) (PDB 7TR3) |
|----------------------|-----------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| **Data collection and processing** |                                               |                                               |                                               |
| Magnification (actual) | 45,872                                           | 58,893                                         | 46,168                                         |
| Voltage (kV)          | 300                                               | 300                                            | 300                                            |
| Electron exposure (e⁻/Å²) | 64.0                                           | 64.6                                           | 64.0                                           |
| Defocus range (µm)    | −0.8; −1.9                                       | −0.8; −1.7                                     | −0.9; −3.2                                     |
| Pixel size (Å)        | 1.090                                             | 0.849                                          | 1.083                                          |
| Symmetry imposed      | Helical                                           | Helical                                        | C14                                            |
| Rise (Å)              | 5.54                                              | 5.55                                           | 5.45                                           |
| Twist (deg)           | 168.08                                            | 168.09                                         | 168.09                                         |
| **Particle images identified as 15R/C14 symmetry (no.)** | 37,146                                          | 12,219                                         | 37,920                                         |
| **Particle images in helical/C14 reconstruction (no.)** | 37,146                                          | 12,219                                         | 37,920                                         |
| **Overall resolution (Å)** | 3.1                                              | 3.0                                            | 3.9                                            |
| **FSC threshold**     | 0.143                                             | 0.143                                          | 0.143                                          |
| **Kinesin resolution (Å)** | 3.4                                              | 3.4                                            | 3.9                                            |
| **Tubulin resolution (Å)** | 3.0                                              | 3.0                                            | 3.9                                            |
| **Refinement**        |                                                   |                                                |                                                |
| **Model composition** |                                                   |                                                |                                                |
| Non-hydrogen atoms    | 9708                                              | 9588                                           | 10,224                                         |
| Protein residues      | 1216                                              | 1207                                           | 1289                                           |
| Ligands               | 6                                                 | 4                                              | 4                                              |
(Fig. 3c, d; left panels). This section interacts with helix-12 and a loop between helix-7 and the β7 strand of α-tubulin via residues Phe136, Ser139, Arg140, and one of the two visible conformations of the side chain of His144 (Fig. 3c, d; right panels). These loop-2-tubulin interactions are more numerous than the loop-2 contacts made by kinesin-13s,24, which are essential for kinesin-13’s microtubule depolymerization activity.24,26,27,47 Several of the CaKip3 loop-2-tubulin helix-12 interactions are electrostatic. For example, Arg140 is close enough to α-tubulin to hydrogen bond with Glu420 and Asp424 in helix-12. These contacts confirm earlier assumptions that loop-2 contributes positively charged residues that associate with the negatively charged surface of α-tubulin.24,48 We observe similar loop-2-tubulin contacts, and overall motor domain conformation in the two microtubule-bound AMP-PNP complexes, MT-CaKip3-MDC-ANP and MT-CaKip3-MDN-ANP (Fig. 4c).

In the MT-CaKip3-MDC-AAF and CT-CaKip3-MDC-ANP complexes, the loop-2 density is much weaker, indicating a more mobile loop structure. We could trace the average path of loop-2 in these complexes by applying a low-pass filter of 9 and 7 Å, respectively (Fig. 3e, f; left panels). In CT-CaKip3-MDC-ANP, stronger density is visible along α-tubulin, especially near the loop-2 tip around α-tubulin residue Y262, compared to MT-CaKip3-MDC-AAF, indicating MT-CaKip3-MDC-AAF has the most mobile loop-2. In both complexes, much of loop-2 is shifted away from helix-12 of α-tubulin to a degree that many of the bonds observed in the APO and AMP-PNP states do not form (Fig. 3e, f; right panels). These changes in the conformation of loop-2 are accompanied by movement of the β1 strand and helix-6 of CaKip3 away from α-tubulin, opening the space needed for the neck-linker to dock against the motor core (Fig. 3e, f; left panels).

Loop-2 modulates CaKip3’s catalytic function according to tubulin protofilament shape. The fact that microtubule-bound CaKip3 showed more extensive loop-2–tubulin interactions in the open APO- and ANP-states compared to the closed AAF-state suggests that the interaction of loop-2 with the microtubule lattice restricts rotation of the minus subdomain of the motor domain (Figs. 2 and 3). To investigate this hypothesis experimentally, we designed and expressed a mutant version of the CaKip3-MDN construct, whose long loop-2 was replaced with the short loop-2 sequence found in human kinesin-1, HsKIF5B (Supplementary Fig. 1b; CaKip3-MDN12-HsKHC), which does not contact α-tubulin in any of the available microtubule-bound or tubulin-bound kinesin-1 structures.22,50,51 In line with our hypothesis, the cryo-EM structure of this CaKip3 construct bound to microtubules has a docked neck-linker and closed nucleotide pocket in the ATP-like state (Fig. 4b; MT-CaKip3-MDN12-HsKHC-AMP-PNP), unlike the CaKip3-MDN construct with a full loop-2 (Fig. 4c; MT-CaKip3-MDN-ANP). These data show that the kinesin-8-specific elongated loop-2 acts as a tubulin tether on the microtubule lattice pre-ATP hydrolysis, preventing the motor from closing, and that removing the CaKip3 loop-2 residues that interact with the microtubule lattice enable the motor domain to form the closed conformation in the ATP-bound state. The MT-CaKip3-MDN12-HsKHC-ANP structure also suggests that loop-2 of kinesin-8 is directly involved in inducing tubulin curvature because the tubulin conformational changes in this structure are less extensive than in the MT-CaKip3-MDC-AAF structure (Fig. 4d, Supplementary Fig. 7d, f; compare blue arrows on α-tubulin). To learn how the loop-2–tubulin interactions and motor domain conformations observed for microtubule-bound CaKip3 relate to its motile activity, we assessed the microtubule-gliding velocity of the CaKip3-MDN and CaKip3-MDN12-HsKHC motors. The gliding data shows that elimination of the CaKip3 loop-2 increased the microtubule-gliding speed of the motor by over threefold (Fig. 4e, g and Supplementary Fig. 8a). Conversely, when loop-2 of CaKip3 was grafted into an equivalent kinesin-1 construct (HsKHC-MDN12-CaKip3), the microtubule-gliding speed slowed twofold (Fig. 4f, g and Supplementary Fig. 8a). One explanation for these results is that the interaction of loop-2 with straight αβ-tubulin protofilaments delays hydrolysis of ATP by the motor domain, thereby limiting the neck-linker docking event that enables forward stepping. An alternative, but not mutually exclusive explanation, is that the loop-2–tubulin interaction limits the stepping velocity of CaKip3 by increasing microtubule affinity. To distinguish between these two possibilities, we assessed the microtubule-stimulated ATPase activity of CaKip3-MDN, CaKip3-MDN12-HsKHC-HsKHC-MDN, and HsKHC-MDN12-CaKip3 at steady state, and then measured the microtubule affinity of these proteins using a sedimentation assay. The data from the ATPase experiments show that CaKip3-MDN12-HsKHC is ~1.6-fold faster at hydrolyzing ATP on microtubules than CaKip3-MDN (Fig. 4h, j). Likewise, the microtubule-stimulated ATP turnover by HsKHC-MDN12-CaKip3 was almost twofold slower than HsKHC-MDN (Fig. 4i, j). Our microtubule-binding
data showed that CaKip3-MDN has ~35-fold higher affinity for microtubules than CaKip3-MDN_{12-HsKHCh} when AMP-PNP occupies the nucleotide-binding pocket (Fig. 4k, m and Supplementary Fig. 8b). The HsKHCh-MDN_{12-CaKip3} construct also had a sevenfold greater affinity for the microtubule in the ATP-like state compared to HsKHCh-MDN (Fig. 4l, m and Supplementary Fig. 8c). These results demonstrate that the extended loop-2 of CaKip3 both increases the lifetime of the microtubule-bound state and limits ATPase activity of the motor domain on the microtubule lattice.

To understand the functional effects of the loop-2-tubulin interactions observed in the curved tubulin-bound structure of CaKip3, we compared the dolastatin-stabilized tubulin-ring (D-ring)-stimulated ATPase activities of CaKip3-MDN and CaKip3-MDN_{12-HsKHCh}. Our data shows that ATP hydrolysis by CaKip3-MDN_{12-HsKHCh} was twofold slower than CaKip3-MDN on D-rings...
Fig. 2 Conformational changes in CaKip3 and tubulin. a Displacement vectors for Ca atoms in CaKip3 when comparing the microtubule-unbound CaKip3-MDN-ADP structure and the MT-CaKip3-MDC-APO complex. The models were aligned to microtubule interacting regions H4, loop-11 and loop-8 of the MT-CaKip3-MDC-AP0 structure, specifically, residues 249-257 and 350-379. Displacement vectors for Ca atoms in CaKip3 and tubulin when comparing b MT-CaKip3-MDC-APO and MT-CaKip3-MDC-ANP, c MT-CaKip3-MDC-ANP and MT-CaKip3-MDC-AAF, and d MT-CaKip3-MDC-ANP and CT-CaKip3-MDC-ANP. All structures in b-d were aligned to the β-tubulin chain of the MT-CaKip3-MDC-APO complex. Displacement vectors for Ca atoms are color-coded to match the segment of the model protein that is inserted, using the color scheme in Fig. 1. Views are from the minus end, down the long axis of the protofilament (left), side view (middle) and close-up of the tubulin interface (right). In each panel, the structure indicated on the left side of the arrow is shown as semi-transparent colored ribbons using the same regional color scheme as in Fig. 1. Only vectors with a magnitude of 1.0 Å or higher are shown.

(Fig. 5a, d). This reduction in ATPase activity without the full loop-2 region does not appear to be limited by D-ring binding because we observed a large proportion of the total CaKip3-MDN_{L1, HSKHC} protein sedimented with D-rings using a co-sedimentation analysis (Supplementary Fig. 9a). Together with our structural data, these results show that the extended CaKip3 loop-2 functions like a tubulin protofilament shape sensor that modulates the motility and ATPase activity of the motor domain according to the shape of the tubulin protofilament it binds.

When we explored the tubulin shape-sensing ability of CaKip3’s loop-2 further using unpolymerized free tubulin, which is also thought to be in the curved conformation,52,53, we observed similar experimental results. CaKip3-MDN_{L2, HSKHC} exhibited almost no free tubulin-stimulated activity, while CaKip3-MDN had a free tubulin-stimulated ATPase activity that is comparable to other kinesin-8s (Fig. 5b, e). Moreover, insertion of the CaKip3 loop-2 into HskHC increased free tubulin-stimulated ATPase activity threefold (Fig. 5c, e), opposite its effect on microtubule-stimulated ATPase activity (Fig. 4i, j). These changes in tubulin-stimulated ATPase activity, brought about by swapping the loop-2 region, do not appear to be fully explained by a lack of tubulin binding because a large proportion of the total CaKip3-MDN_{L2, HSKHC} protein eluted with DARPin-capped α-tubulin during size-exclusion chromatography (SEC) analysis (Supplementary Fig. 9b; fractions 13.5–15 mL). However, loop-2-tubulin contacts observed in our CaKip3 structures do appear to be important tubulin-affinity determinants because insertion of CaKip3’s loop-2 into kinesin-1 (HsKHC-MDC_{L2, CaKip3}) increased the amount of motor that co-eluted with α-tubulin-DARPin relative to HsKHC-MDC (Supplementary Fig. 9c, d; fractions 12–12.5 mL).

Loop-1 and loop-2 are key drivers of microtubule-depolymerization. In addition to the altered loop-2-tubulin interface and closed conformation of the motor domain of CaKip3 bound to curved tubulin (CT-CaKip3-MDC-ANP), a striking finding in this complex is the appearance of map density for loop-1 against the minus end neighboring CaKip3 motor domain (Fig. 1f). This head-to-tail interaction of helix-12 of CaKip3 and the inlet and outlet strands of the loop-8 lobe move towards the nucleotide-binding pocket closes upon binding curved tubulin (Fig. 5b, e) and the inlet and outlet strands of the loop-8 lobe move towards the nucleotide-binding pocket closes upon binding curved tubulin (Fig. 5b, e). Moreover, insertion of the CaKip3 loop-1 into the other motor domain could potentially stabilize its closed conformation.

Loop-1 density is also present in the MT-CaKip3-MDC-AAF complex above the loop-8 lobe and likely represents a similar loop-1-mediated motor-motor interaction (Supplementary Fig. 10). No equivalent loop-1 density is observed in the MT-CaKip3-MDC-AP0 or -ANP structures. Taken together, these data suggest that loop-1 interactions with a minus end neighbor motor domain are stabilized by CaKip3 adopting a closed conformation, and that insertion of loop-1 into the other motor domain could potentially stabilize its closed conformation.

Using a sedimentation assay that separates microtubules from soluble tubulin subunits, we monitored GMP-CP stabilized microtubule depolymerization by CaKip3-MDN, a CaKip3-MDN construct lacking loop-1 (CaKip3-MDN_{L1}), the loop-2 swap construct (CaKip3-MDN_{L2, HSKHC}), and a CaKip3-MDN_{L2, HSKHC} construct lacking loop-1 (CaKip3-MDN_{L2, HSKHC−L1}). Analysis of these reactions by SDS-PAGE revealed that loop-1 and loop-2 are both important for CaKip3’s microtubule-depolymerase activity, and that loss of loop-2 causes the most impairment of this activity (Fig. 6c and Supplementary Fig. 11). CaKip3-MDN was 2.5-fold more active than CaKip3-MDN_{L1} and sevenfold more active than CaKip3-MDN_{L2, HSKHC} at depolymerizing microtubules (Fig. 6c). Loss of loop-1 and loop-2 simultaneously (CaKip3-MDN_{L2, HSKHC−L1}) resulted in a 19-fold decrease in microtubule depolymerization activity (EC_{50}). These dramatic effects of loop-1 and loop-2 truncation support the functional importance of these regions in the microtubule depolymerization activity of CaKip3.

The small amount of microtubule depolymerization activity retained by CaKip3-MDN_{L2, HSKHC−L1} shows that loop-1 and loop-2 are not the only features of the motor domain involved in catalyzing this process. The inability of HsKHC-MDN_{L2, CaKip3} to depolymerize microtubules (Fig. 6d) and the tubulin curvature that we observe in the CaKip3-MDN_{L2, HSKHC}−ANP complex (Fig. 4d) further support this idea. Overall, our structural data show that the closed nucleotide-binding pocket conformation of the motor domain actively induces tubulin curvature, which likely accounts for the residual depolymerization activity of the CaKip3-MDN_{L2, HSKHC−L1} Construct. Cataloging the differential tubulin contacts made by the open and closed conformations of the motor highlights that several closed-conformation-specific contacts are formed besides those in loop-2 (Supplementary Fig. 12). While many of these interactions likely contribute to the bending of tubulin, loop-2 adds to the tubulin-curved ability of the motor. This is supported by the greater degree of α-tubulin displacement relative to β-tubulin between the MT-CaKip3-MDC-ANP and MT-CaKip3-MDC-AAF complexes than between the MT-CaKip3-MDN_{L2, HSKHC−APO} and MT-CaKip3-MDN_{L2, HSKHC−ANP} complexes (Supplementary Fig. 7d, f). Moreover, the loss of microtubule depolymerization activity caused by elimination of loop-1 (CaKip3-MDN_{L1}) indicates that loop-1’s interactions with the minus end neighboring motor domain are also involved in the microtubule depolymerization mechanism of CaKip3. These interactions may allow the linked motor domains to act cooperatively (Fig. 1f and Supplementary Fig. 4b).
Discussion
The central focus of this research was to understand the mechanistic basis for the dual ability of kinesin-8 motors to move processively on microtubules and to depolymerize microtubule plus-ends. To achieve this, we obtained cryo-EM structures that demonstrate how the unique features of a kinesin-8 motor domain interact with αβ-tubulin subunits in the microtubule lattice and on microtubule ends. We also obtained functional data to show how the motor domain conformation and catalytic activity affects, and is affected by, the distinctive shapes of these
tubulin polymers. Our findings demonstrate that, when kinesin-8 encounters curved tubulin protofilaments, or protofilaments that can become curved, which are found at the microtubule plus-end, its motor domain readily forms a pro-depolymerization state (Fig. 7). In this state, the elongated loop-2 region moves toward the motor core to accommodate the position of α-tubulin in curved αβ-tubulin subunits. This displacement of loop-2 is accompanied by closing of the ATP pocket to form a nucleotide-hydrolysis-competent active site and a docked neck-linker. In addition, loop-1 becomes more structurally ordered and inserts into the deep groove between loop-8 and the core β-sheet of the preceding motor domain. By maintaining loop-2 contacts with tubulin, and the loop-1 linkage between motors, the conformational transition of multiple motor domains to a closed conformation could increase tubulin protofilament curvature enough to trigger microtubule depolymerization. Alternatively, when kinesin-8 binds tubulin protofilaments that are constrained to a straight conformation by the lateral protofilament interactions of the microtubule lattice, the interactions between loop-2 and α-tubulin restrict the motor domain from transitioning to the closed conformation with a docked neck-linker. Unable to stably interact with the preceding motor via loop-1, or to curve tubulin enough
Fig. 4 Structures and activity of loop-2 swap mutants on microtubules. Cryo-EM map of a MT-CoKip3-MDN$_{L2-\text{HsKHC}}$ in the APO state, b MT-CoKip3-MDN$_{L2-\text{HsKHC}}$ in the AMP-PNP state, and c MT-CoKip3-MDN in the AMP-PNP state. d Displacement vectors for Ca atoms in CoKip3 and tubulin when comparing the MT-CoKip3-MDN-ANP and MT-CoKip3-MDN$_{L2-\text{HsKHC}}$-ANP complexes. Structures were aligned to the β-tubulin chain of MT-CoKip3-MDC-ANP. Displacement vectors for Ca atoms are colored regionally to match the segment of the protein model that is compared, using the color scheme in Fig. 1. MT-CoKip3-MDN-ANP structure is shown as semi-transparent colored ribbons using the same regional color scheme as in Fig. 1. Only vectors with a magnitude of 1.0 Å or higher are shown. e, f Microtubule-gliding assay velocity distributions for CaKip3-MDN, CoKip3-MDN$_{L2-\text{HsKHC}}$, HsKHC-MDN, and HsKHC-MDN$_{L2-\text{CaKip3}}$ using taxol-stabilized microtubules. n = 3 independent experiments for each protein. Asterisks indicate two-tailed unpaired t-test significance. p = 1.3e-135, p = 3.8e-122 for e and f, respectively. g Summary of motor velocities. Values are presented as mean ± SEM. h Microtubule-stimulated ATPase kinetics of CoKip3-MDN and CaKip3-MDN$_{L2-\text{HsKHC}}$ at steady state (n = 3 independent experiments). The basal rate was 0.13 ± 0.01 s$^{-1}$ for both motors. Mean values (±SD) are shown. i Summary of microtubule-stimulated ATPase activities of the motors. Values are presented as mean ± SEM. Mean values (±SD) are shown. j Summary of the microtubule-stimulated ATPase activities of the motors. Values are presented as mean ± SEM. Data was fit to the Michaelis-Menten equation using GraphPad Prism to obtain $K_{O_{5, MT}}$ and $k_{cat}$ values. k Microtubule-co-sedimentation data for CaKip3-MDN and CaKip3-MDN$_{L2-\text{HsKHC}}$ (1 μM each) in the presence of 2 mM MgAMP-PNP (n = 3 independent experiments). Mean values (±SD) are shown. l Microtubule-co-sedimentation data for HsKHC-MDN and HsKHC-MDN$_{L2-\text{CaKip3}}$ (1 μM each) in the presence of 2 mM MgAMP-PNP (n = 3 independent experiments). Mean values (±SD) are shown. Taxol-stabilized microtubules were pelleted by centrifugation to separate the free kinesin and microtubule-bound kinesin. SDS-PAGE and Coomassie brilliant blue staining were used to determine the fraction of microtubule-bound kinesin. m Microtubule-binding affinities ($K_d$ values) of the motors were calculated using the quadratic equation given in Materials and Methods. Values are presented as mean ± SEM for three independent experiments. Source data are provided in the Source Data file.

Fig. 5 Activity of loop-2 swap mutants on curved tubulin. a Dolastatin-induced tubulin-ring (D-ring)-stimulated ATPase kinetics of CoKip3-MDN and CoKip3-MDN$_{L2-\text{HsKHC}}$ at steady state (n = 3 independent experiments). Mean values (±SD) are shown. b Free tubulin dimer-stimulated ATPase kinetics of CoKip3-MDN and CoKip3-MDN$_{L2-\text{HsKHC}}$ at steady state (n = 3 independent experiments). Mean values (±SD) are shown. c Summary of the D-ring-stimulated ATPase activities of the motors. d Summary of the tubulin-stimulated ATPase activities of the motors. Values are presented as mean ± SEM. Data were fit to the Michaelis-Menten equation using GraphPad Prism to obtain $K_{O_{5, MT}}$ and $K_{cat}$ values. Data sources are provided in the Source Data file.

to disrupt protofilament contacts, some of the loop-2-tubulin bonds eventually break, allowing the motor domain to form the pro-motility state, in which the neck-linker docks and the tethered head is moved to the next αβ-tubulin subunit.

Our structural results demonstrate that the closed conformation of the CaKip3 nucleotide-binding pocket not only accommodates the curved conformation of tubulin but also actively induces tubulin curvature (Figs. 2 - 4 and Supplementary Fig. 7). Comparing the microtubule structures with the CaKip3 motor domain in the open or closed conformations indicates that motor domain closure is accompanied by tubulin structural changes toward the curved conformation. This conformational change occurs with or without an intact loop-2 but appears more extensive in the former case. These results indicate that loop-2 is important but not essential for kinesin-8 induced tubulin bending. This could explain the residual depolymerization activity we and others observe with kinesin-8 constructs with a mutated or deleted loop-2. (Fig. 6c)28,32. Previously, it was reported that loop-11 acts to recognize the curved conformation of tubulin by selectively interacting with an aspartate residue on α-tubulin when tubulin is curved (Asp118—yeast tubulin, Asp116—bovine or porcine tubulin)28. This interaction was proposed to suppress the ATPase activity of the motor at the microtubule plus-end, leading to prolonged plus-end binding and subsequent microtubule depolymerization. In our CT-CaKip3-MDC-ANP structure, loop-11 residues are not within bonding distance of Asp116/118 and we observe no structural evidence of this interaction (Supplementary Fig. 13). While we cannot rule out the possibility...
that the conformation of loop-11 on curved tubulin may limit ATPase activity, our structures suggest Asp116/118 is not a major tubulin contact.

We observe that the mechaehemical cycle of a motile depolymerase is distinct from that of a strictly motile or strictly depolymerizing kinesin. During motility, the CaKip3 nucleotide-binding pocket remains in the open conformation following ATP binding. This is similar to the conformation of the depolymerizing kinesin-13s following ATP binding, and dissimilar from motile motors whose nucleotide pockets close in the equivalent nucleotide state. The fact that kinesin-8s and kinesin-13s both have elongated loop-2 regions that make microtubule contacts is further support of the hypothesis that loop-2 is also responsible for the open nucleotide-binding pocket of AMP-PNP-bound kinesin-13s. On the other hand, a pro-motility microtubule-bound state for kinesin-13s with a closed nucleotide-binding pocket and docked neck linker has not been reported. Differences between the loop-2 of kinesin-8s and kinesin-13s appear well suited for the different functionalities of these kinesins. The rigid kinesin-13 loop-2 inserts into the tubulin inter-dimer interface and makes similar contacts whether tubulin is in the straight or curved tubulin conformations. However, to maintain these contacts the motor domain must be in the open conformation when interacting with straight tubulin and in the closed conformation when interacting with curved tubulin. In the case of kinesin-8s a less structured loop-2 can form alternate contacts with tubulin allowing the motor domain to close and reach the pro-motility state while bound to straight tubulin in the microtubule lattice.

While the loop-2 region of kinesin-13s has a highly conserved motif (KVD) that is critical for microtubule depolymerization, the loop-2 of kinesin-8s is not highly conserved. As we show here, and as others have previously reported, it is important but not essential for microtubule depolymerization (Fig. 6c, d). Our results also agree with previous experiments demonstrating that mutations to the kinesin-8 loop-2 can impart faster motility of monomeric constructs, and that kinesin-1 grafted with enough kinesin-8 parts (loop-2, loop-11, and the neck) has depolymerization activity but is a slower motor. We now build on these previous findings and clarify the role of loop-2 as a structural element that controls whether kinesin takes on a pro-motility or pro-depolymerization conformation, based on tubulin curvature.

Other studies of ScKip3 showed that kinesin-8 acts cooperatively to mediate length-dependent microtubule depolymerization. Our structure of CaKip3 bound to D-rings provides a possible explanation for the cooperative mechanism of kinesin-8 microtubule depolymerization activity. We show that loop-1 (and loop-2) enable formation of linear arrays of motor domains on curved tubulin protofilaments, α-helix-0 extends toward the end of

Fig. 6 Loop-1 and loop-2 are major contributors to microtubule depolymerization activity. Cartoon representation of CoKip3 in the CT-CaKip3-MDC-ANP complex showing the polyasparagine section of loop-1 (magenta) inserted into the preceding motor domain (shown in surface representation). The insert shows "close up" view of the polyasparagine section in order to visualize structural elements involved in complex formation. Sequence alignment shows polyasparagine section of loop-1 in CaKip3 compared to ScKip3. a Cryo-EM map of the CT-CaKip3-MDC-ANP complex. A low-pass filtered map (5 Å) is shown as a black mesh around the polyasparagine track of loop-1. b Microtubule depolymerization by sedimentation dose-response curves for the indicated CoKip3 proteins. 2 μM GMP-CPP-stabilized microtubules were incubated with increasing concentrations of CaKip3-MDN, CaKip3-MDNΔL1, CaKip3-MDNΔL1-Kip3-MDC-ANP, or CaKip3-MDNΔL1-Kip3-MDC-ANP + ΔL1 in the presence of ATP. The data from three independent experiments were analyzed and fit to the four-parameter logistic equation. Mean values (±SD) are shown. EC50 values are presented as the mean ± SEM. d Depolymerization of 2 μM GMP-CPP-stabilized microtubules by 3 μM of HsKHC-MDN, 3 μM HsKHC-MDNΔL2, 1 μM HsKHC-MDN assessed by sedimentation. Reactions were incubated for 20 min in the presence of 20 mM MgATP, then free tubulin and microtubule polymers were separated into supernatant (S) and pellet (P) fractions by ultra-centrifugation. Equal portions of (S) and (P) were loaded and analyzed on a 10% Coomassie blue-stained SDS-PAGE gel. Similar results were obtained from two independent experiments. Source data are provided in the Source Data file.
ADP state

semi-open
PDB: 7LFF

ADP state

open
PDB: 7TQZ

APO state

open
PDB: 7TQZ

APO state

open
PDB: 7TR3

ATP state

open
PDB: 7TQX, 7TR0

ATP state

closed
PDB: 7TR3

ATP state

closed
PDB: 7TQY

kinesin-8 steps onto microtubule lattice

kinesin-8 steps onto microtubule end

- ADP

- ATP

- ADP

- ATP

+ ADP

+ ATP

Fig. 7 Model for tubulin shape-induced alternations between pro-motility and pro-depolymerization states of kinesin-8. When kinesin-8 binds tubulin protofilaments that are constrained to a straight conformation by the lateral protofilament interactions of the microtubule lattice (left), the interactions between loop-2 and α-tubulin restrict the motor domain from transitioning to the closed conformation with a docked neck-linker. Unable to stably interact with the preceding motor via loop-1, or to curve tubulin enough to disrupt protofilament contacts, some of the loop-2-tubulin bonds eventually break, allowing the motor domain to form the pro-motility state, in which the neck-linker docks and the tethered head is moved to the next αβ-tubulin subunit. Alternatively, when kinesin-8 encounters curved tubulin protofilaments, or protofilaments that can become curved, which are found at the microtubule plus-end (right), its motor domain readily forms a pro-depolymerization state. In this state, the elongated loop-2 region moves toward the motor core to accommodate the position of α-tubulin in curved αβ-tubulin subunits. This displacement of loop-2 is accompanied by closing of the ATP pocket to form a nucleotide-hydrolysis-competent active site and a docked neck-linker. In addition, loop-1 becomes more structurally ordered and inserts into the deep groove between loop-8 and the core β-sheet of the preceding motor domain. By maintaining loop-2 contacts with tubulin, and the loop-1 linkage between motors, the conformational transition of multiple motor domains to a closed conformation could increase tubulin protofilament curvature enough to trigger microtubule depolymerization.
the docked neck-linker of the proximal motor domain and then transitions into a turn near Asp55 of loop-1 that inserts into the space between the loop-8 lobe and the core β-sheet. Three segments of loop-1 contact the adjacent motor domain, burying ~1075 Å² of the adjacent motor domain surface. The polyasparagine track in loop-1 (including residues Asn62, Asn64, Asn65, Asn66, Ser69, Asn70, Ser71, Asn78, Gly79) forms the key part of this interaction, and some of this track is conserved in S. cerevisiae.57,59

### Methods

#### C. albicans strain construction.
A complete list of the *C. albicans* strains and primers used for cell biology experiments are presented in Supplementary Tables 1 and 2. All plasmids and DNA cassettes were transformed into *C. albicans* using the lithium acetate/PEG/heat shock method.56

**Cloning.** The genes for Candida albicans Kitp3 and Homo sapiens kinesin heavy chain (HsKHC/Kit58) were codon-optimized for expression in *E. coli* and ordered as chemically synthesized DNA fragments from Integrated DNA Technologies (IDT). For all kinesin constructs used in these studies (Supplementary Fig. 1a), gene fragments of the desired length were PCR amplified and cloned into pET-24d (+) using NcoI and Xhol, yielding a C-terminal 6X-His tag. Mutant genes were generated either through overlap-extension PCR (Grikp3-MDNL2-ΔASK2, Gkmp3-MDNL2-ΔASK2, Gkmp3-MDNL2-ΔARK1/ΔK12, Gkmp3-MDNL2-ΔARK1/ΔK12) or as chemically synthesized DNA (HsKHC-MDCL2-Gakip3). The Gkmp3-MDNL2-ΔARK1 and Gkmp3-MDNL2-ΔASK2 constructs have the His6Flag8 loop-2 sequence “LASK” (residues 141-K44) replacing Gkmp3 residues F116-H144. The Gkmp3-MDNL2-ΔASK2, Gkmp3-MDNL2-ΔARK1 constructs have residues V44-G101 removed. The HsKHC-MDCL2-Gakip3 and HsKHC-MDCL2-Gakip3 constructs have the Gkmp3 loop-2 sequence from F161-H144 replacing the His6Flag8 loop-2 sequence “LASK” (residues 141-K44). Constructs HsKHC-MDC and HsKHC-MDC-L1 contained a cleavable C-terminal TEV-SNAP-6X-His tag (SNAP-tag from NEB).

**Expression and purification of recombinant proteins.** For all kinesin proteins and DARPin D2, the same general purification scheme was used. Plasmids encoding kinesins were transformed into *E. coli* BL21(DE3) cells (Agilent, 200131) and plasmids encoding DARPin D2 were transformed into BL21 RIL cells (Agilent, 230245). All proteins were expressed in Luria-Bertani (LB) media supplemented with the appropriate antibiotic. Protein production was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and cells were grown overnight between 16 and 25 °C. Cell pellets were lysed in lysis buffer (10 mM sodium phosphate, 300 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.2 mM ATP, 5 mM 2-Mercaptoethanol (2-ME), 0.2 mg/mL lysozyme (Bioshop), Pierce Protease Inhibitor Tablets (Thermo), pH 7.2) using sonication. Clarified supernatant was loaded onto Ni-NTA resin (Qiagen) equilibrated with wash buffer (10 mM sodium phosphate, 300 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 20 mM imidazole, 0.2 mM ATP, 5 mM 2-ME, pH 7.2) and resin was thoroughly washed with wash buffer. Target protein was eluted with elution buffer (wash buffer + 300 mM imidazole) then dialyzed overnight into HEPES buffer (20 mM HEPES, 1 mM MgCl₂, 150 mM NaCl, 0.2 mM ATP, 1 mM diasthiothreitol (DTT), pH 7.2). The next morning, dialyzed protein was run over a Superdex 200 26/60 size-exclusion column (GE Healthcare) equilibrated with HEPES buffer. Fractions containing target protein were pooled, concentrated, flash frozen in liquid N₂, and stored at ~80 °C.

**Microscopy of税务-stabilized microtubules and DARpins for biochemistry assays.** Taxol-stabilized microtubules were assembled as previously described with some modifications.62 Soluble tubulin was diluted to 5 mg/mL in BR80 buffer (80 mM PIPES, 1 mM MgCl₂, 1 mM K-EGTA, 1 mM DTT, pH 6.8) supplemented with 50 μM ATP, 1 mM GTP, and 10 μM taxol. Microtubules were polymerized by incubation at 25 °C for 10 min, followed by centrifugation at 312,984 x g (TLA-100; Beckman Coulter) for 5 min at 4 °C, then microtubule-polymerization was incubated by addition of 10% DMSO and incubated at 37 °C. After 40 min, 20 μM taxol (pactaxel-ds, Toronto Research Chemicals) was added to the reaction mixture and incubation was continued at 37 °C for another 40 min. Microtubules were sedimented at 312,984 x g (TLA-100; Beckman Coulter) for 15 min at 25 °C and resuspended in the appropriate reaction buffer supplemented with 20 μM taxol. Unless otherwise specified, microtubule concentration was determined by measuring optical density at 280 nm (A₂₈₀). To avoid the light scattering effect of microtubule polymers, microtubules were depolymerized by incubation on ice and addition of 5 mM CaCl₂ prior to measuring A₂₈₀.

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**Dolastatin-induced tubulin rings (D-rings) were polymerized by diluting tubulin to 40 μM in BR80 buffer containing 80 μM dolastatin-10 (APesB0C) and incubating the reaction for 1 h at 25 °C.**

**Expression and purification of recombinant proteins.** For all kinesin proteins and DARPin D2, the same general purification scheme was used. Plasmids encoding kinesins were transformed into *E. coli* BL21(DE3) cells (Agilent, 200131) and plasmids encoding DARPin D2 were transformed into BL21 RIL cells (Agilent, 230245). All proteins were expressed in Luria-Bertani (LB) media supplemented with the appropriate antibiotic. Protein production was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and cells were grown overnight between 16 and 25 °C. Cell pellets were lysed in lysis buffer (10 mM sodium phosphate, 300 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.2 mM ATP, 5 mM 2-ME, pH 7.2) and resin was thoroughly washed with wash buffer. Target protein was eluted with elution buffer (wash buffer + 300 mM imidazole) then dialyzed overnight into HEPES buffer (20 mM HEPES, 1 mM MgCl₂, 150 mM NaCl, 0.2 mM ATP, 1 mM diasthiothreitol (DTT), pH 7.2). The next morning, dialyzed protein was run over a Superdex 200 26/60 size-exclusion column (GE Healthcare) equilibrated with HEPES buffer. Fractions containing target protein were pooled, concentrated, flash frozen in liquid N₂, and stored at ~80 °C.

**Microscopy of税务-stabilized microtubules and DARpins for biochemistry assays.** Taxol-stabilized microtubules were assembled as previously described with some modifications.62 Soluble tubulin was diluted to 5 mg/mL in BR80 buffer (80 mM PIPES, 1 mM MgCl₂, 1 mM K-EGTA, 1 mM DTT, pH 6.8) supplemented with 50 μM ATP, 1 mM GTP, and 10 μM taxol. Microtubules were polymerized by incubation at 25 °C for 10 min, followed by centrifugation at 312,984 x g (TLA-100; Beckman Coulter) for 5 min at 4 °C, then microtubule-polymerization was incubated by addition of 10% DMSO and incubated at 37 °C. After 40 min, 20 μM taxol (pactaxel-ds, Toronto Research Chemicals) was added to the reaction mixture and incubation was continued at 37 °C for another 40 min. Microtubules were sedimented at 312,984 x g (TLA-100; Beckman Coulter) for 15 min at 25 °C and resuspended in the appropriate reaction buffer supplemented with 20 μM taxol. Unless otherwise specified, microtubule concentration was determined by measuring optical density at 280 nm (A₂₈₀). To avoid the light scattering effect of microtubule polymers, microtubules were depolymerized by incubation on ice and addition of 5 mM CaCl₂ prior to measuring A₂₈₀. Dolastatin-induced tubulin rings (D-rings) were polymerized by diluting tubulin to 40 μM in BR80 buffer containing 80 μM dolastatin-10 (APesB0C) and incubating the reaction for 1 h at 25 °C. When D-rings were used in experiments, the concentration was not determined, and the concentration was lower than the concentration of tubulin and never below a concentration of 20 μM. Assembled D-rings were used within 2 h.
ATPase assay. ATP turnover by kinesin was measured when stimulated by taxol-stabilized microtubules, D-rings, or soluble tubulin. To prepare tubulin for the ATPase assay, tubulin was diluted to 2 mg/mL in BRB80 buffer supplemented with 10% DMSO and 2 mM GTP. The mixture was depolymerized on ice for 10 min, then centrifuged at 312,984 × g at 25 °C for 15 min. The supernatant was supplemented with 100 mM KCl and diluted to 2 mg/mL in BRB80 buffer supplemented with 0.5 mM GMP-CPP. Pellets were resuspended in 100 μL BRB80 buffer containing 4 μM D-rings, 20 μM Dolastatin, and 4 μM kinesin, and 2 mM AMP-PNP. Dolastatin was also present in control reactions that did not contain D-rings. Reactions were incubated for 10 min at 25 °C, then centrifuged at 312,984 × g (TLA-100; Beckman Coulter) for 15 min at 25 °C. The top 30 μL of the reaction supernatant was retained and the remainder was discarded. Pellets were resuspended in 50 μL BRB80 buffer and 30 μL was retained for SDS-PAGE analysis. Supernatant and pellet samples were analyzed on SDS-PAGE gels stained with Coomassie brilliant blue R-250.

Microtubule co-sedimentation assay. Microtubule-kinesin co-sedimentation reactions were performed and analyzed based on an established protocol with the following modifications. Prior to setting up co-sedimentation reactions, kinesin motor was pre-cleared by centrifugation at 312,984 × g (TLA-100; Beckman Coulter) for 5 min at 4 °C. Co-sedimentation reactions were prepared by incubating 10 μL of microtubule solutions, 10 μL of kinesin (0.5 μM) in BRB80 buffer and 50 μL of ATPase assay reaction supernatants were retained and the remainder was discarded. Pellets were resuspended in 50 μL BRB80 buffer and 30 μL was retained for SDS-PAGE analysis. Supernatant and pellet samples were analyzed on SDS-PAGE gels stained with Coomassie brilliant blue R-250.

Crystallization of CaKip3-MDN-ADP. Crystals of CaKip3-MDN-ADP were grown using hanging drop vapor diffusion by mixing the protein in a 1:1 ratio with a precipitant solution of 0.1 M MMT (Malic acid, MES, and TRIS), 25% PEG 1500, pH 8 at 277 K. The total drop volume was 4 μL. Fractions were collected using a 25% cushion of 20% ethylene glycol, 25% PEG 1500, 20% ethylene glycol, pH 8, and were flash frozen in liquid N2. X-ray diffraction data for the CaKip3-MDN-ADP crystals were collected using the synchrotron beamline CMCF 08ID-1 of the Canadian Light Source (Saskatoon, Canada) at 100 K. The in-house software program AutoProcess developed at the CMCF was used to run DSDS, which processed and scaled the diffraction data and produced the reflection output file for structure determination. The initial CaKip3-MDN-ADP structure was solved usingPath averaging from the CIF file. The structure was solved using the program PHENIX (version 1.17). The model was refined using simulated annealing refinement with the program REFMAC5 (version 5.17).

Analytical size-exclusion chromatography. Analytical SEC experiments were performed based on an established protocol with minor changes. A 200 μL reaction was analyzed in HEPES buffer (20 mM HEPES, 1 mM MgCl2, 50 mM NaCl, 1 mM EDTA, pH 7.2) containing 20 μM D-rings, 20 μM kinesin, and 2 mM AMP-PNP. Reactions were incubated on ice for 30 min, then centrifuged at 13,148 × g at 4 °C for 15 min. The top 40 μL of the reaction supernatant was retained and the remainder was discarded. Pellets were resuspended in 50 μL HEPES buffer and 50 μL was retained for SDS-PAGE analysis. Supernatant and pellet samples were analyzed on SDS-PAGE gels stained with Coomassie brilliant blue R-250. For specific samples, immunoblotting using HRP-conjugated anti-His antibody (Abcam, ab1187) was performed based on an established protocol. Following SDS-PAGE of fractions collected from each reaction, 20 μL of each fraction was transferred to a PVDF membrane. Membranes were blocked with 5% milk powder in TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5) for 1 h. Membranes were incubated in anti-His antibody (diluted 1:1000 in blocking buffer) for 1 h. Membranes were washed three times with TBST at room temperature then incubated with Pierce Enhanced Chemiluminescence Western Blotting Substrate (Thermo Fisher) prior to imaging. Blots were imaged using an Azure C300 Digital Imager.

Motility assay. Rhodamine-labeled taxol-stabilized microtubules were assembled based on an established protocol with the following changes. Rhodamine-labeled porcine tubulin (Cytoskeleton Inc.) and unlabelled tubulin were mixed in a 1:7 molar ratio (5 mg/mL total tubulin) in BRB80 buffer supplemented with 10% DMSO and 2 mM GTP. The mixture was depolymerized on ice for 10 min, then centrifuged at 312,984 × g (TLA-100; Beckman Coulter) for 5 min at 4 °C. The supernatant was supplemented with 10 μM kinesin, 4 μM D-rings, and 20 μM AMP-PNP. Reaction mixtures were incubated for 30 min at 25 °C, then centrifuged at 312,984 × g (TLA-100; Beckman Coulter) for 15 min at 25 °C. The top 30 μL of the reaction supernatant was retained and the remainder was discarded. Pellets were resuspended in 100 μL BRB80 buffer and 50 μL was retained for SDS-PAGE analysis. Supernatant and pellet samples were analyzed on SDS-PAGE gels stained with Coomassie brilliant blue R-250.
refinement using PHENIX\textsuperscript{76}. Diffraction data collection and refinement statistics are summarized in Table 1.

**Cryo-EM samples preparation**

The datasets were collected on gold grids (UltraAufoil R22/2 200 mesh) plasma cleaned just before use (Gatan Solarus plasma cleaner, at 15 W for 6 s in a 75% argon/25% oxygen atmosphere).

Fifteen-protofilament-enriched microtubules were prepared from porcine brain tubulin (Cytoskeleton, Inc. CO)\textsuperscript{75}. Microtubules were polymerized fresh the day of the cryo-EM sample preparation. Kinesin aliquots were thawed on ice just before use. All nucleotides stock solutions were used with an equimolar equivalent of MgCl\textsubscript{2} to ADP or AMP-PNP. Four microliters of a microtube solution at 2–5 μM tubulin in BRB80 buffer supplemented with 20 μM paclitaxel were layered onto the EM grid and incubated for 1 min at room temperature. This microtube solution also contained AMP-PNP at 4 μM or ADP at 4 μM plus 2 mM AlCl\textsubscript{3} and 10 mM KF for the AMP-PNP and ADP-AlF\textsubscript{3} conditions respectively. During the incubation time, a fraction of the thawed kinesin aliquot was diluted to prepare a 20-μM kinesin solution containing 20 μM paclitaxel and either of the three nucleotide conditions to be probed: (1) 4 mM AMP-PNP, (2) 4 mM ADP plus 2 mM AlCl\textsubscript{3} and 10 mM KF (ADP-AlF\textsubscript{3} conditions) or (3) apyrase: 5 × 10\textsuperscript{-3} units/μL (Apo conditions). Then the excess microtube solution was blotted from the grid using a Whatman #1 paper. Four microliters of the kinesin solution were then applied on the EM grid, transferred to the chamber of a Vitrobot apparatus (FEI-ThermoFisher MA) at 100% humidity where it was blotted for 1 min at room temperature and blotted for 2.5 min with a Whatman #1 paper and a 2-mm offset before plunge-freezing into liquid ethanol. Grids were clipped and stored in liquid nitrogen until imaging in a cryo-electron microscope.

Dolastatin-10 rings (D-rings) were made by incubating 80 μM porcine brain tubulin (Cytoskeleton, Inc. CO) with 160 μM dolastatin-10 (APExBIO) in BRB80 buffer pH 6.8 and incubating for 1 h at 25 °C. That solution was then diluted to make the cryo-EM sample: 3 μM tubulin D-rings, 20 μM dolastatin-10, 12 μM CaKip3-MDC, 4 mM AMP-PNP in BRB80 buffer, pH 6.8.

**Cryo-EM data collection**

Data were collected at 300 kV on Titan Krios microscopes (Supplementary Table 3) equipped with K2 summit detectors for the microtubule datasets and with K3 detector for the CT-CaKip3-MDC-ANP dataset. Acquisition was controlled using Leginon\textsuperscript{71} with the image-shift protocol and calibration grid with gold crystals was used to estimate the current magnification using the program mag_distortion_estimate v1.0 75.

Acquisition was controlled using Leginon with the image-shift protocol and with K3 detector for the CT-CaKip3-MDC-ANP dataset. Contrast transfer function (CTF) parameters per micrographs were estimated with Gctf\textsuperscript{76} on aligned and non-dose-weighted movie averages. The rest of the processing was done using relion\textsubscript{reconstruct} on the original image-particles without signal subtraction.

To obtain a final locally filtered and locally sharpened map, post-processing of the two largest datasets (MT-dolastatin-10 and CaKip3-MDC-ANP) was performed as follows. One of the two unfiltered half-map was low-pass-filtered to 15 Å and the minimal threshold value that does not show noise around the microtubule fragment was used to generate a mask with relion\textsubscript{mask_create} (low-pass filtration: 15 Å, extension: 10 pixels, soft edge: 10 pixels). This soft mask was used in blocres\textsuperscript{80} on 12-pixel boxes to obtain an initial local resolution estimates. The class averages of the two largest datasets (MT-CaKip3-MDN-ANP and MT-CaKip3-MDC-AAF) were sharpened with local sharpening in localcub\textsuperscript{81} with resolution search up to 25 Å. The localcub\textsuperscript{81} program converged to a filtration appropriate for the tubulin part of the map but over-sharpened for the kinesin part. The maps at every 2–3 Å stage cycle were then used and the map with better resolution for the kinesin part area, with the aim of resolving better the kinesin loops, were selected.

**Processing of the cryo-EM datasets of microtubule-kinesin complexes**

The processing was done as previously described\textsuperscript{75}. Movie frames were aligned with motioncor\textsubscript{2}\textsuperscript{24} generating dose-weighted and non-dose-weighted and correcting for magnification anisotropy (Supplementary Table 3). Before each of the corresponding cryo-EM session, a series of ~20 micrographs on a cross-grating calibration grid with gold crystals was used to estimate the current magnification anisotropy of the microscope using the program mag_distortion_estimate v1.0 75.

Magnification anisotropy correction was performed within motioncor\textsubscript{2} using the obtained distortion estimates (Supplementary Table 3). Contrast transfer function (CTF) parameters per micrographs were estimated with Gctf\textsuperscript{76} on aligned and non-dose-weighted movie averages.

Helical reconstruction on 15R microtubules was performed using a helical-single-particle 3D and helical-workflow in FrealignX\textsuperscript{78}, as described previously\textsuperscript{21,24} with each half filament contributing to a distinct half dataset. The box size used are indicated in Supplementary Table 3. Per-particle CTF refinement was performed with FrealignX\textsuperscript{78}.

To select for tubulins bound to kinesin motors and to improve the resolution of the kinesin-tubulin complexes the procedure HSARC\textsuperscript{21} was used for these one-headed states. The procedure follows these steps:

1. Relion helical refinement. The two independent FrealignX helical refined half datasets were subjected to a single helical refinement in Relion 3.1\textsuperscript{79} where each dataset was assigned to a distinct half-set and using as priors the Euler angle values determined in the helical-single-particle 3D reconstruction (initial resolution: 8 Å, sigma on Euler angles sigma Ang: 1.5, no helical parameter search).

2. Asymmetric refinement with partial signal subtraction. An atomic model of a kinesin-tubulin complex was used to generate two soft masks using EMAN pdb2mrc and relion\textsubscript{mask_create} (low-pass filtration: 30 Å, initial threshold: 0.05, extension: 0.15 pixels, soft edge: 6 or 8 pixels). One mask (mask\textsubscript{ANP}) was generated from a kinesin model bound to one tubulin dimer and two longitudinally flanking tubulin subunits while the other mask (mask\textsubscript{kinesin}) was generated with the kinesin coordinates. The helical dataset alignment file was symmetry expanded using the 15R microtubule symmetry of the dataset. Partial signal subtraction was then performed using mask\textsubscript{ANP} to retain the signal within that mask. During this procedure, images were re-centered on the projections of 3D coordinates of the center of mass of mask\textsubscript{ANP} (CaKip3-MDC-ANP) and mask\textsubscript{kinesin} (Kip3-MDC) datasets relative to the Relion 3.1 helical single-particle Dataset Table 3. The partially signal subtracted dataset was then used in a Relion 3D refinement procedure using as priors the Euler angle values determined from the Relion helical refinement and the symmetry expansion procedure (unit cell resolution: 8 Å, sigma: 0.05, offset range corresponding to 3.5 Å, hepalix_order and auto_local_hepalix_order set to 5). The CTF of each particle was corrected to account for their different position along the optical axis.

3. 3D classification of the kinesin signal. A partial signal subtraction procedure identical to the first one but using mask\textsubscript{kinesin} and with particles re-centered on the projections of CTF was performed to select all but the kinesin signal. The images obtained were resampled to 3.5 Å/pixel and the 3D refinement from step 2 was used to update the Euler angles and shifts of all particles. A 3D focused classification without images alignment and using a mask for the kinesin generated like mask\textsubscript{kinesin} was then performed on the resampled dataset (8 classes, tau2_fudge 4, padding 2, iterations: 175).

4. 3D reconstructions with original images (not signal subtracted). To avoid potential artifacts introduced by the signal subtraction procedure, final 3D reconstructions were obtained using relion\textsubscript{reconstruct} on the original image-particles without signal subtraction.

A dataset collected with no stage tilt was analyzed with 2D classifications after picking ring-like structures to observe the different structures present. Representative class averages of the different structures that were detected are presented in Supplementary Fig. 4. The samples of CaKip3 mixed with tubulin, dolastatin-10 and AMP-PNP contained both rings of various diameters and spiral/spiral-like flexible structures (Supplementary Fig. 4a, b). Because the dolastatin-10 rings was long preferentially orientation (Supplementary Fig. 4c), we focused on obtaining a 3D structure was done on a dataset collected at a 40-degree stage tilt. The flexible spiral/spiral-like structures were avoided during the particle picking to focus on the rings. A subset of ~2k rings with a wide range of out of plane tilt were first picked manually. On data resampled to ~4 Å/pix, a series of 3 cycles of 2D classification followed by re-centering on the ring centers was applied to generate the class averages subsequently used as template for automated picking with a low-pass filtration of 50 Å. Using the resulting automatically picked particles, 3 cycles of 2D classification, re-centering, and particle extraction were performed to select rings. The predominant rings type that has 14 asymmetric units (called C14 rings) from here) was selected. Representative rings obtained at the end of the third 2D classification are provided in Supplementary Fig. 4c. Local CTF parameters estimation for each of these C14 rings was performed using Gctf. These particles were resampled at 1.5 Å/pixel with 312-pixel size box particle images and then used in a 3D refinement with C1 symmetry (initial resolution 60 Å) using the helical model generated with Relion\textsubscript{reconstruct} with its handedness corrected. A 3D classification on data resampled at ~3.5 Å/pixel (6 classes, tau2_fudge 2, 25 iterations) was then used to select the rings
contribution to the main class characterized by the most regular ring structure (71% of the particles). A 3D refinement was then performed with a C1 symmetry, the initial model mentioned above, low-pass filtered at 30 Å, and without polishing. Duplicated particles were then removed, leading to ~38 k C14 rings (Table 2). No further particle classification was used from that stage of the processing, with a homogeneous set of C14 rings. A 3D refinement with C14 symmetry was then performed with an initial resolution of 20 Å, a soft mask around the ring (from the previous cycle, with relion_mask_create: low-pass filtration 30 Å, threshold 0.01, extension 25 pixels, soft edge 10 pixels), the solvent_correct_fsc option and local constrains (offset_range 2, offset_step 1, sigma_ang 2) and led to a 6.2 Å map of the full ring structure. A CTF refinement step using only brute-force defocus search over a range of 3000 Å was performed followed by a 3D refinement with identical parameters as the previous one, which improved the resolution to 5.1 Å. A step of particle polishing (with box size of 700 pixels and scaled down to 512 pixels, 1.5 Å/pixel) without training and followed by a 3D refinement (initial resolution 10 Å, a soft mask around the ring made as before, with the solvent_correct_fsc option and local constrains offset_range 2, offset_step 1 & sigma_ang 2) enabled to improve the resolution and led to the final map of C14 ring (Fig. 1a).

The processing was continued by doing local alignments focused on fragments of the C14 rings as described in the following. First, to continue the processing locally without losing the benefit from the particle polishing done on the full rings, the polished particles from the previous polishing step were regenerated on larger boxes (size polished particles-images as input and with re-centering on the center of a mask covering a single asymmetric unit). This enabled to extract from each of these C14 rings in 900-pixel boxes, 14 particles each in 416-pixel boxes and each centered on the electron density of the respective rotational group (Table 2). The defocus of these particles was updated, considering their different position along the optical axis. A preliminary PDB was used to create with EMAN pdb2mrc and relion_mask_create a soft mask covering 3 asymmetric units (low-pass filtration 30 Å, initial threshold 0.3, extension 25 pixels, soft edge 6 Å). That mask was used in a focused refinement with fixed priors on the Euler angles and shifts based on the previous alignment data, local constrains (offset_range 6, offset_step 1, sigma_ang 5) and started with the reconstruction of this expanded data filtered at 10 Å. This focused refinement produced a refined 4.0 Å map (Fig. 1f, top structure). Repeating the same focused refinement but with a mask made similarly covering only the central asymmetric unit (low-pass filtration 30 Å, initial threshold 0.3, extension 20 pixels, soft edge 6 Å) and with tighter local constrains (offset_range 3, offset_step 1, sigma_ang 2) produced a 3.9 Å map. To obtain a final locally filtered and locally sharpened map, the same procedure as the one used for the microtubule datasets was used, leading to the final map (Fig. 1f, bottom structure).

Cryo-EM resolution estimation. The final resolutions for each cryo-EM reconstruction were estimated by fitting WCS curves generated with Relion 3.1 post processing (FSCw0.15 criteria, Table 2, Supplementary Fig. 3). To estimate the overall resolution, these curves were computed from the two independently refined half maps (gold standard) using soft masks that isolate a single asymmetric unit containing a kinesin and a tubulin dimer. The soft masks were created with Relion 3.1 relion_mask_make: create (for microtubule datasets: low-pass filtration 15 Å, threshold 0.1, extension 2 pixels, soft edge 5–6 pixels; for the CT-CaKip3-MDC-ANC dataset: low-pass filtration 20 Å, threshold 0.1, extension 4 pixels, soft edge 10 pixels) applied on the correctly positioned EMAN pdb2mrc density map generated against the cryo-EM density maps using the coordinates of the respective refined atomic models. WCS curves for the tubulin or kinesin parts of the maps were generated similarly using the corresponding subset of the PDB model to mask only a kinesin or a tubulin dimer (Supplementary Fig. 3, Table 2). The final cryo-EM maps together with the corresponding half maps, masks used for resolution estimation, masks used in the partial signal subtraction for the microtubule and kinesin datasets, and the WCS curves are deposited in the Electron Microscopy Data Bank (Table 2).

Model building from cryo-EM densities. Atomic models of the cryo-EM density maps were built as follows. First, atomic models for the kinesin chains were generated from their amino-acid sequence by homology modeling using Modele28. The initial tubulin structures were taken from25. Second, the protein chains were manually placed into the cryo-EM densities and fitted as rigid bodies using UCSF Chimera62. The tubulin models were flexibly fitted into the density maps using Rosetta for cryo-EM relax protocol84,85 and the models with the best scores (match to the cryo-EM density and best molProbity scores) were selected. Fourth, the Rosetta-refined models were further refined against the cryo-EM density maps using PHENIX real space refinement tools79 and/or ISOLDE80. Fifth, the models were exported using Coot73 for several iterations of Phenix real space refinement and Coot editing were performed to reach the final atomic models. Atomic models and cryo-EM map figures were prepared with UCSF-Chimera83 and ChimeraX85.

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**Author contributions**

B.H., C.F., and I.S. constructed the wild type and kip3 mutant Candida albicans strains used to understand CaKip3 protein functions in vivo. B.H., C.F., and I.S. conducted the cell growth, cell imaging, and phenotypic analysis of these strains. B.H., C.D., and D.T. cloned, expressed, and purified the kinesin proteins; B.H. and J.S.A. collected X-ray diffraction data for the CaKip3-MDN crystal, determined the structure, built and refined the atomic model, and analyzed the structure; M.P.M.H.B. and A.B.A. assembled kinesin-microtubule and kinesin-curved tubulin complexes and made cryo-EM grid samples; A.B.A. performed sample screening and optimization for cryo-EM imaging and performed microtubule selection; M.P.M.H.B. and A.B.A. designed the cryo-EM experiments and performed cryo-EM data collection; M.P.M.H.B. designed and performed the cryo-EM data processing of kinesin-microtubule and kinesin-ring complexes, mechanistically interpreted the cryo-EM structures and proposed several of the biochemical experiments; M.P.M.H.B. and H.S. built and refined the atomic models, and analyzed the structures; B.H. designed the biochemical analyses; B.H. and C.D. performed ATPase assays; B.H. performed all other biochemical experiments; J.S.A. and H.S. supervised the project; B.H., M.P.M.H.B., A.B.A., H.S., and J.S.A. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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