Kinesin-binding protein remodels the kinesin motor to prevent microtubule binding

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Kinesins are regulated in space and time to ensure activation only in the presence of cargo. Kinesin-binding protein (KIFBP), which is mutated in Goldberg-Shprintzen syndrome, binds to and inhibits the catalytic motor heads of 8 of 45 kinesin superfamily members, but the mechanism remains poorly defined. Here, we used cryo–electron microscopy and cross-linking mass spectrometry to determine high-resolution structures of KIFBP alone and in complex with two mitotic kinesins, revealing structural remodeling of kinesin by KIFBP. We find that KIFBP remodels kinesin motors and blocks microtubule binding (i) via allosteric changes to kinesin and (ii) by sterically blocking access to the microtubule. We identified two regions of KIFBP necessary for kinesin binding and cellular regulation during mitosis. Together, this work further elucidates the molecular mechanism of KIFBP-mediated kinesin inhibition and supports a model in which structural rearrangement of kinesin motor domains by KIFBP abrogates motor protein activity.

INTRODUCTION

Kinesins comprise a superfamily of microtubule-based motor proteins that play essential roles in virtually every aspect of cell physiology, including mitotic spindle assembly, regulation of microtubule dynamics, ciliogenesis, and transportation of cargoes throughout the cell (1–4). A signature protein fold shared among all members of the kinesin superfamily is a catalytic “motor” domain. The kinesin motor domain contains binding sites for both microtubules and adenosine 5′-triphosphate (ATP), enabling these proteins to convert energy from ATP hydrolysis into mechanical force (5). In most kinesin motors, this catalytic cycle powers motility of the proteins along microtubule tracks. While the motor domain exhibits structural and high sequence conservation among the superfamily, sequence differences imbue each kinesin with unique characteristics and are responsible for diversifying motor functions within the cell. In addition, the nonmotor regions of different kinesin family members have diverged to confer specificity for cargo binding and regulation.

Kinesins are regulated at many levels to ensure that they become activated at the right time and place. Autoinhibition, wherein kinesins adopt a conformation that prevents microtubule binding (6–8), as well as sequestration within the nucleus (9–14) and cell cycle–dependent protein expression (15) are common strategies to prevent untimely motor-track interactions. Kinesins are also regulated by posttranslational modifications, e.g., phosphorylation, which can serve to activate microtubule binding (16–18). Last, kinesin-interacting proteins such as adaptor proteins and light chains, and their phosphorylation, can regulate the ability of transport kinesins to engage cargo (1, 19–24) or target them to specific locations within the cell (25, 26). KIFBP, a unique class of kinesin-binding protein, has emerged as an important negative regulator of a subset of kinesin motors (27, 28).

KIFBP was found as a disease-causing gene associated with the neurological disorder Goldberg-Shprintzen syndrome [GOSHS (29–31)], an autosomal disease characterized by facial dysmorphism, mental retardation, and congenital heart disease [OMIM (Online Mendelian Inheritance in Man) #609460]. In mice and zebrafish, loss of KIFBP function leads to neuronal migration and maturation defects in the developing brain (32, 33). Emerging data demonstrate a compelling role for KIFBP in regulating motor-microtubule interactions for 8 of the 45 kinesin motors encoded by the human genome. KIFBP interacts directly with the motor head of Kinesin-2 (KIF3A), Kinesin-3 (KIF1A, KIF1B, KIF1C, KIF13B, and KIF14), Kinesin-8 (KIF18A), and Kinesin-12 (KIF15) family members, resulting in inhibition of motor-microtubule binding both in vitro and in cells (27, 28). How the regulation of kinesin motors by KIFBP is linked to specific biological processes is largely unexplored, although neuronal microtubule dynamics appear to be controlled through KIFBP-dependent regulation of KIF18A (27). Moreover, recent work has shown that KIFBP is critical for ensuring proper mitotic spindle assembly by regulating the mitotic kinesins KIF15 and KIF18A (28).

Recently, a 4.6-Å structure for KIFBP and a 6.9-Å structure for KIFBP bound to KIF15 were reported (34). These studies indicated that KIFBP alters the KIF15 kinesin motor to prevent microtubule binding (34). Despite the advances afforded by this study, many open questions remain. First, the resolution of the published structures did not fully define the KIFBP-interaction interface with KIF15 given the large degree of uncertainty for the atomic model. For example, at the resolutions reported, the atomic models may have incorrect helical placement or helical registries. Without an accurate atomic model of KIFBP, the molecular mechanism of kinesin regulation by KIFBP remains unclear. Second, the generality of the previously proposed inhibition mechanism is unknown. The earlier work also included an analysis of KIFBP in complex with KIF1A, but the low resolution of the KIFBP–KIF1A complex and heterogeneity of binding poses did not allow firm conclusions regarding the binding mechanism. Third, the previous study did not address the importance of

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residue-residue contacts between KIFBP and kinesin motors (KIF15 and KIF1A) in the context of the specific biological processes in which these motors participate.

To understand how KIFBP engages kinesin motors, we leveraged an interdisciplinary approach to generate a high-confidence atomic model of the KIFBP–kinesin motor interface by combining cryo-electron microscopy (cryo-EM) with cross-linking mass spectrometry (XL-MS). We show that KIFBP is a tandem repeat protein constructed of nine helix pairs that assemble into a solenoid-like structure. When complexed with KIF15 and KIF18A, three helices in KIFBP [helical pair 4a/b (HP4a/b)–HP5] associate closely with the kinesin α4 helix, an interaction that requires a 15-Å displacement of α4 from its resting position. Using molecular dynamics (MD) simulations, we find that kinesin α4 is immobile when a motor head is not bound to KIFBP, suggesting that allosteric changes drive the repositioning of α4 required for binding HP4a/b–HP5. Using our high-confidence KIFBP: kinesin atomic model, we identify two regions in KIFBP that are responsible for the interaction in vitro and show that mutations in these regions disable the ability of KIFBP to regulate KIF15 and KIF18A during mitosis. Collectively, our work describes the molecular mechanism of KIFBP-mediated inhibition of KIF15 and KIF18A via binding to and stabilizing a conformation of the kinesin motor head that is incompatible with microtubule binding.

RESULTS

KIFBP adopts a solenoid structure composed of TPR motifs

To determine an atomic model of KIFBP, we used cryo-EM to determine the overall structure of KIFBP and a higher-resolution structure of the N terminus of KIFBP (Fig. 1 and movie S1). Reconstructions of the full KIFBP molecule at 4.6 Å showed that KIFBP is almost entirely α-helical, having nine HPs along with one long helix throughout the 621–amino acid sequence, similar to previous reports (34) (Fig. 1A, figs. S1 and S2, and table S1). The α helices are arranged into a right-handed superhelical twist, giving KIFBP an appearance analogous to other tetratricopeptide repeat (TPR) proteins (35).

Given that the resolution of 4.6 Å is not sufficient to build an atomic model, we wanted to improve the resolution of our reconstruction. To this end, we performed masking and three-dimensional (3D) classification on the N-terminal two-thirds of KIFBP to obtain a higher-resolution structure at 3.8 Å (Fig. 1B). We could unambiguously identify amino acid side chains at this resolution, allowing us to construct an atomic model for amino acids 5 to 403 (Fig. 1C, figs. S1 and S3 to S5, and table S2). Our atomic model provides a high-confidence positioning of KIFBP residues, allowing us to map the structure onto the KIFBP sequence (Fig. 1D), confirming α-helical positions, and showing the locations of loops connecting HPs throughout the structure.

KIFBP inhibits KIF15 microtubule binding and remodels the motor domain of KIF15

After determining the structure of KIFBP alone, we used cryo-EM to determine the structure of KIFBP bound to KIF15. To prepare cryo-EM samples, we incubated the purified KIF15 motor domain (amino acids 1 to 375) with KIFBP and subjected the sample to size exclusion chromatography in the presence of ATP (fig. S6). SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis determined that KIF15 comigrated with KIFBP in a 1:1 complex, and fractions containing the complex were used for cryo-EM sample preparation.

We used cryo-EM to determine a ~4.8-Å-resolution structure of KIFBP bound to KIF15 (Fig. 2A, figs. S7 and S8, table S3, and movie S1). At this resolution, we could unambiguously identify the regions of density corresponding to KIFBP in addition to the motor domain of KIF15 (Fig. 2A). Unexpectedly, when we docked the structure of KIF15 into the reconstruction, we noticed that the α4 helix of KIF15 was missing. Instead, we noticed the presence of an additional α-helical density within a cleft of KIFBP, suggesting the displacement of KIF15-α4 into this cleft during complex formation (Fig. 2B). In the structure, KIFBP occupies the microtubule-binding surface of KIF15, sterically blocking access to the microtubules by KIF15.

To understand how KIFBP affected the overall architecture of KIF15, we used both manual building and Rosetta comparative modeling (36) to develop a model for the KIFBP-engaged KIF15

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Fig. 1. KIFBP adopts a solenoid structure composed of TPR motifs. (A) Overview of KIFBP structure at 4.6 Å. Dotted lines indicate the masked region for the higher-resolution KIFBP core. (B) Structure of KIFBP core at 3.8 Å. (C) Combined atomic model of KIFBP. (D) Structural features and nomenclature for KIFBP.
motor (Fig. 2C). Our analysis of KIFBP:KIF15 revealed structural rearrangements of the KIF15 motor by KIFBP to disrupt KIF15’s microtubule-binding interface. The most notable structural change involved the repositioning of KIF15-α4 away from the kinesin motor domain, placing KIF15-α4 15 Å away from the location found in the crystal structure of KIF15 (37). Notably, kinesin motors require the α4 helix for engaging microtubules during the kinesin mechanochemical cycle (38). The adjoining loops on each side of KIF15-α4, Loop-11 (“KIF15-L11”) and Loop-12 (“KIF15-L12”), accommodated the repositioning of KIF15-α4 by facilitating the extension of KIF15-α4 away from the body of the motor domain (Fig. 2C). Whereas KIF15-L12 remains extended in solution, KIF15-L11 is positioned away from the motor domain and binds along KIFBP-HP4a.

In addition to seeing changes in KIF15-L11, KIF15-α4, and KIF15-L12, we saw that the overall structure of KIF15 adopted a more open conformation (Fig. 2, D and E). The structure showed the shift of α helices KIF15-α1, KIF15-α3, and KIF15-α6 away from the core of the motor. We observed large movements for β-strand pairs KIF15-β1β-1c and KIF15-β5β-5 in addition to loop KIF15-L5. These changes indicate that KIFBP stabilizes several structural changes in KIF15 to block microtubule binding. Thus, KIFBP blocks microtubule binding by sterically preventing microtubule interaction in addition to allosterically altering the KIF15 motor.

**KIFBP binds to KIF15-α4 in a distinct manner relative to αβ-tubulin**

Given that KIFBP binds along the microtubule-binding interface of KIF15, we sought to compare the interaction interface between KIF15:KIFBP and KIF15:αβ-tubulin (34). First, we noticed that the length of the α4 helix is shorter for KIF15-KIFBP compared to the microtubule-engaged α4 helix (Fig. 3, A to F). The length of α4 in KIFBP:KIF15 is similar to the crystal structure of KIF15 when not bound to microtubules (37). Second, KIF15-L11 is bent relative to α4 at an angle of ~120° (Fig. 3B), whereas KIF15-L11 on the microtubule adopts a helical structure to extend the length of α4 (Fig. 3E) (34). These two observations indicate that KIFBP holds KIF15-α4 in a conformation that is incompatible with microtubule binding.

Comparing the hydrophobicity and electrostatic charge surfaces on KIFBP versus αβ-tubulin shows that KIFBP binds KIF15-α4 via hydrophilic and hydrophobic helices (Fig. 3, B, C, E, and F, and movie S1). The strong electrostatic nature of αβ-tubulin results in minimal hydrophobic residues contributing to KIF15 binding. Unlike β-tubulin, KIFBP uses a composite binding site stretching across three helices to bind both hydrophobic and polar residues to interact with KIF15-α4. Comparing the overall hydrophobicity and charge distribution indicates that KIFBP binds KIF15-α4 in a manner distinct from αβ-tubulin.

**KIFBP engages the microtubule-binding interface of KIF15 using multiple contact points**

To obtain further insight into the regions of KIFBP and KIF15 that interact with each other, we performed XL-MS. Recombinant KIFBP and KIF15 (1 to 375) were incubated with the 11-Å lysine-targeting cross-linker BS3, digested with trypsin, and analyzed using tandem mass spectrometry (MS/MS). We identified cross-linked peptides using pLink software (see Materials and Methods). We present all high-confidence cross-links between KIFBP and KIF15 peptides (ε value of >0.05) in table S5 and have displayed them on the primary and secondary structures of KIFBP:KIF15 as well (Fig. 4, A and B, and movie S1).
We observed the highest density of cross-links between three residues of KIFBP-L1 (K26, K30, and K36) and the microtubule-binding interface of KIF15 (K273, K283, K319, and K361) (Fig. 4, A and C). These same KIF15 residues also cross-linked to regions in the middle of KIFBP [HP4a, LH (long helix), and LL (long loop)] (Fig. 4D) and toward the C terminus (L17, HP9a, and HP9b) (Fig. 4E). Two KIF15 residues that cross-linked multiple KIFBP sites (K273 and K283) are located in KIF15-L11, adjacent to KIF15-α4. In addition, residues KIF15-K273 and KIF15-K319 form the kinesin motor microtubule-binding interface (34). The high density of cross-links involving these KIF15 residues supports a mechanism of inhibition where KIFBP directly binds the microtubule-binding domain of kinesins, occluding interactions with the microtubule lattice.

KIFBP:KIF15 cross-links span nearly the entire length of KIFBP to bind the KIF15 microtubule-binding surface. When superimposed onto the KIFBP:KIF15 structure (Fig. 4, B to E), these cross-links bridge distances greater than the 11-Å BS3 can reach. This suggests that the cross-linked regions of KIFBP may associate transiently with the microtubule-binding interface of KIF15 at different time points during complex formation (see Discussion).

**KIFBP inhibits KIF18A via a similar mechanism as KIF15**

After characterizing how KIFBP inhibits KIF15 (kinesin-12 family), we next aimed to establish whether KIFBP uses the same mode of inhibition for a kinesin motor from a different kinesin family, KIF18A (kinesin-8 family). To determine how KIFBP inhibits KIF18A, we first purified recombinant KIF18A (1–363) motor domain, incubated KIF18A with KIFBP, and performed size exclusion chromatography to confirm the formation of a 1:1 complex (fig. S9). After preparing cryo-EM grids with the complex, we obtained 2D class averages that appeared similar in shape and features as seen previously for KIF15 (fig. S10), further confirming the formation of a KIFBP-KIF18A complex.

After performing further single-particle analysis, the cryo-EM structure of KIFBP-KIF18A revealed that KIFBP binds KIF18A similar to KIF15 (Fig. 5, A and B, fig. S11, table S4, and movie S1). In the structure, KIFBP N- and C-terminal domains engage both sides of the motor domain, while KIF18A-α4 is displaced away from the motor into the central cavity of KIFBP. To highlight the similarities between KIFBP engagement of KIF15 and KIF18A, we segmented the motor density from either KIFBP:KIF18A (Fig. 5, C and D) or KIFBP:KIF15 (Fig. 5, E and F). This comparison shows for both motors that (i) α4 is held within the central cleft of KIFBP, (ii) Loop-11 and Loop-12 are extended away from the motor, and (iii) KIFBP-L11 adopts a curved shape as it makes a ~120° turn to follow helix KIFBP-HP4a within the KIFBP cleft. Thus, KIFBP stabilization of kinesin α4 helix away from the motor is a shared mode of kinesin inhibition by KIFBP for KIF18A and KIF15.

**KIFBP uses Loop-1 and Loop-14 to bind kinesin in vitro**

Our structural data and XL-MS results identified multiple KIFBP-motor interactions that may be important for robust binding and
inhibition of motor activity. In particular, our XL-MS results revealed that the most extensively cross-linked residues in KIFBP occurred within Loop-1 and the C-terminal helix pairs of KIFBP (Fig. 4A and table S5). Our cryo-EM structure of KIFBP complexed with the motor domains of both KIF15 and KIF18A supports a prominent role for Loop-1 in motor engagement. We therefore targeted both KIFBP-L1 and KIFBP-HP9b for mutagenesis, selecting positively and negatively charged residues in those regions and substituting them with alanine or glycine residues (Fig. 6B). We also constructed a third KIFBP mutant by mutating residues 460 to 465 in KIFBP-L14 to alanine (Fig. 6B) because of the proximity of KIFBP-L14 to KIF15 and previous work indicating that it is important for kinesin motor binding (34). All proteins were purified for in vitro binding studies (fig. S12).

First, we tested the ability of these three KIFBP mutants to bind the motor domains of KIF15 and KIF18A in vitro. We performed in vitro pull-down assays using hexahistidine-tagged motor domains of either KIF15 (1 to 375) or KIF18A (1 to 363) immobilized on nickel resin and analyzed the ratio of recombinant wild-type (WT) or mutant KIFBP to kinesin motor domain in the pellet. We used glutathione S-transferase (GST) as a negative control, which showed little to no nonspecific interaction with nickel resin or immobilized kinesin. When incubated with KIF15, we observed a fourfold reduction in binding of the KIFBP-L1mutant and a twofold reduction in binding of the KIFBP-L14mutant compared to KIFBP-WT, indicating that mutations in these regions abrogate the ability of KIFBP to interact with the motor robustly. Unexpectedly, KIFBP-HP9bmut showed no difference in binding compared to KIFBP-WT, suggesting that the charged residues that mutated are not essential for motor binding. Thus, although both Loop-1 and the C terminus of KIFBP were implicated as potentially important for binding in our XL-MS experiment (Fig. 4, C and E), mutations to only one of these regions, Loop-1, biochemically affected binding.

Next, we repeated pull-down assays with KIF18A immobilized on the resin and analyzed the binding of the same panel of mutants. Similar to KIF15, the KIFBP-L1mutant showed a threefold reduction in binding compared to KIFBP-WT. Intriguingly, the KIFBP-L14mutant showed only a slight 10% reduction in binding to KIF18A compared to KIFBP-WT, in contrast to the 50% decrease in binding to KIF15.
Mutations in KIFBP-L1 and KIFBP-L14 disrupt the regulation of mitotic kinesins

Overexpression of KIFBP in HeLa cells leads to defects in chromosome alignment and an increase in spindle length (28). To determine whether mutations that block KIFBP interaction in vitro also reduce KIFBP effects during mitosis, we transfected N-terminally mCherry-tagged KIFBP constructs into HeLa Kyoto cells and measured chromosome alignment and spindle length in metaphase-arrested cells. Consistent with previous results, overexpression of mCherry-KIFBP-WT decreased chromosome alignment, quantified by an increase in full width at half maximum (FWHM) of centromere fluorescence distribution along the length of the spindle (Fig. 7, A and B) (28, 39). Overexpression of mCherry-KIFBP-WT also increased spindle lengths, as shown previously (28) (Fig. 7, A and B). These mitotic effects scaled with the mCherry-KIFBP-WT expression level, where cells expressing higher levels of mCherry-KIFBP-WT had longer spindles and more severe chromosome alignment defects (fig. S13).

To test the mitotic effects of the KIFBP mutants, we generated mCherry-KIFBP overexpression mutant constructs mCherry-KIFBP-L1m, mCherry-KIFBP-L14m, and mCherry-KIFBP-HP9bm (Fig. 6B). Cells expressing mCherry-KIFBP-L1m and mCherry-KIFBP-L14m mutants displayed similar chromosome alignment and spindle lengths as cells overexpressing the mCherry control (Fig. 7, A and B). In contrast to mCherry-KIFBP-WT, mitotic effects of mCherry-KIFBP-L1m did not scale with expression level, suggesting that mCherry-KIFBP-L1m does not inhibit kinesin activity even when expressed at higher levels (fig. S13). Spindle length and chromosome alignment defects increased at high expression levels of mCherry-KIFBP-L14m, suggesting that mCherry-KIFBP-L14m may inhibit kinesin activity at high expression levels (fig. S13).

In contrast to mCherry-KIFBP-L1m and mCherry-KIFBP-L14m, mCherry-KIFBP-HP9bm showed similar effects as mCherry-KIFBP-WT overexpression, decreasing chromosome alignment and increasing spindle length (Fig. 7, A and B). Mitotic defects did not scale with expression level for mCherry-KIFBP-HP9bm, in contrast to mCherry-KIFBP-WT (fig. S13). Cells expressing lower levels of mCherry-KIFBP-HP9bm displayed mitotic defects, suggesting that mCherry-KIFBP-HP9bm may be a more potent inhibitor than mCherry-KIFBP-WT. These findings are consistent with the in vitro observations that KIFBP-L1m and KIFBP-L14m reduce KIFBP’s interaction with KIF15 and KIF18A, whereas the KIFBP-HP9bm does not block interaction (Fig. 6, C to E).

To further investigate the cellular effects of the KIFBP mutations, we measured KIF18A localization in HeLa Kyoto cells overexpressing WT KIFBP or the KIFBP mutants. KIF18A accumulates at the plus ends of microtubules during metaphase, and we have previously shown that overexpression of mCherry-KIFBP-WT alters KIF18A spindle localization (28). Overexpression of mCherry-KIFBP-WT abolishes KIF18A plus-end enrichment and leads to a more uniform spindle localization (Fig. 8A), consistent with previous observations (28). Line scan analysis confirmed the loss of KIF18A from microtubule plus-end enrichment along individual kinetochore microtubules (Fig. 8B).

We predicted that mutations that abolished KIFBP interaction with kinesins in vitro would not disrupt KIF18A localization. Overexpression of mCherry-KIFBP-L1m and mCherry-KIFBP-L14m did not disrupt KIF18A plus-end enrichment on kinetochore microtubules (Fig. 8, A and B). In contrast, overexpression of mCherry-KIFBP-HP9bm showed similar effects to overexpression of mCherry-KIFBP-WT (Fig. 8, A and B). This is especially interesting considering that KIFBP-L14m binds KIF18A in vitro, suggesting that the interaction...
To analyze the dynamics and conformational spaces explored by these proteins, we performed a series of MD simulations using a number of kinesin family members. We selected two members that bind KIFBP (KIF15 and KIF18A) and two that show little or no interaction with KIFBP (KIF5C and KIF11) (27). Using only the motor domains, we performed 500+ nanosecond simulations of all-atom MD for each protein in the unbound, adenosine 5′-diphosphate (ADP) state (see Materials and Methods).

To analyze the dynamics and conformational spaces explored by these proteins, we compared MD trajectories between KIFBP-binding motors (KIF15 and KIF18A) and motors that do not bind KIFBP (KIF5C and KIF11). We performed principal components analysis (PCA) using the KIF15 simulation as the reference for the other proteins to reduce dimensionality. To minimize noise in the PCA that comes from fluctuations in unstructured regions of the protein, we only analyzed amino acids that were in stable secondary structure elements (α helices of β strands) at least 80% of the time. Last, we removed α4 from the PCA because this helix remained stably bound to the motors in the simulations (fig. S14) but is extended in the cryo-EM structure and would thus skew the PCA results.

Comparison of PCA for these kinesin motors shows that (i) all four proteins have similar ranges of motion and explore comparable regions of conformational space and (ii) there are specific motions that are only present in KIFBP-binding kinesins (KIF15 and KIF18A) (Fig. 9A). Close inspection of the amino acids that contribute to PC1 (i.e., the largest motions of KIF15) revealed that splaying of both the C-terminal end of α3 and the N-terminal end of α6 is the dominant contributor to this mode. These structural differences are consistent with the KIFBP:KIF15 cryo-EM structure (Fig. 2D) where movements of these helices are a defining feature of the bound complex. Thus, MD simulations indicate that only KIF15 and KIF18A are able to reach the conformation found in the bound complex (Fig. 9A).

The MD KIF15 solution structure differs in specific structural regions with KIFBP-bound KIF15. First, the KIF15 solution structure shows that KIF15-α4 remains closely bound to the motor body throughout each simulation. This would suggest that the release and translocation of KIF15-α4 would only occur upon interaction
with KIFBP or on a much longer time scale than that sampled during the simulation. Second, although we saw that the movement of KIF15-a3 was a key feature from the PCA, we see in the cryo-EM structure that the C-terminal end of KIF15-a3 loses its structure over the final eight to nine residues, forming an extended loop with a short antiparallel β sheet (Fig. 9, B and C). This part of KIF15 makes contact with KIFBP, and it appears that this structural change would allow better contact between KIF15 and KIFBP.

Last, although α4 has a nearly identical conformation for all kinesins, L11 is much more dynamic, and its conformation is kinesin dependent. For KIF5C, L11 tends to be extended away from α4, and when superimposed on the cryo-EM complex, L11 has a steric clash with KIFBP (Fig. 9, B and D). Conversely, both KIF15 and KIF18A adopt more compact L11 structures, and these fit well within the cavity of the KIFBP structure (KIF15 structure shown in Fig. 9D). Together, this suggests that the overall conformation of the motor head and the dynamics or conformation of more flexible parts of each protein may act in concert to determine which kinesins will bind to KIFBP and which will not.

**DISCUSSION**

Our work presents a previously undescribed mode of kinesin motor protein regulation via a multivalent interaction between KIFBP and the kinesin motor domain (movie S1). Using a combination of cryo-EM, XL-MS, MD simulations, biochemical assays, and cell biology, we describe a model in which KIFBP stabilizes the microtubule-binding α4 helix (5, 40–42) away from the kinesin motor domain in addition to sterically inhibiting the microtubule-binding interface. KIFBP does not mimic the negatively charged microtubule surface (43) to engage kinesin motors. Instead, KIFBP uses a hydrophobic cleft to hold α4 and Loop-11 in a conformation that is incompatible with microtubule binding while simultaneously engaging and sterically inhibiting the kinesin microtubule-binding interface (Fig. 3).

Determining the binding mechanism of KIFBP relied on cryo-EM structural data for relatively small macromolecular samples. We determined a near-atomic structure of KIFBP corresponding to ~40 of 72 kDa, using this reconstruction for de novo model building. This places the KIFBP reconstruction and model among the smallest– molecular weight macromolecules to be built de novo by cryo-EM.
The relatively small size of KIFBP may be the driving factor limiting the overall resolution of KIFBP alone to ~3.8 Å instead of obtaining higher-resolution reconstructions.

During the preparation of this work, another study used cryo-EM and cell biology assays to propose a mechanism of KIFBP-mediated kinesin inhibition (34). In this study, Atherton et al. (34) determined lower-resolution structures of KIFBP (4.8 Å) and KIFBP-KIF15 (6.9 Å) to arrive at a similar model of kinesin motor inhibition, where KIFBP stabilizes the α4 helix away from KIF15. In our work, our higher-resolution structures of KIFBP alone (3.8 Å) and KIFBP-KIF15 (4.8 Å) allowed us to (i) build a high-confidence atomic model for KIFBP and (ii) map conformational changes in the KIF15 motor when bound to KIFBP. We showed that KIFBP uses a similar mode of inhibition for KIF18A, indicating that our proposed model is likely a general mode of kinesin inhibition. Last, we used our structures alongside XL-MS to map the interaction of KIFBP with kinesin motors and showed that blocking the interaction between KIFBP and KIF15 and KIF18A via mutagenesis minimizes its ability to regulate motor activity in the physiologically relevant context of mitosis (Figs. 7 and 8).

Kinesin motor recognition by KIFBP

Our cryo-EM reconstruction of KIFBP reveals that KIFBP contains a 9-TPR array, which folds into a solenoid with a concave kinesin-interacting surface. Unlike continuous TPR proteins such as LGN (44), KIFBP is punctuated by a centrally located helix and loop (Fig. 1). The binding of KIFBP to kinesin motor heads is notably different from the interaction of many TPR proteins to their ligands (35). Many TPR proteins bind a short sequence, e.g., HOP binds the motif MEEVD in Hsp90 (45). In contrast, our cryo-EM and XL-MS show that the interaction of KIFBP with kinesin motors is highly multivalent. First, KIFBP-L1, localized at its N terminus, engages α3 and β6 of the kinesin motor head. Second, KIFBP-L14 contacts β4-β5 of KIF15 and KIF18A with amino acid E168 in the KIF15 motor domain (or E161 in KIF18A) positioned to play a key role in this interaction. Third, α4 helices of KIF15 and KIF18A...
become nestled into a multihelix groove created by KIFBP-HP4a, KIFBP-HP4b, and KIFBP-HP5a. The binding of KIF15, KIF18A, and KIF-HP-α4 to KIFBP is notable because it requires a 15-Å displacement of the helix from its resting position within the kinesin motor head.

Our structure- and XL-MS-guided mutagenesis study of KIFBP revealed that KIFBP-L1 is especially important for motor binding. Charge neutralization of Loop-1 renders KIFBP incompetent for binding both KIF15 and KIF18A in vitro (Fig. 6). Consistent with these data, KIFBP-L1 mmutant failed to produce phenotypes associated with KIFBP overexpression in cells, i.e., disruption of chromosome alignment and increased spindle length (Figs. 7 and 8). Unlike KIFBP-WT, the KIFBP-L1 mmutant was also incapable of disrupting KIF18A localization.

In contrast to KIFBP-L1, the role of KIFBP-L14 in motor inhibition remains less clear. Previous work using an artificial peroxisome transport assay suggested that KIFBP-L14 is important for the ability of KIFBP to inhibit the motor activities of KIF1A and KIF15 (34). However, mutation of KIFBP-L14 more strongly affected the ability of KIFBP to retrieve KIF1A from cell lysates than KIF15. In our experiments with purified proteins, we observed reduced binding of KIFBP-L14 m to KIF15 but only minimal reduction in binding to KIFBP-HP9b (residues K610 and K617) and the microtubule-binding interface of KIF15 (Fig. 4). These structural elements are not within the cross-linking range of BS3 in our cryo-EM structures (Figs. 2 and 5), and the significance of these cross-links is therefore not clear. Perhaps in line with this, our analysis of the charge neutralization mutant KIFBP-HP9b mmutant revealed that this mutation had little effect on in vitro interaction with KIF15 or KIF18A or on the mitotic phenotypes that we quantified, suggesting that electrostatic interactions with amino acids 610 to 617 of KIFBP are not critical for kinesin interaction. A role for the C terminus in KIFBP-motor interactions should not be dismissed, as a recent study identified a novel nonsense KIFBP mutation in a patient with GOSHs that truncates the protein at position 593 (46). It will be interesting to determine whether the C terminus of KIFBP is generally important for its interaction with all kinesins or whether it instead drives interactions with kinesins that are more clinically relevant to GOSHs.

KIFBP remodels the kinesin motor head to displace kinesin α4
Kinesin α4 helix plays a critical role in motor-KIFBP binding. Lysine residues within KIFBP-HP4a (K205) and KIFBP-LH (K307) cross-link residues located in KIF15-L11 (K273 and K283). Unexpectedly, residues in KIFBP-L1 (K26, K30, and K36) also cross-linked KIF15 residues K273 and K283. The significance of these cross-links is not clear, but these data may suggest that an intermediary...
complex between KIFBP and kinesin motor domains, driven by the interaction of KIFBP-L1 with kinesin Loop11, may form before the acquisition of the final bound state.

One outstanding question concerns the mechanism by which KIF15/KIF18A a4 undergoes long-range motion to achieve KIFBP binding. The simplest possibility is that a4 is positionally unstable. If sufficiently compliant, then the adjacent loops, i.e., Loop-11 and Loop-12, may allow a4 excursions that eventually result in “capture” of a4 by KIFBP. Long-range motions of a4 are not without precedent. For example, Wang et al. (47) observed by x-ray crystallography that a4 of KIF19 is positioned much farther from the motor head than is typical. Our MD work, however, suggests that a4 remains closely associated with the motor head (fig. S14), leading us to speculate that the binding of a motor head by KIFBP results in allosteric changes in the structure of both proteins, inducing motions of a4 that predispose it to KIFBP binding. Comparison of KIF15 in apo versus bound states supports this possibility (Fig. 2, D and E). When bound to KIFBP, KIF15 showed a shift of several α helices (α1, α3, and α6) away from the core of the motor as well as large movements of several β-strand pairs. The movement of these structural elements causes the motor to assume a more open conformation.

Another unresolved issue is the functional relevance of a4 extraction by KIFBP. a4 is displaced a substantial distance from its position in the motor head (Fig. 2, A to C), but it is unclear why this displacement is advantageous for the mechanism of action by KIFBP. In principle, steric inhibition of the KIF15/KIF18A microtubule-binding domain should be sufficient to prevent motor-microtubule binding. One possibility is that a4 extraction is necessary for the formation of a stable, long-lived complex; however, as mentioned above, our MD analysis suggests that a4 is only available for extraction due to allosteric changes caused by KIFBP binding. In addition, mutating KIFBP Loop-1 disables both in vitro interaction (Fig. 6) and motor inhibition in cells (Figs. 7 and 8), indicating that a4 displacement is not sufficient for complex formation. Another possibility is that a4 extraction is necessary for complex maintenance, perhaps serving to lock the motor in a bound conformation. It is also yet unknown how KIFBP dissociates from kinesin motor domains; it is possible that the interaction of a4 with HP4α/b-HP5 may be stable enough to require some form of active regulation for disengagement. Future work is required to elucidate the functional implications of a4 extraction.

In summary, our work establishes a structural mechanism by which KIFBP inactivates the microtubule-binding activity of mitotic kinesins KIF15 and KIF18A. Unlike common TPR tandem proteins, KIFBP uses multivalency to form a complex with the kinesin motor head. Multivalency may explain why it has not been possible to identify a consensus sequence for kinesin motors that bind KIFBP versus those that do not (34). Our MD simulations and PCA also indicate that motor-specific steric clashes may serve as a mechanism that prevents certain motors from binding KIFBP. Specifically, we observed that L11 of KIF5C would sterically clash with KIFBP-HP2, whereas L11 of KIF15 and KIF18A fits within the cavity between HP3 and HP4 of KIFBP. Further work is required to test the generality of this idea. An additional area for future work is to reveal the mechanism by which KIFBP dissociates from a kinesin motor. The multivalency with which KIFBP interacts with a kinesin motor, in particular the interaction of HP4α/b-HP5 with a4, suggests that a motor will not readily disengage from KIFBP. Motor recycling may require active regulation, e.g., phosphorylation, as proposed in earlier work (27).

MATERIALS AND METHODS

Plasmid construction

The following plasmids that were used in this study were previously described elsewhere: GST-KIFBP (28), mCherry, and mCherry-KIFBP expression plasmids (28). The construction of the other plasmids used in this study is described as follows.

His6-KIF15-N375 was created through isothermal assembly where the first 375 amino acids of the KIF15 open reading frame were amplified from pEGFP-C1-Kif15-FL (48) and inserted into the pET15b vector. Correct insertion was confirmed by sequencing.

His6-KIF18A-N363 was created through isothermal assembly where a block gene fragment of the first 363 amino acids of KIF18A codon-optimized for expression in Escherichia coli (IDT) was inserted into the pET15b vector. Correct insertion was confirmed by sequencing.

GST-KIFBP-L1α was created by site-directed mutagenesis of GST-KIFBP, replacing amino acids 21 to 40 with the altered amino acid sequence described in Fig. 6B. Similarly, GST-KIFBP-HP9βm was created by site-directed mutagenesis of GST-KIFBP, replacing amino acids 610 to 617 with the altered amino acid sequence described in Fig. 6B. Mutagenesis was confirmed by sequencing of the open reading frame. mCherry-KIFBP-L1α and mCherry-KIFBP-HP9βm were created in the same manner by site-directed mutagenesis of the mCherry-KIFBP WT plasmid. To create the mCherry-KIFBP-L14α plasmid, a GeneStrand containing KIFBP base pairs 1092 to 1588 with the L14α mutations was synthesized (Eurofins). This gene fragment was then inserted into the mCherry-KIFBP expression vector by iso thermal assembly using the commercially available Gibson Assembly Master Mix (New England Biolabs) after PCR amplification of the mCherry-KIFBP expression vector.

Protein expression and purification

Expression of GST-KIFBP, GST-KIFBP-L1α, GST-KIFBP-L14α, and GST-KIFBP-HP9βm was induced in BL21-DE3 cells with 0.4 M isopropyl-β-d-thiogalactopyranoside (IPTG) overnight at 16°C. Cells were pelleted and resuspended in lysis buffer [1× phosphate-buffered saline (PBS), 0.5 mM NaCl, 5 mM β-mercaptoethanol, 1% NP-40, and protease inhibitors [1 mM phenylmethylsulfon fluoride (PMSF), 1 mM benzamidine, and lysophosphatidylcholine (LPC; 10 μg/ml)]] after which they were incubated with lysozyme (1 mg/ml) for 30 min on ice followed by sonication. The lysate was clarified by centrifugation for 30 min at 35,000 rpm at 4°C in a Type 45 Ti rotor (Beckman). Cleared lysate was incubated with 2 ml of glutathione-Sepharose (Thermo Fisher Scientific) for 1 hour and washed with 50 ml [25 CV (column volume)] of wash buffer (1× PBS, 0.5 M NaCl, and 5 mM β-mercaptoethanol). Resin was incubated with 200 μl of PreScission Protease (Cytiva) in 2 ml of cleavage buffer [50 mM tris-HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA, and 1 mM diithiothreitol (DTT)] for 4 hours at 4°C to cleave the GST tag. Protein was eluted with 50 mM tris-HCl (pH 8.0), and peak fractions were combined and clarified by centrifugation for 5 min at 20,000 rpm at 4°C, after which they were subjected to size exclusion chromatography on a Superdex 200 column (GE Healthcare) equilibrated in 10 mM K-Hepes (pH 7.7), 50 mM KCl, and 1 mM DTT. Protein concentration of fractions after gel filtration was estimated using a Bradford assay, after which peak fractions were combined, concentrated
to >1 mg/ml using Amicon 10-kDa centrifugal filter units (Millipore), and either used immediately for cryo-EM or flash-frozen and stored at −80°C.

Expression of His<sub>6</sub>-KIF15-N375 and His<sub>6</sub>-KIF18A-N363 was induced in BL21-DE3 cells with 0.4 M IPTG overnight at 16°C. Cells were pelleted and resuspended in lysis buffer (1× PNI (50 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole), 1% NP-40, 1 mM MgATP, and protease inhibitors [1 mM PMSF, 1 mM benzamidine, and LPC (10 μg/ml)], after which they were incubated with lysis buffer (1 mg/ml) for 30 min on ice followed by sonication. The lysate was clarified by centrifugation for 30 min at 35,000 rpm at 4°C in a Type 45 Ti rotor (Beckman). Cleared lysate was incubated with 2 ml of Ni-NTA (nitriotriacetic acid) agarose (Qiagen) for 1 hour and washed with 50 ml of wash buffer (1× PNI, 100 μM MgATP, and 5 mM β-mercaptoethanol). Protein was eluted with elution buffer (1× PNI, 100 μM MgATP, 5 mM β-mercaptoethanol, and 200 mM imidazole), and peak fractions were combined and clarified by centrifugation for 5 min at 20,000 rpm at 4°C, after which they were subjected to size exclusion chromatography on a Superdex 200 column equilibrated in gel filtration buffer [10 mM K-Hepes (pH 7.7), 50 mM KCl, 1 mM DTT, and 0.2 mM MgATP]. Protein concentration of fractions after gel filtration was estimated using a Bradford assay, after which peak fractions were combined and concentrated to >1 mg/ml using Amicon 10-kDa centrifugal filter units (Millipore). Before cryo-EM grid preparation, the protein was then mixed with equimolar KIFBP and subjected to size exclusion chromatography for a second time on a Superose 6 column (GE Healthcare) equilibrated with gel filtration buffer. Peak fractions were analyzed by SDS-PAGE and stained with Coomassie blue. Fractions containing only the two proteins of interest were then combined, concentrated to >1 mg/ml, and used immediately for cryo-EM.

**Cryo-EM grid preparation and data collection**

For cryo-EM grid preparation, after size exclusion chromatography, KIFBP was concentrated to 4 mg/ml, whereas KIFBP·KIF15 and KIFBP·KIF18A complexes were each concentrated to 1 mg/ml. Aliquots of 4 μl were applied on glow-discharged UltrAuFoil R(1.2/1.3) 300-mesh gold grids (Electron Microscopy Sciences). The grids were then blotted with filter paper and plunge-frozen into liquid ethane cooled by liquid nitrogen using a Vitrobot Mark IV (Thermo Fisher Scientific) set to 4°C, 100% humidity, 1.5-s blot, and a force of 20.

For KIFBP and KIFBP·KIF15 samples, datasets were collected using Leginon (49) on a Thermo Fisher Scientific Glacios transmission electron microscope operating at 200 keV equipped with a Gatan K2 Summit direct electron detector (Gatan Inc.) in counting mode. For KIFBP, a total of 11,086 micrographs were collected through three data collection sessions with total doses of 60 e<sup>−</sup>/Å<sup>2</sup> during 6 to 7 s of exposure time with dose fractionated into 35 to 40 movie frames.

Data collection for the KIFBP·KIF18A sample was automatically collected using Leginon (49) on an FEI Talos Arctica transmission electron microscope operating at 200 keV equipped with a Gatan K2 Summit direct electron detector in counting mode. Three datasets were collected, resulting in a total of 4669 micrographs in a physical pixel size of 0.91 Å per pixel. The total dose ranges from 52 to 62 e<sup>−</sup>/Å<sup>2</sup> in 7 to 8 s of exposure time with dose fractionated into 35 to 40 movie frames.

**Cryo-EM data processing**

The data processing diagram for KIFBP is shown in figs. S2 and S3. Movie alignment, Contrast Transfer Function (CTF) parameter estimation, and particle picking were performed using Warp (50). The resulting particles were imported into cryoSPARC (51) and underwent iterative 2D classification to remove incorrect particle picks. We initially analyzed sample heterogeneity in “dataset3” using ab initio reconstruction with three classes. Particles from the one higher-resolution class were then subjected to another round of ab initio reconstruction with two classes, resulting in two very similar classes. After careful examination, we believe that the first class is the full-length KIFBP map, while the other is the KIFBP lacking the C-terminal helices, explaining why some class averages are missing the C-terminal helix pairs of the KIFBP.

To resolve the structure of the full KIFBP, we deliberately selected the class averages from “dataset1” and “dataset2” that resemble the full KIFBP molecule using cryoSPARC (51). These particles were then combined with the particles from the full-length KIFBP class in dataset3 and subjected to ab initio reconstruction with two classes. Particles from the higher-resolution classes were selected for nonuniform refinement (52) to obtain a 4.7-Å-resolution map. The map quality was improved to 4.6 Å using local refinement with a static mask. The resulting map was manually sharpened for the visual inspection purpose using a B-factor of −50 Å<sup>2</sup>.

Next, we focused on analyzing the N terminus of KIFBP lacking the C-terminal helices to try to improve the resolution. Particles from dataset2 were chosen because CTF fit resolution in dataset2 was the best among all three datasets. A total of 913,455 particles from Warp (50) were imported into cryoSPARC (51). We then performed iterative heterogeneous refinement with one class from the KIFBP lacking the C-terminal helices and two low-resolution classes from failed ab initio reconstruction jobs. A total of 301,491 particles corresponding to KIFBP were enriched after extensive heterogeneous refinement and 2D classification. These particles were then subjected to homogeneous refinement and local refinement, resulting in a 3.8-Å-resolution map. However, the quality of the map was not satisfactory. To improve the map quality, the particles were exported into RELION-3.1 (53) and underwent one round of 3D autorefinement to obtain a reconstruction at 4.2 Å. Subsequently, two rounds of CTF refinement were performed to correct for beam tilt (54). 3D autorefinement with refined beam tilt yielded an estimated resolution of 4.1 Å. We then exported micrograph motion trajectories from Warp and performed Bayesian polishing (55) to optimize per-particle motion tracks. 3D autorefinement from the polished particles resulted in a 3.8-Å map. Following this step, one round of 3D classification with six classes was performed to further remove heterogeneity. Five classes corresponding to KIFBP lacking the C terminus were selected and underwent another round of refinement, yielding a 3.8-Å-resolution map. The particles were then reextracted and recentered. The following 3D autorefinement yielded a 4-Å map. Bayesian polishing was performed on this particle stack, resulting in an improved map.
quality. 3D classification without alignment using $T = 12$ resulted in one high-resolution class. 3D autorefinement using the 128,190 particles from this high-resolution class gave a 3.8-Å map with improved map quality.

For KIFBP:KIF15, all the data processing steps were performed in cryoSPARC (51), as presented in fig. S8. To generate an initial map for the KIFBP:KIF15 complex, 1007 movies from dataset1 were imported into cryoSAPRC (51). Patch motion correction and patch CTF estimation were used to correct beam-induced motion and estimate CTF parameters. A total of 235,721 particles were automatically picked using the Topaz general model (56). These particles were then subjected to iterative 2D classification to remove incorrect particle picks. The resulting 32,262 particles were used for ab initio reconstruction with one class and also retraining Topaz. The model from the ab initio reconstruction was refined to ~7 Å and used as a template for the heterogeneous refinement in the following steps.

Movies from dataset1, dataset2, dataset3, and dataset4 were imported into cryoSPARC (51) and processed separately at the beginning steps. Movies were aligned using patch motion correction with dose weighting. CTF parameters were estimated with patch CTF estimation. Micrographs with CTF fit resolution below 5 Å were selected and subjected to particle picking using a restrained Topaz model. The picked particles underwent one round of 2D classification to remove incorrect particle picks. We then performed iterative heterogeneous refinement with one class from the initial template and two low-resolution classes from the early terminated ab initio reconstruction jobs to enrich particles corresponding to KIFBP:KIF15 complex. The resulting particles were further cleaned by 2D classification and ab initio reconstruction with multiclasses. Particles from the individual dataset were then reextracted, recentered, and combined, resulting in 189,984 particles. These particles were subsequently classified into three classes using ab initio reconstruction. Two classes showing KIFBP and KIF15 density were merged and further classified into two classes with ab initio reconstruction. One class with the better KIF15 motor domain density was selected and subjected to homogeneous refinement, resulting in a 4.8-Å-resolution map. Then, local refinement with a user-defined mask was performed to improve the map quality.

For KIFBP:KIF18A, the data processing diagram is presented in fig. S11. A total 4669 micrographs were collected through three datasets. For each dataset, motion correction, CTF estimation, and particle picking were performed in Warp (50), resulting in 71,529 particles (dataset1), 94,716 particles (dataset2), and 638,186 particles (dataset3). These particles were imported into cryoSPARC (51) and underwent interactive 2D classification to remove incorrect particle picks. The remaining 156,159 particles were used for ab initio reconstruction into four classes in cryoSPARC. A total of 54,801 particles from the class with clear KIF18A density were selected for homogeneous refinement to obtain a 5-Å-resolution structure of the KIFBP:KIF18A complex. The quality of the map was further improved by local refinement in cryoSPARC with a user-defined mask.

Model building
To construct an atomic model of KIFBP, first, we began by de novo building of the 3.8-Å KIFBP reconstruction using Coot (57) on the RELION (58) postprocessed reconstruction. To guide model building, we used density modification with DeepEMhancer (59) that was run on the COSMIC$^2$ science gateway (60) to help interpret the cryo-EM density. From this process, we built amino acids 5 to 403 of KIFBP, and the RELION postprocessed map was used for model refinement and validation using Phenix (61). After building this high-resolution part of our reconstruction, we built polyalanine models for the C-terminal helices KIFBP-HP6b, KIFBP-HP7, KIFBP-HP8, and KIFBP-HP9 using Coot (59). The manual build model was then subjected to real-space refinement in Phenix (61).

Because of the moderate resolution (4.8 Å) of KIF15:KIFBP, we built the model of KIF15:KIFBP using a combination of Rosetta-CM (36), Rosetta-Relax, and manual building in Coot (57). For the KIFBP model, we manually docked the KIFBP model into the density using Chimera (62), after which we fit the model into the density Rosetta-Relax. To fit KIF15 into the density, we manually docked KIF15 [Protein Data Bank (PDB): 4BN2] (37) into the cryo-EM density, removing KIF15-L11, KIF15-α4, and KIF15-L12 from the model. With this docking, we then ran Rosetta-CM (36), using atomic models 1V8K (Chain A) (63), 2OWM (Chain B) (64), 3U06 (Chain A) (65), 4BN2 (Chain C) (37), 5G5Z (Chain A) (66), 5MIO (Chain C) (67), 5MLV (Chain D) (68), 5MM4 (Chain K) (69), 5MM7 (Chain K) (69), and 6B01 (Chain K) (70) as the library of fragments for re-building. After running Rosetta-CM to calculate 5000 models, we used the lowest scoring model for the final step of Rosetta-Relax. To build KIF15-L11, KIF15-α4, and KIF15-L12, we built a polyalanine model manually using Coot (57).

For the KIF18A:KIFBP model, we used Rosetta-Relax to fit the KIFBP model into the density. For the KIF18A motor, we manually docked the crystal structure of KIF18A (PDB: 3LRE) (71) into the density with the exception of KIF18A-L11, KIF18A-α4, and KIF18A-L12. To build KIF18A-L11, KIF18A-α4, and KIF18A-L12, we built a polyalanine model manually using Coot (57).

The efficiency (cryoEF) (72) for each reconstruction was calculated on the COSMIC$^2$ science gateway (60). Figures were prepared using Chimera (62) and Chimera X (73).

Cross-linking mass spectrometry
H$_{13}$-KIF15-N375 and KIFBP were purified as described above. An equimolar solution of both proteins was prepared in a cross-linking buffer [40 mM Hepes (pH 7.4)] where the total protein concentration was 10 μM and the amount of each protein was at least 20 μg. A 50 mM solution of the 11-Å lysine-targeting cross-linker BS3 was prepared in water and added to the reaction in a 100 M excess. The reaction proceeded for 30 min while rotating at 4°C, after which it was quenched with tris-HCl (pH 7.5) at a final concentration of 50 mM. As an uncross-linked control, a separate reaction was prepared and quenched the same way, but no cross-linker was added.

The cross-linking reactions were resuspended in 50 μl of 0.1 M ammonium bicarbonate buffer (pH ~8). Cysteines were reduced by adding 50 μl of 10 mM DTT and incubating at 45°C for 30 min. Samples were cooled to room temperature, and alkylation of cysteines was achieved by incubating with 65 mM 2-chloroacetamide, under darkness, for 30 min at room temperature. Overnight digestion with 1:50 enzyme:substrate modified trypsin was carried out at 37°C with constant shaking in a ThermoMixer. Digestion was stopped by acidification, and peptides were desalted using SepPak C18 cartridges using the manufacturer's protocol (Waters). Samples were completely dried via Vacufuge. Resulting peptides were dissolved in 9 μl of 0.1% formic acid/2% acetonitrile solution, and
2 μl of the peptide solution was resolved on a nano-capillary reverse-phase column (Acclaim PepMap C18, 2 μm, 50 cm, Thermo Fisher Scientific) using a 0.1% formic acid/2% acetonitrile (buffer A) and 0.1% formic acid/95% acetonitrile (buffer B) gradient at 300 nl/min over a period of 180 min (2 to 25% buffer B in 110 min, 25 to 40% in 20 min, and 40 to 90% in 5 min followed by holding at 90% buffer B for 10 min and equilibration with buffer A for 30 min). The eluent was directly introduced into a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, San Jose, CA) using an EasySpray source. MS1 scans were acquired at 60 K resolution [automatic gain control (AGC) target, 3 × 10⁶; max Injection time (IT), 50 ms]. Data-dependent collision-induced dissociation MS/MS spectra were acquired using the Top speed method (3 s) following each MS1 scan.

24-well plate format, ~75,000 cells in 500 μl of MEM-α medium (Gibco) containing 10% fetal bovine serum (Gibco). For plasmid transfections in a 24-well plate format, ~75,000 cells in 500 μl of MEM-α medium were seeded onto acid-washed glass coverslips and subsequently transfected with 375 ng of mCherry alone or mCherry-KIFBP plasmid DNA (containing WT KIFBP sequence or indicated KIFBP mutant). Cells were treated with mCherry and indicated mCherry-KIFBP plasmids that were preincubated for 10 min in 50 μl of Opti-MEM (Gibco) and 1 μl of Lipofectamine LTX reagent (Invitrogen). Plasmid transfections were incubated for 24 hours before fixation for immunofluorescence.

Cell fixation and immunofluorescence
For metaphase observations of spindle length and chromosome alignment, cells expressing mCherry and mCherry-KIFBP (WT or indicated mutants) were treated with 20 μM MG132 (N-carbobenzyloxy-t-leucyl-t-leucyl-t-leucinal) (Selleck Chemicals) for 2 hours before fixation. Cells were fixed on coverslips in −20°C methanol (Thermo Fisher Scientific) with 1% paraformaldehyde (Electron Microscopy Sciences) for 10 min on ice. Coverslips were then washed three times for 5 min each in tris-buffered saline [TBS; 150 mM NaCl and 50 mM tris base (pH 7.4)]. Coverslips were blocked for 1 hour at room temperature in 20% goat serum in antibody dilution buffer [Abdil: TBS (pH 7.4), 1% bovine serum albumin, 0.1% Triton X-100, and 0.1% sodium azide]. Coverslips were then washed two times in TBS for 5 min each before the addition of primary antibodies. Primary antibodies were diluted in Abdil. For KIF18A localization analyses, the following primary antibodies were used at the indicated dilutions: rabbit anti–α-tubulin 1:500 (MAB1864; Sigma-Aldrich), rabbit anti-KIF18A 1:100 (A301-080A; Bethyl), and mouse anti-Hecl 1:500 (GTX70268; GeneTex). All mCherry images for KIF18A localization analyses are direct mCherry fluorescence. For KIF18A localization analyses, the following secondary antibodies were used at 1:500 dilution: goat anti-rabbit immunoglobulin G (IgG) conjugated to Alexa Fluor 488 (A11034; Invitrogen), goat antiserum IgG conjugated to Alexa Fluor 405 (A31553; Invitrogen), and goat anti-rat IgG conjugated to Alexa Fluor 647 (A21247; Invitrogen). For spindle length and chromosome alignment analyses, the following primary antibodies were used at the indicated dilutions: mouse anti–γ-tubulin 1:500 (T5326; Sigma-Aldrich), rabbit anti-mCherry 1:500 (ab167453; Abcam), and human anti-centromere antibody (ACA) 1:250 (15-235; Antibodies Inc.). All primary antibodies were incubated for 1 hour at room temperature with the exception of the human ACA antibody, which was incubated at 4°C overnight. For spindle length and chromosome alignment analyses, the following secondary antibodies were used at 1:500 dilution: goat anti-human IgG conjugated to Alexa Fluor 488 (A11013; Invitrogen), goat antimouse IgG conjugated to Alexa Fluor 405 (A31553; Invitrogen), and goat anti-rabbit IgG conjugated to Alexa Fluor 594 (A11037; Invitrogen). Coverslips were washed two times in TBS for 5 min each between primary and secondary antibody incubations. Coverslips were washed three times in TBS for 5 min each before mounting coverslips with ProLong Gold Antifade mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (spindle length and chromosome alignment analyses) (P36935, Invitrogen) or ProLong Gold Antifade mounting medium without DAPI (KIF18A localization analyses) (P36934, Invitrogen). Coverslips were imaged on a Ti-E inverted microscope (Nikon Instruments) using a Plan Apo λ 60× 1.42 numerical aperture objective, environmental chamber at 37°C, a Clara cooled charge-coupled device camera (Andor), and Nikon Elements Software (Nikon Instruments).

Cell culture and transfections
HeLa Kyoto cells were cultured at 37°C with 5% CO₂ in MEM-α medium (Gibco) containing 10% fetal bovine serum (Gibco). For plasmid transfections in a 24-well plate format, ~75,000 cells in 500 μl of MEM-α medium were seeded onto acid-washed glass coverslips and subsequently transfected with 375 ng of mCherry alone or mCherry-KIFBP plasmid DNA (containing WT KIFBP sequence or indicated KIFBP mutant). Cells were treated with mCherry and indicated mCherry-KIFBP plasmids that were preincubated for 10 min in 50 μl of Opti-MEM (Gibco) and 1 μl of Lipofectamine LTX reagent (Invitrogen). Plasmid transfections were incubated for 24 hours before fixation for immunofluorescence.
above. As described previously (28, 39), single focal plane images with both spindle poles in focus were acquired. A boxed region of interest with a fixed height and width defined by the length of the spindle was used to measure the distribution of ACA-labeled kinetochore fluorescence using the Plot Profile command in Fiji. The ACA signal intensity was normalized internally to its highest value and plotted as a function of distance along the pole-to-pole axis. These plots were then fitted to a Gaussian curve, and the FWHM for the Gaussian fit and the spindle length are reported for each cell analyzed. Means and SDs are reported from a minimum of three independent experiments for each construct. The following cell numbers were analyzed for the indicated mCherry and mCherry-KIFBP constructs: (i) mCherry (control) = 132 cells, (ii) mCherry-KIFBP WT = 165 cells, (iii) mCherry-KIFBP-L14m = 102 cells, (iv) mCherry-KIFBP-L14m = 99 cells, and (v) mCherry-KIFBP-HP9b = 89 cells.

KIF18A line scan analysis
Cells expressing mCherry or indicated mCherry-KIFBP constructs were fixed and stained for endogenous KIF18A, α-tubulin, and Hec1 as described above. Cells were imaged with 0.2-μm z-stacks throughout the entire cell. Within these z-sections, 2-μm line scans were manually drawn in Fiji for individual kinetochore microtubules (one to three line scans per cell), and the profile intensities along those lines were measured and recorded for the KIF18A, α-tubulin, and Hec1 channels. Each of these profile intensities for KIF18A, α-tubulin, and Hec1 was normalized internally to its highest value. These normalized line scans were then aligned by peak Hec1 intensity and averaged for each pixel distance. Means and SDs are reported from a minimum of three independent experiments for each construct. The following cell numbers and line scans were analyzed for the indicated mCherry and mCherry-KIFBP constructs: (i) mCherry (control) = 40 cells (64 lines), (ii) mCherry-KIFBP WT = 34 cells (64 lines), (iii) mCherry-KIFBP-L14m = 34 cells (64 lines), (iv) mCherry-KIFBP-L14m = 32 cells (68 lines), and (v) mCherry-KIFBP-HP9b = 33 cells (63 lines).

MD simulations and analysis
The structures of KIF5C, KIF15, KIF18A, and KIF11 bound to ADP and Mg2+ were taken from PDB structures 1BG2 (74), 4BN2 (37), 3LRE (71), and 1II6 (75), respectively. The missing residues of KIF5C were filled in as previously described (76). For all other proteins, I-TASSER was used to fill in the gaps of the remaining structures. AmberTools was then used to prepare all systems for simulation (80). Each system was solvated with a box of TIP3P water molecules with 10-Å padding around the protein. Na+ and Cl− were added to both neutralize the charge and cause oculomotor axon stalling. As described previously (85), single focal plane images with both spindle poles in focus were acquired. A boxed region of interest with a fixed height and width defined by the length of the spindle was used to measure the distribution of ACA-labeled kinetochore fluorescence using the Plot Profile command in Fiji. The ACA signal intensity was normalized internally to its highest value and plotted as a function of distance along the pole-to-pole axis. These plots were then fitted to a Gaussian curve, and the FWHM for the Gaussian fit and the spindle length are reported for each cell analyzed. Means and SDs are reported from a minimum of three independent experiments for each construct. The following cell numbers were analyzed for the indicated mCherry and mCherry-KIFBP constructs: (i) mCherry (control) = 132 cells, (ii) mCherry-KIFBP WT = 165 cells, (iii) mCherry-KIFBP-L14m = 102 cells, (iv) mCherry-KIFBP-L14m = 99 cells, and (v) mCherry-KIFBP-HP9b = 89 cells.

All analysis was carried out using the Bio3d package (v2.4.1) in R (85). We first aligned our four kinesins of interest and then restricted analysis to the amino acids that appeared in all the motors. To focus on large-scale rearrangements in the motor head and remove the noise from fluctuating loops, we next restricted analysis to amino acids in stable secondary structures, leaving us with 154 amino acid positions in each protein. We performed PCA using KIF15 as the reference structure. The remaining three kinesin simulations were then projected onto this KIF15 PCA space for direct comparison with each other and the cryo-EM structure.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.aby9812

View/request a protocol for this paper from Bio-protocol.

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