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1        **The mechanism of kinesin inhibition by kinesin binding protein**

2

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24

25

26 **Abstract**

27

28 Subcellular compartmentalisation is necessary for eukaryotic cell function. Spatial  
29 and temporal regulation of kinesin activity is essential for building these local  
30 environments via control of intracellular cargo distribution. Kinesin binding protein  
31 (KBP) interacts with a subset of kinesins via their motor domains, inhibits their  
32 microtubule (MT) attachment and blocks their cellular function. However, its  
33 mechanisms of inhibition and selectivity have been unclear. Here we use cryo-  
34 electron microscopy to reveal the structure of KBP and of a KBP-kinesin motor  
35 domain complex. KBP is a TPR-containing, right-handed  $\alpha$ -solenoid that sequesters  
36 the kinesin motor domain's tubulin-binding surface, structurally distorting the motor  
37 domain and sterically blocking its MT attachment. KBP uses its  $\alpha$ -solenoid concave  
38 face and edge loops to bind the kinesin motor domain, and selected structure-guided  
39 mutations disrupt KBP inhibition of kinesin transport in cells. The KBP-interacting  
40 motor domain surface contains motifs exclusively conserved in KBP-interacting  
41 kinesins, suggesting a basis for kinesin selectivity.

42

43

## 44 **Introduction**

45

46 Kinesins are a superfamily of microtubule (MT)-based molecular motors that play  
47 important roles in cellular functions such as mitosis, cell motility and intracellular  
48 transport<sup>1-3</sup>. Kinesins are categorised into 14 sub-classes (kinesin-1 to kinesin-14<sup>4</sup>) by  
49 motor domain conservation and within these sub-classes individual family members  
50 (a total of 45 'KIF' or 'Kif' genes in humans and mice respectively) have a wide  
51 range of functional characteristics and biological roles<sup>1,5</sup>. Dysfunction of kinesin  
52 family members has been implicated in a number of pathological conditions<sup>6,7</sup>. The  
53 kinesin motor domain is the microtubule-binding engine that drives these activities,  
54 converting the chemical energy of ATP binding and hydrolysis into mechanical force.  
55 While these mechanical forces are classically used to generate motility in transport  
56 kinesins, some kinesin family members drive MT organisation or depolymerisation of  
57 MTs.

58 Kinesins are highly regulated in order to prevent both waste of ATP and to  
59 spatially and temporally control kinesin function. This is particularly important in  
60 highly polarised and compartmentalised cells such as neurons. Kinesin regulation via  
61 inhibition of their motor domains can occur through a number of mechanisms that  
62 limit ATPase activity and/or block track binding - these include intramolecular  
63 inhibition by kinesin tail domains, post-translational modification of the motor, or  
64 through interactions with regulatory binding partners. Recently, it has been  
65 demonstrated that a subset of kinesin superfamily members, including kinesin-2s, -3s,  
66 -8s and -12s, are sequestered by kinesin binding protein (KBP; KIF1BP; KIAA1279),  
67 which inhibits MT track attachment by their motor domains and, thus, blocks their  
68 MT-related functions<sup>8-10</sup>.

69           KBP is expressed in multiple human tissues including brain and heart<sup>8</sup>.  
70   Mutations in the KBP have been identified as causing autosomal recessive Goldberg-  
71   Shprintzen syndrome (GOSHS)<sup>11-14</sup>, which presents as congenital facial dysmorphism,  
72   nervous system pathology and dysfunction and heart defects<sup>15</sup>. In addition, KBP gene  
73   copy number has been recently reported as predictive in paediatric neuroblastoma  
74   prognosis, prompting its suggestion as a drug target<sup>16</sup>. KBP was originally identified  
75   as a kinesin-3 binding partner that modulated its mitochondrial transport function<sup>8</sup>;  
76   however, KBP has since been shown to interact with a subset of other kinesin family  
77   members to regulate diverse cellular processes including mitosis<sup>17,18</sup>,  
78   spermatogenesis<sup>19</sup> and neuronal differentiation, growth and cargo distribution<sup>10,20-24</sup>.

79           We do not currently know what the structure of KBP is, nor understand the  
80   mechanism of KBP-kinesin inhibition. It is also completely unknown how KBP  
81   differentiates between particular kinesin family members. KBP is a 72 kDa protein, is  
82   predicted to contain several tetratricopeptide repeats (TPRs) and to be mainly  $\alpha$ -  
83   helical in secondary structure content<sup>8,9</sup>. Here we present cryo-electron microscopy  
84   (cryo-EM) structures of KBP alone and of KBP bound to the motor domain of the  
85   human mitotic kinesin KIF15 (a 110 kDa complex). We show that KBP is a TPR-  
86   containing, right-handed  $\alpha$ -solenoid protein composed of 9 antiparallel  $\alpha$ -helix pairs  
87   interrupted by a linker region. We also show that KBP's concave face binds KIF15  
88   via its MT-binding elements and induces a large displacement of the kinesin  $\alpha$ 4 helix,  
89   sterically inhibiting MT association. Finally, we show that KBPs kinesin selectivity is  
90   associated with specific kinesin sequences spread across the interaction surface.

91

92 **Results**

93

94 ***KBP is a TPR-containing, right-handed  $\alpha$ -solenoid***

95

96 The 3D structure of the ~72 kDa KBP at 4.6 Å resolution (Figure 1 and Figure 1–  
97 figure supplement 1a,b) was determined using cryo-EM data collected using a Volta  
98 Phase Plate (VPP), and an atomic model was calculated (see Methods). Our structure  
99 revealed that KBP is a right-handed  $\alpha$ -solenoid protein (Figure 1a,b and Figure 1–  
100 figure supplement 1c-e). Nine pairs of anti-parallel  $\alpha$ -helices ( $\alpha$ HP1 ( $\alpha$ -helical pair 1)  
101 to  $\alpha$ HP9) are broken by a single ‘linker  $\alpha$ -helix’ (L $\alpha$ H) and ‘linker loop’ (LL) in the  
102 centre of the fold separating KBP into N-terminal and C-terminal subdomains (Figure  
103 1 and Figure 1–figure supplement 1c-e, Figure 1–figure supplement 2). The four  
104 predicted TPR motifs contribute exclusively to  $\alpha$ -helical pairs in the N-terminal  
105 subdomain (Figure 1a,d,e).

106 The supercoiling  $\alpha$ -helical pairs form concave and convex faces linked by short  
107 and long loops that constitute the two edges of the  $\alpha$ -solenoid (Figure 1–figure  
108 supplement 1c-e, Figure 1–figure supplement 2). In contrast to the shorter loops, the  
109 longer loops (>7 residues) tend to be partially disordered, show low sequence  
110 homology between KBP orthologues in different species and are mainly found in the  
111 N-terminal subdomain (e.g. L2, L6 and L10, Figure 1–figure supplement 1d-e, Figure  
112 1–figure supplement 2). The linker loop is the longest (62 residues) and is thus unique  
113 in the KBP structure because it is reasonably conserved and mainly ordered, with  
114 visible corresponding density clearly bridging the N and C-terminal subdomains  
115 (Figure 1a-b, Figure 1–figure supplement 1d-e, Figure 1–figure supplement 2 and  
116 Figure 1–figure supplement 3). Despite this clear ordered density, this loop was not

117 modelled due to low homology to available structures and a lack of consensus in  
118 secondary structure prediction (see Methods). In spite of this lack of consensus,  
119 density in this region suggests that part of this loop may form further  $\alpha$ -helical  
120 structures. Other TPR-containing  $\alpha$ -solenoid proteins form important regulatory  
121 interactions in numerous contexts, and the structure we describe is indicative of  
122 similar properties for KBP.

123

124 ***KBP conformationally adapts to bind KIF15's motor domain using both***  
125 ***subdomains***

126

127 To elucidate the mechanism of kinesin inhibition by KBP, we determined the  
128 structure of KBP in complex with the human KIF15 (kinesin-12) motor domain  
129 (KIF15\_MD, 1-375). This construct, which has six of its eight cysteine residues  
130 mutated to serine (C5S, C50S, C162S, C294S, C314S, C346S) and two additional  
131 cysteines were inserted (S250C, G375C), has comparable steady state ATPase  
132 activity to previously published reports<sup>25</sup> (Figure 2–figure supplement 1a) and we  
133 refer to it as KIF15\_MD6S. The overall resolution of this KBP-KIF15\_MD6S  
134 complex was 6.9 Å, with KBP and KIF15\_MD6S determined to similar local  
135 resolutions (Figure 2–figure supplement 1b-c). We built a model of the complex via  
136 flexible fitting using our KBP model and the KIF15\_MD crystal structure (Figure  
137 2a,b and see Methods). The complex is arranged such that KIF15\_MD6S sits in the  
138 concave face of the KBP  $\alpha$ -solenoid, analogous to a baseball enclosed in a baseball  
139 glove. The kinesin MD is positioned centrally between the N and C-terminal  
140 subdomains and contacts the KBP concave face and loops at the  $\alpha$ -solenoid edges.

141           When the structure of KBP-alone is superimposed onto KBP in the KBP-  
142 KIF15\_MD6S complex, it is clear that KBP undergoes a conformational change in the  
143 presence of its kinesin motor domain binding partner, with the largest differences  
144 resulting from an unfurling motion of its N-terminal subdomain (Figure 2c,d and  
145 Video 1). The KBP-alone model is incompatible with KIF15\_MD6S binding, due to  
146 clashes with L14 in the C-terminal subdomain and  $\alpha$ HP3a,  $\alpha$ HP4a and L8 in the N-  
147 terminal subdomain. The conformational changes in KBP upon KIF15\_MD6S  
148 binding relieves these clashes in the complex (Video 1).

149           To establish whether the KBP-KIF15\_MD6S mode of interaction applied to  
150 other kinesins, we also collected data of the complex formed by KBP with the motor  
151 domain of the human kinesin-3 KIF1A (KIF1A\_MD). 2D classification of these  
152 images revealed a number of classes with an extra-density corresponding the size of a  
153 kinesin motor domain bound to the concave face of KBP, consistent with what was  
154 observed in the KBP-KIF15\_MD6S dataset (Figure 2–figure supplement 1c).  
155 However, in contrast to the KBP-KIF15\_MD6S sample, these KBP-KIF1A\_MD 2D  
156 classes provided only limited views of the complex (Figure 2–figure supplement  
157 1c,d), such that a reliable 3D structure could not be calculated. Intriguingly, in  
158 addition, the extra kinesin density in the 2D classes appeared to have a somewhat  
159 flexible position relative to KBP. However, these data did allow us to confirm that  
160 indeed KIF1A\_MD also interacts with KBP on its concave face in the same way as  
161 KIF15\_MD6S and suggests a common mechanism of kinesin inhibition by KBP.

162

163 ***KIF15\_MD6S binds KBP via rearrangement of its tubulin-binding subdomain***

164



165 We examined the effect of KBP binding on the conformation of KIF15\_MD6S.  
166 Kinesin motor domains can be structurally divided into three distinct subdomains<sup>26,27</sup>  
167 which undergo coordinated conformational changes during the MT-based kinesin  
168 ATPase cycle. MT binding stabilises the tubulin-binding subdomain of the MD while  
169 the P-loop and Switch 1/2 subdomains – which contain the conserved nucleotide-  
170 coordinating P-loop and Switch 1 and 2 motifs - move relative to each other in  
171 response to the nucleotide state of the MD<sup>26-28</sup>. We determined the structure of the  
172 MT-bound, AMPPNP state of KIF15\_MD6S, which shows that this MD adopts a  
173 canonical conformation (Figure 3–figure supplement 1). Comparison of this  
174 conformation with an ADP-bound Kif15\_MD crystal structure (PDB: 4BN2<sup>25</sup>)  
175 illustrates the scale of these MT- and nucleotide-dependent subdomain  
176 rearrangements in KIF15, which are similar to those seen in other kinesins MDs<sup>26,28,29</sup>  
177 (Figure 3–figure supplement 1d,e and Figure 3c,d).

178 The structure of the KBP-KIF15\_MD6S complex revealed that KBP binds the  
179 kinesin motor domain via the tubulin-binding subdomain (Figure 3). While the P-loop  
180 and Switch 1/2 subdomains of the KIF15\_MD crystal structure and associated Mg<sup>2+</sup>-  
181 ADP generally fitted well into density of the KBP-KIF15\_MD6S complex, a large  
182 portion of the tubulin-binding subdomain did not (Figure 3a; Figure 3–figure  
183 supplement 2a,b). In particular, there is a striking lack of density in the expected  
184 position for helix  $\alpha$ 4 (Figure 3a; Figure 3–figure supplement 2b). Instead, there was  
185 strong density of length and width consistent with helix  $\alpha$ 4 displaced by  $\sim 15$  Å into  
186 the concave face of KBP, and which we modelled as such (Figure 3b,e, Figure 2 and  
187 Figure 3–figure supplement 2b-e). This displacement of helix  $\alpha$ 4, which lies close to  
188 the TPR-repeat region of the N-terminal subdomain of KBP, is accompanied by  
189 additional rearrangements of the flanking L11 and L12 in KIF15\_MD6S (labelled

190 KL11 and KL12, Figure 3c-e: Figure 3–figure supplement 2b-e). A number of other  
191 TPR-containing  $\alpha$ -solenoids are known to bind peptide motifs with  $\alpha$ -helical content  
192 within their concave faces<sup>30-32</sup> (Figure 3–figure supplement 3), and our structure  
193 shows that KBP binds helix  $\alpha$ 4 of KIF15\_MD6S in a similar way.

194 The KBP-bound conformation of the KIF15\_MD6S tubulin-binding subdomain  
195 is also radically different from its MT-bound conformation (Figure 3d-e). The  
196 tubulin-binding subdomain forms the majority of the MT-binding surface in the  
197 KIF15\_MD6S-MT complex (Figure 3d, Figure 3–figure supplement 1) such that KBP  
198 and MTs cannot simultaneously bind KIF15\_MD6S due to extensive steric overlap  
199 (Figure 3d-e). In summary, KBP sequesters and blocks the MT-interacting surface of  
200 kinesin motor domains via a mechanism that involves significant conformational  
201 change within the motor domain.

202

203 ***KBP binds kinesin motor domains via conserved motifs in the  $\alpha$ -solenoid edge loops***  
204 ***and  $\alpha$ -helices at the concave face***

205

206 KBP contacts the KIF15\_MD6S both via 1) loops connecting the  $\alpha$ -solenoid edges  
207 and 2) TPR-containing  $\alpha$ -helices at the concave face (Figure 4, Figure 4–figure  
208 supplement 1 and Video 2). At the  $\alpha$ -solenoid edges, L1, L3, L5 and L10 in the N-  
209 terminal subdomain and L12, L14, L16 and L18 in the C-terminal subdomain are  
210 close enough to KIF15\_MD6S to be involved in binding. The closest interaction of  
211 these were KBP L12 and L14, which contact both K $\beta$ 5-KL8 and KL12-K $\alpha$ 5-KL13  
212 regions of the KIF15 tubulin-binding subdomain (Figure 4). KBP's disordered L1 lies  
213 close to KIF15\_MD6S's KL9, while the shorter, ordered L3 and L5 are situated near  
214 but not contacting KL11 and K $\alpha$ 6 (Figure 4–figure supplement 1a,b). KBP's C-

215 terminal L16 and L18 are close enough to KIF15\_MD6S that they may interact with  
216 the flexible KL12, N-terminus or neck-linker. At the TPR-containing region of the  
217 concave face of KBP,  $\alpha$ HP4a,  $\alpha$ HP4b and  $\alpha$ HP5a contact the K11-K $\alpha$ 4-KL12 region  
218 of KIF15\_MD6S (Figure 4c-d).

219 To test the functional significance of this interface, we investigated KBP-  
220 kinesin interactions in cells, and examined the activities of mutant KBP constructs in  
221 which the predicted interacting amino acids within potentially kinesin-contacting  
222 loops were substituted for Ala, Gly or Pro residues (Figure 1–figure supplement 2,  
223 and Table 2). Ala-substitutions in the TPR-containing  $\alpha$ -helices at the KBP concave  
224 face were also introduced at particularly inter-species conserved polar residues  
225 predicted to interact with the KIF15\_MD K11-K $\alpha$ 4-KL12 region (Tyr-213 and Gln-  
226 216 in  $\alpha$ HP4a, Gln-238 in  $\alpha$ HP4b, Thr-255 and Gln-258 in  $\alpha$ HP5a; Figure 4c,d and  
227 Figure 1–figure supplement 2). All mutant constructs exhibited roughly equivalent  
228 expression patterns which were also comparable to WT KBP (Figure 5 – figure  
229 supplement 1).

230 We first used pull-down assays. Mouse Kif15 or Kif1A constructs consisting of  
231 only the motor domain and the first coiled-coil region (Kif15\_MDC or Kif1A\_MDC)  
232 were fused to bioGFP and co-expressed with various HA-tagged human KBP  
233 constructs in HEK293T cells, followed by pull-down of HA-KBP by the bioGFP-  
234 KIF\_MDC<sup>9</sup> (Figure 5–figure supplement 2). Although there are moderate qualitative  
235 differences in binding by the two motors, the effects of KBP mutations on motor  
236 binding – described in following - are essentially the same. Ala-substitutions in the  
237 TPR-containing  $\alpha$ -helices at the KBP concave face ( $\alpha$ HP4a,  $\alpha$ HP4b), which lie at the  
238 heart of the KBP- KIF15\_MD6S structural interface, strongly reduced KBP's  
239 interaction with both KIF15\_MDC and KIF1A\_MDC.  $\alpha$ HP5a mutants had a similar

240 but less pronounced effect (Figure 5–figure supplement 2b,c). In contrast, mutation of  
241 L1, L3 or L5 in the KBP N-terminal subdomain or L10 or L16 in the C-terminal  
242 subdomain – none of which form directly visualised interactions with KIF15\_MD6S  
243 in the cryo-EM reconstruction - has no effect on KBP’s interaction with either  
244 KIF15\_MDC or KIF1A\_MDC (Figure 5–figure supplement 2b,c). Mutation of L12  
245 (to some extent) and of L14 (to a greater extent) – which contact both K $\beta$ 5-KL8 and  
246 KL12-K $\alpha$ 5-KL13 - reduced KBP interaction with KIF15\_MDC and KIF1A\_MDC  
247 (Figure 5–figure supplement 2 b,c). Mutation of L12 + L14 additively disrupted the  
248 KBP-motor interaction, consistent with the structural proximity of these two loops in  
249 the kinesin-KBP complex. L10 + L12 and L10 + L14 mutants also had weaker  
250 interactions with KIF15\_MDC/KIF1A\_MDC (Figure 5–figure supplement 2 b,c),  
251 again pointing to the additive contributions of loops in the KBP C-terminal  
252 subdomain to kinesin binding. Strikingly, mutation of L18 appears to enhance the  
253 interaction between KBP and both KIF15\_MDC and KIF1A\_MDC, suggesting that it  
254 may somehow contribute to negative regulation of binding in the context of WT KBP.

255 We then used a previously described inducible peroxisome translocation assay  
256 in COS-7 cells<sup>9</sup>. In this assay, dimeric mouse Kif15\_MDC or Kif1A\_MDC constructs  
257 with an FRB-tag (Kif15\_MDC-FRB or Kif1A\_MDC-FRB) are expressed together  
258 with PEX-mRFP-FKBP, a peroxisome-binding construct, along with the various KBP  
259 constructs. Addition of rapalog induces FRB-FKBP heterodimerisation and motor-  
260 driven peroxisome translocation to the cell periphery, but when the motor is inhibited  
261 by KBP, peroxisome translocation is blocked (Figure 5a-c). Kinesin-mediated  
262 translocation was measured, first by quantifying the number of cells in which  
263 peroxisome translocation is seen (Figure 5 d, e), and second, by quantifying  
264 peroxisome intensities above a threshold value in the cell periphery (Figure 5 f, g).

265 Because of observed differences in peroxisome translocation within the time-frame of  
266 rapalog treatment, different peroxisome intensity threshold values and peripheral  
267 areas were used for KIF1A and KIF15; this is probably due to differences in motor  
268 properties (Figure 5 – figure supplement 3).

269 Intriguingly, while the overall trends in perturbation of KBP inhibition by  
270 mutagenesis seen in the pulldown assay are recapitulated in the translocation assay,  
271 some differences are also observed. As with the pull-down assay, Ala-substitutions in  
272 the TPR-containing  $\alpha$ -helices  $\alpha$ HP4a and  $\alpha$ HP4b at the KBP concave face, as well as  
273  $\alpha$ HP5a, all strongly reduced KBP's inhibition of both KIF15\_MDC and KIF1A\_MDC  
274 peroxisome translocation activity (Figure 5d-g). This can be seen by the extent of  
275 peroxisome translocation and in an increase of peroxisomes in the cell periphery after  
276 rapalog addition, similar to the control condition without KBP (Figure 5d-g). Also, as  
277 observed in the pull-down assay, mutation of L1, L3 or L5 had no effect on KBP's  
278 inhibition of KIF15\_MDC or KIF1A\_MDC (Figure 5d-g). This reinforces the  
279 conclusion that while these elements are close enough to form contacts with the parts  
280 of the kinesin motor domain in our reconstruction, they do not contribute significantly  
281 to KBP inhibition of kinesin-mediated translocation in cells.

282 Mutation of either of L12 or L14 strongly abrogated KBP inhibition of  
283 KIF15\_MDC/KIF1A\_MDC-based translocation, a more pronounced effect than was  
284 seen in the pull-down assay. Similarly, although mutation of L10 and L16 had no  
285 effect on KBP-kinesin interaction in the pull-down assay, mutation of these loops  
286 disrupted KBP inhibition of KIF1A\_MDC and KIF15\_MDC in the translocation  
287 assay (Figure 5d-g). A subset of the above described mutations were also combined to  
288 assess additive effects. Here we observed that KBP constructs containing mutations in  
289 both L10 + L12, L10 + L14 or L12 + L14 had similar effects to KBP with only one of

290 the loops mutated (Figure 5d-g), suggesting that KBP inhibition is more readily  
291 disrupted in the translocation assay.

292 Interestingly, while all the above described regions affected KBP inhibition of  
293 KIF15\_MDC and KIF1A\_MDC equivalently, the L18 KBP mutant inhibited  
294 KIF15\_MDC equivalent to wild type, but only exhibited partial inhibition of  
295 KIF1A\_MDC-mediated translocation. It should, however, be noted that fewer cells  
296 show peroxisome translocation by KIF1A\_MDC when L18 is co-expressed (Figure  
297 5e), suggesting that the L18 KBP mutant also inhibits KIF1A\_MDC to some extent.  
298 The L18 mutant is the single example of contradictory behaviours between the assays,  
299 because it appeared to enhance KBP-kinesin binding in the pull-down assay. Our  
300 structural data do not provide a clear rationale for this, and future studies will  
301 investigate the role of this region of KBP further and, for example, whether it is  
302 subject to post-translational regulation that could regulate KBP's inhibitory activity.

303 However, taken together, the translocation and pull-down assays both  
304 demonstrate the functional importance of the kinesin interaction with the TPR-  
305 containing  $\alpha$ -helices at the KBP concave face and the set of loops in the KBP C-  
306 terminal subdomain. The translocation and pull-down assays also reveal differences in  
307 the sensitivity of the KBP-kinesin interaction to perturbation, where translocation is  
308 more readily disrupted than the interactions detected by pull-down. These differences  
309 likely reflect the greater complexity of motor regulation during active translocation  
310 and could be a function of FRB-FKBP-mediated motor dimerization. It might also  
311 reflect the fact that cellular MTs in the translocation assay can directly compete with  
312 KBP for kinesin binding. Overall, these mutation studies support the idea that KBP  
313 interacts with different kinesin family members in a similar way via an extended

314 interface at KBP's concave face that is composed of TPR-containing  $\alpha$ -helices and  $\alpha$ -  
315 solenoid edge loops, particularly in the C-terminal subdomain.

316

317 *Specific sequences in the tubulin-binding subdomain are conserved across KBP-*  
318 *binding kinesin family members*

319

320 Given that KBP selectively binds and inhibits only a subset of kinesins<sup>9</sup>, we used our  
321 structural data to investigate the basis of this selectivity. Although the resolution of  
322 our reconstruction and the flexibility of some loops do not provide a detailed  
323 molecular description of the interaction interface, our structure shows that the kinesin  
324 tubulin-binding subdomain is the key KBP-interacting region. Analysis of the  
325 sequences of this region in KBP-binding and KBP-non-binding kinesins (Figure 6a)  
326 revealed patterns of sequence conservation across the entire subdomain in all KBP-  
327 binding kinesins; this included both the K $\beta$ 5-KL8 and KL11-K $\alpha$ 4-KL12-K $\alpha$ 5-KL13  
328 regions. In contrast, the equivalent regions are more variable in kinesins that are not  
329 inhibited by KBP. The length of KL8, which joins the two K $\beta$ 5 strands, was also  
330 consistently 5 residues long in KBP-binding kinesins, while it was variable in KBP-  
331 non-binding kinesins. From our KBP- KIF15\_MD6S structure, the sensitivity of the  
332 KBP interaction to KL8 length makes sense considering the tight fit of this loop  
333 between KBP L12 and L14 (Figure 6b). In summary, two consensus motifs in KL11-  
334 K $\alpha$ 4-KL12-K $\alpha$ 5-KL13 and K $\beta$ 5-KL8 regions of the tubulin-binding subdomain are  
335 found in KBP-binding kinesins and these are likely to form the basis of KBP's kinesin  
336 family member selectivity. We therefore propose a model where KBP selects and  
337 inhibits target kinesins through binding and remodelling a compatible tubulin-binding  
338 subdomain, obstructing the kinesin MT-binding surface (Figure 6c).

339

340 **Discussion**

341

342 In this study, we reveal the TPR-containing right-handed  $\alpha$ -solenoid structure of the  
343 ~72 kDa KBP using VPP cryo-EM. At the time of writing and to our knowledge,  
344 structures of only a few macromolecular complexes <80 kDa have been determined  
345 using cryo-EM<sup>33-37</sup>. The structure of the KBP-KIF15\_MD6S complex shows how  
346 KBP binds the KIF15\_MD6S via its concave face and undergoes subtle remodelling  
347 of its N-terminal domain to accommodate kinesin binding. This further reinforces the  
348 idea that the TPR-containing structures are not simply static scaffolds but can flexibly  
349 respond to ligand binding<sup>38</sup>. In contrast, the KIF15 motor domain undergoes a radical  
350 conformational change in forming a complex with KBP, in which helix  $\alpha$ 4, the major  
351 component of the motor's tubulin-binding subdomain, is displaced from the main MD  
352 body by ~15 Å into the KBP concave face. This is consistent with previous  
353 observations that this region of the kinesin motor domain being rather malleable and  
354 able to move independently of the core structure of kinesin motor domains<sup>39,40</sup>. Our  
355 evidence suggests this observation is not due to use of a cysteine-substituted KIF15  
356 motor domain construct because: i) this protein exhibited equivalent MT-stimulated  
357 ATPase activity compared to non-substituted KIF15\_MD (Figure 2 – figure  
358 supplement 2a), ii) it exhibited structurally canonical MT binding and response to  
359 nucleotide (Figure 3 – figure supplement 1), and iii) the substituted residues are not  
360 well conserved among KBP-binding kinesins (Figure 6a). The large displacement of  
361 helix  $\alpha$ 4 expands the surface area over which the normally compact tubulin-binding  
362 subdomain of the kinesin motor domain can interact with KBP, and it is through the



363 sequence and shape of this interface that the selectivity of KBP for a subset of kinesin  
364 motors is presumably defined.

365         Analysis of the KBP-KIF1A\_MD complex (Figure 2–figure supplement 1c)  
366 supports the idea of a conserved mode of interaction between a subset of kinesins and  
367 the concave face of KBP. Interestingly, KIF1A\_MD exhibited flexibility in its  
368 interaction with KBP, which was not observed in the KBP-KIF15\_MD6S complex.  
369 Whether this reflects a physiological reality, or a result of the EM preparation method  
370 is uncertain at present, although we think it unlikely to be due to our use of  
371 KIF15\_MD6S. However, our 2D classifications combined with mutation studies  
372 strongly suggest KIF15\_MD6S and KIF1A\_MD share an overall similar KBP binding  
373 mode.

374         Targeted mutations to the various kinesin-binding KBP elements reduced  
375 complex affinity, yet no single mutation completely disrupted the interaction in our  
376 pull-down assays (Figure 5- figure supplement 2). In contrast, in the context of active  
377 MT-based cargo translocation in the cellular environment, KBP inhibitory activity  
378 appeared more sensitive to disruption (Figure 5 d-g). Although most KBP mutations  
379 that have been reported in GOSHS result in total loss of protein<sup>11-14</sup>, a recent study  
380 details missense mutations that only partially reduce protein expression (MacKenzie  
381 et al, 2020). Our data illustrate that KBP’s activity is additionally sensitive to  
382 mutations in key regions that affect its ability to bind kinesins.

383         Our structure shows that KBP binds exclusively to the tubulin-binding  
384 subdomain of KIF15\_MD6S, sterically preventing MT attachment. The interaction of  
385 kinesin motor domains with the MT surface via its tubulin-binding subdomain  
386 stimulates nucleotide exchange and kinesin ATPase activity. In contrast, on  
387 interaction with KBP, the displacement of helix  $\alpha 4$  from the kinesin nucleotide-

388 binding site, together with the absence of MT-mediated ordering of KL9 and KL11,  
389 means that its catalytic site is distorted and the structural changes associated with MT-  
390 stimulated ATPase cannot occur. This could be an important facet of the role of KBP  
391 in the energy economy of the cell in addition to directly blocking kinesin-MT  
392 interactions.

393         The concave face of TPR-containing  $\alpha$ -solenoids commonly serve as a  
394 recognition platform for specific peptide motifs, including those forming  $\alpha$ -helical  
395 structures<sup>41</sup>. Specificity and affinity for target motifs are determined in part by the  
396 shape of the  $\alpha$ -solenoid concave face, which in turn is defined by the fold's  
397 supertwist. In addition, particular amino-acid arrangements at the concave face  
398 contribute to partner binding affinity and specificity, together with additional  
399 interfaces formed at the convex surface or  $\alpha$ -solenoid edge<sup>42</sup>. KBP kinesin specificity  
400 and affinity is defined by the interaction of its concave face with the large surface area  
401 of the kinesin L11-K $\alpha$ 4-KL12-K $\alpha$ 5-KL13 region, in addition to binding the kinesin  
402 K $\beta$ 5-KL8 region at its  $\alpha$ -solenoid edge. Interestingly, distal to the N-terminal MT-  
403 binding region of kinesin-1, C-terminally-associated kinesin light chain use their  
404 unique TPR-containing  $\alpha$ -solenoid concave face to select cargos via recognition of  
405 specific peptide motifs<sup>43</sup>. Therefore, peptide selectivity by TPR-containing  $\alpha$ -  
406 solenoids is a facet of both kinesin MT-binding and cargo-binding regulatory  
407 mechanisms. Such protein-protein interactions may be selectively targeted for  
408 disruption<sup>44</sup>, and the insights arising from our work provide future avenues to disrupt  
409 KBP-kinesin interactions and thereby explore KBP interactions and regulatory roles.

410         The effective and selective kinesin inhibitory mechanism of KBP revealed by  
411 our work may fulfil specific roles in the kinesin regulatory toolbox employed by cells  
412 to spatially and temporarily orchestrate kinesin activity. Future studies will be aimed

413 at understanding how KBP interacts with kinesins in dimeric and/or autoinhibited  
414 forms. For example, while KBP does not interact with some target kinesins in  
415 autoinhibited conformations<sup>18</sup>, others that retain a structurally available tubulin-  
416 binding subdomain, such as autoinhibited kinesin-3 monomers<sup>45</sup>, are feasible binding  
417 partners. KBP can bind to constitutively active dimeric kinesin constructs that lack  
418 autoinhibitory regions<sup>9,18</sup>, although the stoichiometry and structural details in this  
419 context are unclear. Furthermore, any additional effects of kinesin cargo binding on  
420 their susceptibility to KBP inhibition are not well understood. It will also be of key  
421 importance to elucidate the mechanisms of KBP activity regulation, for example, by  
422 phosphorylation of KBP and/or kinesins<sup>9</sup> and KBP acetylation and targeted  
423 degradation by the ubiquitin system<sup>46</sup>. Our structural characterisation of the KBP-  
424 kinesin inhibitory interaction provides an important mechanistic platform from which  
425 to expand our understanding of KBP's biological roles in neuronal function and  
426 cancer.

427

428

429 **Methods**

430

431 *Protein expression and purification for cryo-EM*

432

433 Full length human KBP residues 1-621 in a PSTCm1 expression vector (with  
434 kanamycin resistance and a N-terminal thrombin cleavable 6 x His-tag) was expressed  
435 in Rosetta2 cells (Novagen) as previously described<sup>9</sup>. Following immobilised metal-  
436 affinity chromatography with Ni-NTA resin (Qiagen), the 6 x His-tag was removed  
437 via incubation with thrombin protease overnight at 4 °C. The protein was then  
438 subjected to reverse IMAC and further purified using size exclusion chromatography  
439 (SEC) into a buffer of 20mM TrisHCL (pH7.4), 150mM NaCl, 2.5mM CaCl<sub>2</sub>, 1mM  
440 DTT. Protein was snap-frozen and stored in at -80 °C.

441 A human KIF15 motor domain and neck linker construct (residues 1-375) in a  
442 pET21a vector with a C-terminal 6 x His-tag was generated by chemical synthesis  
443 (GenScript, Piscataway, NJ). Six of the eight cysteine residues (C5S, C50S, C162S,  
444 C294S, C314S, C346S) were mutated and two cysteines were inserted (S250C,  
445 G375C) for orthogonal experiments not described further here. We refer to this  
446 construct as KIF15\_MD6S. KIF15\_MD6S was expressed and purified using methods  
447 previously described<sup>47</sup>, then buffer exchanged into 25 mM HEPES pH 7.5, 100 mM  
448 KCl, 2 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM DTT, 0.1 mM ATP, snap frozen in liquid  
449 nitrogen and stored at -80°C.

450 A human KIF1A motor domain and neck linker construct (KIF1A\_MD residues  
451 1-362) in a pFN18a vector (with a TEV protease-cleavable N-terminal Halo-tag and a  
452 C-terminal 6 x His-tag) was expressed in BL21-Gold (DE3) cells, as previously  
453 described<sup>28</sup>. Following a first IMAC with Ni-NTA resin, the Halo-tag was removed

454 via incubation overnight with TEV protease at 4 °C. The protein was then isolated  
455 from TEV via a second IMAC with Ni-NTA resin and further purified by SEC into a  
456 storage buffer of (20 mM HEPES, pH 7, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.1 mM ADP  
457 and 1 mM TCEP).

458 KIF15\_MD6S or KIF1A\_MD complexes with KBP were purified via IMAC  
459 using the 6 x His tag on the kinesin constructs. Briefly, His-tagged kinesins were  
460 incubated with a 10 times excess of KBP in 20 mM TrisHCL (pH7.5), 150 mM NaCl,  
461 1 mM MgCl<sub>2</sub>, 10 mM Imidazole, 1 mM DTT, 0.2 mM ADP for 5 minutes at 4 °C.  
462 Following IMAC, complexes were eluted from the Ni-NTA resin (Qiagen) by  
463 addition of 200 mM imidazole, then dialyzed at 4 °C for 4 hours into 20 mM  
464 TrisHCL (pH7.5), 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 0.2 mM ADP.

465

#### 466 *Steady-state ATPase assay*

467

468 ATPase activity of KIF15\_MD6S was measured in ATPase buffer (50 mM potassium  
469 acetate, 25 mM HEPES, 5 mM magnesium acetate, 1 mM EGTA, pH 7.50) by  
470 measuring phosphate production in the presence of a minimum of a 5-fold molar  
471 excess of paclitaxel-stabilised MTs, using a commercially-available kit (EnzChek,  
472 Molecular Probes) at 20°C.

473

#### 474 *Sample preparation for cryo-EM*

475

476 KBP was prepared for cryo-EM using three different approaches. In the first  
477 approach, KBP was diluted to 0.15 mg/ml in KBP dilution buffer (20 mM TrisHCL,  
478 pH7.5, 150 mM NaCl, 2 mM DTT) and 4 µl were applied to glow-discharged C-

479 flat<sup>TM</sup> 2/2 holey carbon EM grids (Protochips, Morrisville, NC). For the second  
480 approach, KBP was diluted to 0.3 mg/ml in KBP dilution buffer and 4 µl were applied  
481 to glow-discharged 1.2/1.3 AuFoil gold grids (Quantifoil<sup>®</sup>). For the third approach,  
482 glow-discharged C-flat<sup>TM</sup> 2/2 holey carbon EM grids were coated with graphene-  
483 oxide (GO) according to the protocol described by Cheng and colleagues<sup>48</sup> then 4 µl  
484 of KBP diluted to 0.02 mg/ml in KBP dilution buffer were added.

485 Kinesin motor domain-KBP complexes were diluted to 0.03 mg/ml in KBP-  
486 kinesin dilution buffer (20 mM TrisHCL (pH7.5), 50 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM  
487 DTT, 0.2 mM ADP) and 4 µl were added to the GO-coated gold grids described  
488 above. After a 30 second incubation of samples on the EM grid in a Vitrobot Mark IV  
489 (FEI Co., Hillsboro, OR) set at 4 °C and 80 % humidity, samples were blotted (6-8  
490 seconds, blot force -10) and vitrified in liquid ethane. All steps were performed at 4  
491 °C.

492 For preparation of the KIF15\_MD6S-MT complex, porcine tubulin (>99%  
493 pure, Cytoskeleton Inc.) was polymerised in MES polymerisation buffer (100 mM  
494 MES, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM DTT, pH 6.5) with 5 mM GTP at 37 °C then  
495 stabilised with 1 mM paclitaxel. ~70 µM KIF15\_MD6S was pre-incubated for 5 min  
496 with 5 mM of AMPPNP in BRB80 at room temperature, and then mixed with 20 µM  
497 stabilised MTs. After a further incubation of 15 min, a 4 µl droplet was applied to a  
498 pre-glow discharged holey carbon grid (2/2 C-flat, Protochips Inc.), blotted for 3.5 s  
499 and then vitrified in liquid ethane using a Vitrobot Mark IV at ambient temperature  
500 and 80% humidity.

501

502 *Cryo-EM data collection*

503

504 For dataset of KBP alone or KBP-KIF15\_MD6S, low-dose movies were collected  
505 automatically using EPU software (Thermo Fisher, MA, USA) on a Titan Krios  
506 electron microscope (Thermo Fisher) operating at 300 kV, with a K2 summit direct  
507 electron detector (Gatan, CA, USA) and a quantum post-column energy filter (Gatan)  
508 operated in zero-loss imaging mode.

509 Datasets of KBP alone were collected either at eBIC or the ISMB, Birkbeck  
510 using a Volta phase plate (VPP), a sampling of  $\sim 1.05$  Å/pixel and a nominal defocus  
511 range of 0.5-0.7  $\mu\text{m}$ . The total dose was  $42 \text{ e}/\text{Å}^2$  over 40 frames, with the detector  
512 operating in counting mode at a rate of  $\sim 5 \text{ e}/\text{pixel}/\text{second}$ .

513 Datasets of KBP-kinesin complexes were collected at the ISMB, Birkbeck  
514 without a phase plate and a nominal defocus range of 1.5-4  $\mu\text{m}$ . KIF1A\_MD-KBP  
515 complexes were collected at a sampling of 0.85 Å/pixel, whereas KBP-KIF15\_MD6S  
516 complexes were collected at a sampling of 1.047 Å/pixel. For KIF1A\_MD-KBP  
517 complexes, the total dose was  $88 \text{ e}/\text{Å}^2$  over 36 frames, with the detector operating in  
518 counting mode at a rate of  $7.1 \text{ e}/\text{pixel}/\text{second}$ . For KBP-KIF15\_MD6S complexes,  
519 the total dose was  $80 \text{ e}/\text{Å}^2$  over 64 frames, with the detector operating in counting  
520 mode at a rate of  $5.7 \text{ e}/\text{pixel}/\text{second}$ .

521 The KIF15\_MD6S-MT dataset was collected manually on a Tecnai Polara  
522 microscope (Thermo Fisher) at the ISMB, Birkbeck, operating at 300 kV, with a K2  
523 summit direct electron detector (Gatan, CA, USA) and a quantum post-column energy  
524 filter (Gatan) operated in zero-loss imaging mode. A nominal defocus range of 1.0-3.5  
525  $\mu\text{m}$  and a final pixel size of 1.39 Å was used. The total dose was  $32 \text{ e}/\text{Å}^2$  over 50  
526 frames, with the detector operating in counting mode at a rate of  $6.2 \text{ e}/\text{pixel}/\text{second}$ .

527

528 ***Cryo-EM data processing***

529

530 Low-dose movies were motion-corrected using MotionCor2<sup>49</sup> with a patch size of 5,  
531 generating full-dose and dose-weighted sums. CTF determination was performed on  
532 full-dose sums with gCTF<sup>50</sup> and then dose-weighted sums were used for all further  
533 processing. Data were cleaned at this stage by first excluding all micrographs with  
534 gCTF resolutions worse than 4.5 Å, as estimated with a custom cross-correlation  
535 coefficient cutoff (Python script kindly shared by Radostin Danev), then manually  
536 removing micrographs with poor appearance (ice contamination, protein aggregation  
537 or poor GO coverage) in real or reciprocal space. For KBP alone data, micrographs  
538 with calculated phase shifts outside the expected phase shift progression at each plate  
539 position were also excluded.

540 Particles were first picked using Eman2's neural network picker<sup>51</sup>, with a 180  
541 pixel box size for KBP-alone and KBP-KIF15\_MD6S datasets, or a 220 pixel box  
542 size for the KBP-KIF1A\_MD datasets. Good 2D classes were then used as templates  
543 to pick the data with Gautomatch (<http://www.mrc-lmb.cam.ac.uk/kzhang/>).

544 For Eman2 neural network picker or Gautomatch-derived particles from each  
545 dataset, separate multiple rounds of 2D classification were performed in RELION  
546 v3.0<sup>52</sup>, cryoSPARC2<sup>53</sup> or cisTEM<sup>54</sup>. This resulted in a total of six sets of good 2D  
547 classes showing clear secondary structure for each dataset, two produced by each  
548 programme for each picking method. For each dataset, these six good 2D class sets  
549 for each dataset were then combined and duplicate particles removed. At this stage,  
550 for each sample (KBP-alone, KBP-KIF15\_MD6S or KBP-KIF1A\_MD) good 2D  
551 classes from their constituent datasets were combined.

552 KBP-KIF15\_MD6S or KBP-KIF1A\_MD datasets, composed of their respective  
553 constituent datasets were easily combined, being from the same microscope and



554 optical set up. However, KBP-alone data were collected on different microscopes and  
555 had a range of pixel sizes (<2% difference). KBP-alone data therefore was combined  
556 at this stage using the optics grouping protocol in RELION v3.1<sup>52</sup>.

557 KBP-alone and KBP-KIF15\_MD6S data were taken to 3D processing at this  
558 stage, while multiple attempts to process KBP-KIF1A\_MD data in 3D gave no  
559 reliable results. For KBP-alone and KBP- KIF15\_MD6S data, *de novo* initial 3D  
560 models were created in cryoSPARC2. For KBP- KIF15\_MD6S data, a single round of  
561 3D classification was performed in RELION v3.0 and the best class selected and  
562 auto-refined. For KBP-alone data, 3D classification in RELION v3.1 or cryoSPARC2  
563 did not reveal different 3D structures or improve reconstructions over sorting only in  
564 2D; therefore, particles selected with 2D classification were used as direct input for  
565 auto-refinement. The final KBP-alone map was sharpened with a B-factor of -200 to  
566 the gold-standard FSC 0.143 cutoff (4.6 Å). The KBP- KIF15\_MD6S map was  
567 sharpened locally with a B-factor of -495, according to local resolutions determined  
568 using RELION v3.1's inbuilt local resolution software.

569 The KIF15\_MD6S-MT dataset was processed using our MT RELION-based  
570 pipeline (MiRP) as described previously, using low-pass filtered KIF5B\_MD-  
571 decorated MTs as references<sup>55,56</sup>. KIF15\_MD6S 13-protofilament-MTs were the  
572 most common MT architecture and were selected after supervised 3D classification in  
573 MiRP for analysis. The symmetrised asymmetric unit (KIF15\_MD6S plus a tubulin  
574 dimer) was locally sharpened in UCSF Chimera with a B-factor of -134 according to  
575 local resolutions determined using RELION v3.0's inbuilt local resolution software.

576 All displayed 3D molecular representations were made in UCSF Chimera or  
577 ChimeraX software<sup>57,58</sup>. Data collection and model refinement statistics can be found  
578 in Table 1.

579

580 *Cryo-EM model building and refinement*

581

582 Due to low overall homology to available structures in the protein data bank (PDB),  
583 structure prediction of KBP produced poor models with little resemblance to the cryo-  
584 EM density. KBP was therefore modelled using a combination of secondary structure  
585 prediction, TPR prediction, fragment homology information, prior knowledge of  
586 right-handed alpha-solenoid proteins and with reference to the cryo-EM density.

587 TPR motifs were identified in the KBP sequence using the TPRpred server<sup>59</sup>  
588 available in the MPI Bioinformatics Toolkit<sup>60</sup>. Secondary structure predictions using  
589 Raptor X<sup>61</sup>, iTasser<sup>62</sup>, JPred<sup>63</sup>, Spider2<sup>64</sup>, PSSpred<sup>65</sup> and SOPMA<sup>66</sup> were then run on  
590 the sequence and consensus between these multiple predictions used to assign likely  
591  $\alpha$ -helical content. To identify regions dispensable for the overall fold and likely  
592 disordered loop regions, disorder prediction was performed with Raptor X and inter-  
593 species low homology regions in KBP (from early Metazoans to humans) were  
594 determined via Clustal Omega multiple sequence alignment<sup>67</sup>. Finally, weak  
595 homology models for overlapping fragments of the structure were identified using the  
596 HHpred<sup>68</sup> server in the MPI Bioinformatics Toolkit.

597 With the information described above a sequence alignment was built with KBP  
598 and the following fragment homology model PDBs; 5OJ8, 4A1S, 3QC1, 4NQ0,  
599 4AIF, and 5MX5. This sequence alignment was used as a basis for multiple rounds of  
600 modelling and flexible fitting with Modeller<sup>69</sup> and Flex-EM<sup>70</sup> respectively, using  $\alpha$ -  
601 helical secondary structure restraints. This modelling process was guided by  
602 consistency with the cryo-EM density and secondary structure and TPR predictions  
603 described above. Finally, the structure was refined against the cryo-EM density in

604 real-space with 5 macro-cycles in Phenix<sup>71</sup>. All 19 predicted and modelled helices  
605 were accounted for by rod-like cryo-EM density in the reconstruction and at 4.6 Å  
606 resolution, density was discernible for bulky side chains in the TPR regions (Figure  
607 1–figure supplement 1a,b), providing a validation of the assigned sequence  
608 directionality in the fold.

609         The KBP KIF15\_MD6S model was built as follows: the final KBP model  
610 described above and the KIF15\_MD x-ray crystallographic model (PDB  
611 code:4BN2<sup>25</sup>) were rigid fitted into the KBP-KIF15\_MD6S density in Chimera.  
612 Density for an extended α6-helix and docked neck-linker in KIF15\_MD6S were  
613 absent; therefore, Modeller was used to model a short α6-helix and the neck-linker  
614 removed. A model for the L11, α4-helix and L12 region in KIF15\_MD6S were then  
615 created using Coot<sup>72</sup> and Modeller. The model was refined into the cryo-EM density  
616 in real-space using Phenix<sup>7</sup> with secondary structure restraints. A first refinement of  
617 15 cycles used rigid bodies describing α-helical hairpins in KBP to get a good rough  
618 fit. Following this, the whole complex was further refined without rigid bodies for  
619 another 5 macro-cycles.

620         The KIF15\_MD6S-MT model was built as follows: the KIF15\_MD,  
621 KIF11\_MD and KIF5B\_MD-tubulin x-ray crystallographic models (PDB  
622 codes:4BN2<sup>25,27,73</sup>) were used as homology models in Modeller to build the KIF15  
623 part of the complex. The KIF15\_MD6S model and the paclitaxel-MT tubulin dimer  
624 model<sup>74</sup> were then rigid fitted into KIF15\_MD6S -MT density, combined then refined  
625 in real-space with 5 macro-cycles in Phenix with peptide backbone restraints.

626

627 *Antibodies, reagents and expression constructs for cell biology*

628

629 The following antibodies were used for immunofluorescence staining: mouse anti-HA  
630 (1:500, Roche), and goat-anti-mouse Alexa 488 (1:400, Thermo Fisher Scientific).

631 The following antibodies were used for western blot: mouse anti-HA (1:2,000,  
632 BioLegend), rabbit anti-GFP (1:10,000, Abcam), goat anti-mouse IRDye800CW  
633 (1:15,000, LI-COR), and goat-anti-rabbit IRDye680LT (1:20,000, LI-COR). A  
634 reagent used in this study is rapalog (AP21967, TaKaRa).

635 The following DNA expression constructs in this study have been described  
636 before: GW1-PEX3-mRFP-FKBP1,  $\beta$ actin-Kif1A\_MDC-FRB<sup>9</sup> (mouse cDNA), BirA  
637 coding vector<sup>75</sup> and pebioGFP<sup>75</sup>. pGW1-HA-KBP contained a linker  
638 (GGATCCCCGGAATTCGGCACGAGGGAGGCCGCT) between the HA tag and  
639 KBP and was cloned using PCR based strategies with human KBP cDNA  
640 (KIAA1279, IMAGE clone 4550085) as template and ligation into the pGW1-HA  
641 backbone. A similar strategy was used to generate the mutated KBP constructs, listed  
642 in Table 2.  $\beta$ actin-KIF15\_MDC-FRB was cloned using a PCR based Gibson  
643 Assembly strategy with mouse KIF15 cDNA as template into the  $\beta$ actin-  
644 KIF1A\_MDC-FRB backbone. PebioGFP-KIF1A\_MDC and pebioGFP-KIF15\_MDC  
645 were cloned into the pebioGFP backbone using PCR based strategies with MDC-FRB  
646 constructs as templates.

647

#### 648 *Cell culture, transfection and immunofluorescence staining*

649

650 COS-7 cells were purchased from ATCC and routinely checked for mycoplasma  
651 contamination using LT07-518 Mycoalert assay (Lonza). Cells were cultured in 50/50  
652 DMEM (Lonza)/Ham's F10 (Lonza) medium supplemented with 10% FCS (Sigma)  
653 and 1% penicillin/streptomycin (Sigma). One day before transfection cells were

654 diluted and plated on 18-mm glass coverslips. COS-7 cells were transfected using  
655 FuGENE6 (Roche) following the manufacturer's protocol. Next day, rapalog (final  
656 concentration 1  $\mu$ M) was added and cells were incubated for 3 hours. Cells were then  
657 fixed with 4% formaldehyde/4% sucrose in phosphate-buffered saline (PBS) for 10  
658 minutes at room temperature, washed three times PBS-CM (PBS supplemented with 1  
659 mM MgCl<sub>2</sub> and 0.1 mM CaCl<sub>2</sub>), permeabilized in 0.2% TritonX-100 for 15 minutes  
660 and washed one time with PBS-CM. Cells were first incubated with 0.2% gelatin for  
661 30 minutes at 37 °C, and then with primary antibodies, diluted in 0.2% gelatin, for 30  
662 minutes at 37 °C. After washing three times with PBS-CM, cells were incubated for  
663 30 minutes at 37 °C with secondary antibody diluted in 0.2% gelatin, washed three  
664 times in PBS-CM, and finally mounted using Fluoromount (Invitrogen).

665

#### 666 *Cell biology image analysis and quantification*

667

668 Fixed cells were imaged on a Carl Zeiss LSM 700 confocal laser scanning microscope  
669 running ZEN2011 software, using a Plan-Apochromat 40x/1.30 oil DIC objective and  
670 image settings were maintained the same for all images within one experiment.

671 Images were acquired of cells that express similar levels of HA-KBP constructs based  
672 on immunostaining (Figure 5 – figure supplement 1). Cells were selected on a first-  
673 come-first served basis. Images were processed and analysed using Fiji software<sup>76</sup>. To  
674 calculate the percentage of cells in which translocation of peroxisomes was observed,  
675 imaged cells were classified as either translocating, when peroxisomes re-localized  
676 into the cell periphery, or not translocating, when peroxisomes remained in the cell  
677 centre. For quantification of PEX translocation, an ROI of the cell area was drawn  
678 and from this a second ROI at 5 (KIF1A\_MDC) or 7.5 (KIF15\_MDC)  $\mu$ m from the

679 outer cell area was created. Images were thresholded at 7500 (KIF15\_MDC) or 10000  
680 (KIF1A\_MDC). Different peripheral areas and threshold values were defined for the  
681 two kinesins, due to observed differences in translocation properties between the  
682 kinesins (compare Figure 5 – figure supplement 3a-c). For the two selected ROIs, the  
683 area with fluorescent intensity above threshold was determined in the RFP channel.  
684 From these values the percentage of cell area above threshold in the cell periphery  
685 from the total area above threshold was calculated.

686

### 687 *Pull-down experiments and western blotting*

688

689 HEK293T cells were purchased from ATCC and routinely checked for mycoplasma  
690 contamination using LT07-518 Mycoalert assay (Lonza). Cells were cultured in 50/50  
691 DMEM (Lonza)/Ham's F10 (Lonza) medium supplemented with 10% FCS (Sigma)  
692 and 1% penicillin/streptomycin (Sigma). One day before transfection cells were  
693 diluted and plated into 6-well plates. Cells were co-transfected with pCl-Neo-BirA,  
694 HA-tagged constructs and bioGFP-tagged constructs using MaxPEI (Polysciences) in  
695 a ratio of 3/1 PEI/DNA, according to the manufacturer's protocol. After 24-hours of  
696 expression, cells were washed in ice-cold PBS and lysed in lysis buffer (100 mM  
697 TrisHCl pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitors (Roche)) for 30  
698 minutes on ice. Lysates were cleared by 30-minute centrifugation at 13.2 krpm at 4°C  
699 and supernatants were incubated with blocked (incubation for 30 minutes at RT in 50  
700 mM Tris-HCl pH 7.5, 150 mM KCl, 0.2 µg/µl chicken egg albumin) Streptavidin  
701 Dynabeads M-280 (Invitrogen) for 1.5 hours at 4°C. Beads were then washed five  
702 times with washing buffer (100 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.5% Triton X-  
703 100) and proteins were eluded from the beads by boiling for 10 minutes at 95°C in 2x

704 DTT+sample buffer (20% glycerol, 4% SDS, 200 mM DTT, 100 mM Tris–HCl pH  
705 6.8, bromophenol blue).

706 Protein samples were run on 10% SDS-PAGE gels and transferred to  
707 nitrocellulose membranes (Bio-Rad) by semi-dry blotting at 16V for 1 hour.  
708 Membranes were blocked by incubation in 3% bovine serum albumin (BSA) in PBST  
709 (PBS supplemented with 0.02% Tween20) for 1 hour at room temperature. This was  
710 followed by overnight incubation with primary antibodies in 3% BSA-PBST.  
711 Membranes were washed three times with PBST, incubated with secondary antibody  
712 in 3% BSA-PBST for 1 hour at room temperature, and washed three times with  
713 PBST. Membranes were scanned using an Odyssey Infrared Imaging system (LI-COR  
714 Biosciences) and blots were acquired at 680 nm and 800 nm.

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716

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- 959

960

961 **Figures**

962

963 **Figure 1. KBP is a TPR-containing right-handed  $\alpha$ -solenoid.**

964 (a) Model of KBP (ribbon representation) displayed in experimental cryo-EM density.

965 The N-terminal (olive) and C-terminal (gold) subdomains are separated by a linker

966 region (black). Semi-transparent density is coloured regionally as per the fitted model.

967 The N- and C-termini are shown, with a dotted line representing the disordered C-

968 terminus (not modelled). The linker loop region was not modelled but its density is

969 shown in semi-transparent black. (b) The same as panel a, but rotated 180° around the

970 axis indicated. (c) The same view as in panel a, but with the density removed and  $\alpha$ -

971 helices displayed as pipes with their directionality indicated by arrows. The 9

972 antiparallel  $\alpha$ -helical pairs ( $\alpha$ HP1 to  $\alpha$ HP9) are each coloured separately and labelled,

973 as is the linker  $\alpha$ -helix (L $\alpha$ H) and linker loop (LL, dotted line). (d) Ribbon

974 representation of KBP showing the 4 tetratricopeptide repeat (TPR) motifs and the

975 L $\alpha$ H coloured according to the labels. View related to panel c, by a 90° rotation

976 around the indicated axis. (e) Schematic of the KBP showing the position of the TPR

977 motifs between residue 95 and 283 of the N-terminal subdomain and position of the

978 linker region (L $\alpha$ H and LL) between residues 305 and 392.

979

980 **Figure 2. KBP conformationally adapts to bind KIF15's motor domain via both**

981 **subdomains**

982 (a) Model of the KBP-KIF15\_MD6S complex (ribbon representation) displayed in

983 experimental cryo-EM density. The N-terminal (olive) and C-terminal (gold)

984 subdomains and the linker helix (black) are shown in KBP, while kinesin is coloured

985 in magenta. Semi-transparent density is coloured regionally as per the fitted model

986 and additional density for the linker loop is shown in semi-transparent black. (b) The  
987 same as panel a, but rotated 180° around the axis indicated. (c) The KBP-alone model  
988 (light grey ribbons) was superimposed on the KBP-KIF15\_MD6S model (opaque  
989 ribbons) using Chimera's matchmaker<sup>57</sup>. Colouring and view as in panel b. (d) RMSD  
990 in Å for KBP comparing KBP-KIF15\_MD6S and superimposed KBP-alone models  
991 as in panel c, shown on KBP from the KBP- KIF15\_MD6S model. Parts of the KBP  
992 model coloured black are disordered/missing in the KBP alone model. The  
993 KIF15\_MD6S is shown in transparent magenta.

994

995 **Figure 3. The KIF15 motor domain binds KBP via rearrangement of its tubulin-**  
996 **binding subdomain.**

997 (a) The crystallographic model of the KIF15\_MD alone (PDB: 4BN2<sup>25</sup>) was  
998 superimposed on the KIF15 region of the KBP-KIF15\_MD6S complex, with the  
999 KIF15 part of the KBP- KIF15\_MD6S complex model hidden. The KIF15\_MD6S  
1000 Switch 1/2 subdomain (Switch 1/2 subdomain) is coloured sienna, the P-loop  
1001 subdomain (Kin-PLsd) is coloured light pink. The TBsd of the KIF15\_MD  
1002 crystallographic model is shown as pale magenta to illustrate poor fit into density.  
1003 The KBP subdomains are coloured as labelled. Black arrows indicate unaccounted-  
1004 for cryo-EM density. Individual secondary structure elements in the tubulin-binding  
1005 subdomain are labelled. The cryo-EM density for the KBP-KIF15\_MD6S complex is  
1006 shown in mesh and is coloured by proximity ( $\leq 3.5$  Å) to the fitted model. (b) Same as  
1007 in panel a, but the whole fitted KBP-KIF15\_MD6S complex model is shown. The  
1008 KIF15\_MD6S tubulin-binding subdomain (TBsd) is now coloured magenta to  
1009 indicate good fit into density. (c) Zoomed view of just the TBsd (corresponding to the  
1010 boxed region in Figure 3–figure supplement 2d), showing just the KIF15\_MD-alone

1011 crystallographic model. (d) The TBsd in the KIF15\_MD6S-MT model, same view as  
1012 in panel c. The MT is shown in light grey surface representation. (e) The TBsd in the  
1013 KBP-KIF15\_MD6S model, same view as in panel c. KBP is shown in light grey  
1014 surface representation and the  $\sim 15$  Å displacement of helix  $\alpha 4$  is indicated by the  
1015 dashed grey arrow.

1016

1017 **Figure 4. KBP binds kinesin MDs via conserved motifs in the  $\alpha$ -solenoid edge**  
1018 **loops and  $\alpha$ -helices at the concave face.**

1019 (a) Pseudo-atomic model of the KBP-KIF15\_MD6S complex (ribbon representation)  
1020 displayed in cryo-EM density, using the same viewpoint as Figure 2a, but with the  
1021 KIF15\_MD6S now coloured by subdomain as in Figure 3. The KIF15\_MD6S Switch  
1022 1/2 subdomain (Kin S1/2 sd) is coloured sienna, the P-loop subdomain (Kin-PLsd) is  
1023 coloured light pink. The KIF15\_MD6S tubulin-binding subdomain (TBsd) is coloured  
1024 magenta. The KBP subdomains are coloured as labelled. The nine helix pairs of KBP  
1025 are labelled. Semi-transparent density is coloured regionally as per the fitted model  
1026 and additional density for the linker loop is shown in semi-transparent black. (b) The  
1027 same as panel a, but rotated  $45^\circ$  and  $15^\circ$  respectively around the axes indicated. (c)  
1028 Zoomed view of the region indicated in panel a, with density removed and selected  
1029 KIF15\_MD6S and KBP secondary structure elements labelled. (d) Zoomed view of  
1030 the region indicated in panel b, with density removed and selected KIF15\_MD6S and  
1031 KBP secondary structure elements labelled.

1032

1033 **Figure 5. Disruption of cryo-EM defined KBP-kinesin interface perturbs KBP**  
1034 **inhibition of KIF15 and KIF1A-mediated cargo translocation in cells.**



1035 (a) Schematic depiction of the inducible peroxisome motility assay, with the kinesin  
1036 motor domain fused to an FRB domain and PEX fused to an FKBP domain. Addition  
1037 of rapalog (Rap) links FRB and FKBP and induces peroxisome translocation by  
1038 kinesin dimers. Expression of KBP inhibits kinesin movement, such that addition of  
1039 rapalog cannot induce peroxisome translocation. (b) Schematic representation of the  
1040 inducible peroxisome motility assay in cells. Without rapalog or KBP, peroxisomes  
1041 localize in the cell center, whereas kinesin moves towards the cell periphery. Rapalog  
1042 induces peroxisome translocation into the cell periphery, which is inhibited in  
1043 presence of KBP. (c) Representative images of peroxisomes in COS-7 cells  
1044 expressing KIF15\_MDC-FRB, PEX-mRFP-FKBP and HA (left panels) or HA-KBP  
1045 (right panels) without and with addition of rapalog . Scale bar, 10  $\mu\text{m}$ . (d, e)  
1046 Quantification of the percentage of cells in which peroxisome translocation is  
1047 observed after rapalog treatment in cells expressing KIF15\_MDC-FRB (d) or  
1048 KIF1A\_MDC-FRB (e), PEX-mRFP-FKBP, and HA-KBP constructs including the  
1049 indicated mutants. Data are displayed as mean  $\pm$  s.e.m. (n=28-35 cells from two  
1050 independent experiments). (f, g) Quantification of the area above threshold intensity  
1051 in the outer 5  $\mu\text{m}$  (KIF1A\_MDC) or 7.5  $\mu\text{m}$  (KIF15\_MDC) of the cell from the total  
1052 area above threshold intensity in cells expressing KIF15\_MDC-FRB (f) or  
1053 KIF1A\_MDC-FRB (g), PEX-mRFP-FKBP, and HA-KBP constructs including the  
1054 indicated mutants without and with rapalog treatment. Data are displayed as mean  $\pm$   
1055 s.e.m. (n=28-35 cells from two independent experiments).

1056

1057 **Figure 6. Conserved motifs in KBP-binding kinesin MDs.**

1058 (a) Sequence alignment of the tubulin-binding subdomain from kinesin motor  
1059 domains, made using Clustal Omega multiple sequence alignment<sup>67</sup>. Residues are

1060 coloured according to standard Clustal X colouring (dependent on residue type and  
1061 conservation, see  
1062 [http://bioinfolab.unl.edu/emlab/documents/clustalx\\_doc/clustalx.html#C](http://bioinfolab.unl.edu/emlab/documents/clustalx_doc/clustalx.html#C)). Kinesin  
1063 MD constructs experimentally assessed for KBP interactivity are taken from<sup>9</sup>;  
1064 strongest interactors are in rows highlighted in darker shades of green, weaker  
1065 interactors in lighter shades of green and non-interactors in red. Secondary structure  
1066 element, conservation and charge variation columns, as well as a ‘binding consensus’  
1067 column indicating residues/loop length conserved at the interface (according to the  
1068 KBP-KIF15\_MD6S complex) in KBP-binding but not non-binding kinesins are  
1069 shown above the alignment. Non-conservation relative to this consensus is shown in  
1070 boxed sequence; red boxes, non-conservative substitutions, orange boxes,  
1071 conservative substitutions (general charge/polarity/hydrophobicity retention), cyan  
1072 boxes, extended loop region, dark blue boxes, truncated loop region. (b) Top panel;  
1073 view of the K $\beta$ 4-KL8 region of the tubulin-binding subdomain in the KBP-  
1074 KIF15\_MD6S model, coloured as in Figure 4. Bottom panel; as in upper panel, but  
1075 with the KIF11\_MD cryo-EM model (PDB: 6TA4<sup>77</sup>) superimposed onto the now  
1076 hidden KBP-KIF15\_MD6S. Note steric clash introduced by KIF11\_MD’s extended  
1077 KL8. c) Schematic model of KBP’s hypothesised selective kinesin inhibition  
1078 mechanism. KBP (olive) binds the compatible TBsd of recognised kinesins (magenta)  
1079 but is incompatible with the TBsd of non-binding kinesins (salmon). For its target  
1080 kinesins, KBP therefore sterically blocks the TBsd interaction with MTs (grey),  
1081 preventing activation of kinesin ATPase and motility.

1082

1083 **Figure 1–figure supplement 1. KBP reconstruction, structure and loop lengths.**

1084 (a) Gold-standard FSC curves between independent masked, unmasked, phase-  
1085 randomised and corrected half-maps<sup>78</sup> of KBP as calculated by RELION v3.1<sup>52</sup> (4.6 Å  
1086 resolution at the ‘gold-standard’ 0.143 FSC cutoff). (b) Density and fitted model for  
1087 TPR3 of KBP, showing exemplar bulky side chain density that guided modelling. (c)  
1088 Same view as Figure 1c (upper panel), or rotated 90° around the indicated axis (lower  
1089 panel), showing only  $\alpha$ -helices (semi-transparent white tubes) with their terminal  
1090 residues coloured, illustrating the edges and faces (concave and convex) of the  $\alpha$ -  
1091 solenoid respectively. (d) Same view as panel c, but now with loops shown and  
1092 coloured (semi-transparent tube helices have their directionality represented by  
1093 arrows). Each loop or terminus label has a superscript number indicating their length.  
1094 (e) Same as panel d, rotated 180° around the indicated axis.

1095

1096 **Figure 1–figure supplement 2. KBP loops, sequence, inter-species conservation**  
1097 **and experimental mutations.**

1098 The human KBP sequence (numbering above), with residues coloured by intra-  
1099 species sequence identity as indicated in the key. The following species were included  
1100 in the Clustal Omega multiple sequence alignment<sup>67</sup>; *Homo sapiens*, *Mus musculus*,  
1101 *Gallus gallus*, *Xenopus tropicalis*, *Alligator mississippiensis*, *Danio rerio*, *Drosophila*  
1102 *melanogaster*, *Amphimedon queenslandica*, *Stylophora pistillata*, *Trichoplax*  
1103 *adhaerens*, *Spizellomyces punctatus* and *Salpingoeca rosetta*. Above the sequence,  
1104 secondary structure elements are indicated, coloured to delineate the nine  $\alpha$ -helical  
1105 pairs and connecting loops. Mutation sites are indicated within each boxed sequence  
1106 region, labelled to coordinate with Figure 5. Within these boxes, the mutated  
1107 sequence is shown below the original wild-type sequence.

1108

1109 **Figure 1–figure supplement 3. Approximate path of the KBP linker loop.**

1110 View of KBP’s linker loop, using the same KBP model subdomain colouring and  
1111 representation as in Fig. 1a-b. Only density for the linker loop is shown and a rough  
1112 path for the linker loop is indicated with a solid black line. KBP  $\alpha$ -helices contacting  
1113 the linker loop region are labelled.

1114

1115 **Figure 2–figure supplement 1. KBP-KIF15\_MD6S reconstruction resolution**  
1116 **estimation and 2D class analysis of KBP-KIF1A\_MD and KBP-KIF15\_MD**  
1117 **complexes.**

1118 (a) KIF15\_MD6S MT-activated steady-state ATPase velocity plotted as a function of  
1119 [MT]. Data were fit to a Michaelis Menten kinetic (pink curve) yielding values for  
1120  $k_{cat} = 2.9 \pm 0.5 \text{ sec}^{-1}$  and  $K_{0.5,MT} = 4.8 \pm 1.4 \mu\text{M}$ ;  $R^2 = 0.97$ , which are very similar  
1121 to previously published values for KIF15\_MD of  $k_{cat} = 2.1 \text{ sec}^{-1}$  and  $K_{0.5,MT} = 3.1$   
1122  $\mu\text{M}^{25}$ . (b) Gold-standard FSC curves between independent masked, unmasked, phase-  
1123 randomised and corrected half-maps<sup>78</sup> of the KBP-KIF15\_MD6S complex as  
1124 calculated by RELION v3.0<sup>52</sup>. The resolution at the ‘gold-standard’ 0.143 FSC cutoff  
1125 is 6.9 Å. (c) Local resolution as calculated by RELION v3.0, shown on the same view  
1126 as in Figure 2a with coloured density corresponding to the local resolutions indicated  
1127 in the key. (d) Selected RELION v3.0<sup>52</sup> 2D classes of KBP-KIF15\_MD6S (left) and  
1128 KIF1A\_MD-KBP (4 to the right). Densities for the kinesin motor domain and KBP  
1129 are pseudo-coloured pale magenta and pale orange respectively. Classes have been in-  
1130 plane rotated such that KBP is seen from roughly the same orientation. Note poor  
1131 resolution and a variable relative position in the KIF1A\_MD. (d) A representative  
1132 subset of KBP-KIF15\_MD6S complex 2D classes, showing multiple orientations.

1133

1134 **Figure 3–figure supplement 1. KIF15\_MD6S adopts a canonical MT-bound**  
1135 **kinesin conformation.** (a) Gold-standard FSC curves between independent masked,  
1136 unmasked, phase-randomised and corrected half-maps<sup>78</sup> of the KIF15\_MD6S-MT  
1137 complex as calculated by RELION v3.0<sup>52</sup>. The resolution at the ‘gold-standard’ 0.143  
1138 FSC cutoff is 4.5 Å. (b) Local resolution as calculated by RELION v3.0, with  
1139 coloured density corresponding to the local resolutions indicated in the key. (c) The  
1140 KIF15\_MD6S-MT asymmetric unit model in corresponding density. The  
1141 KIF15\_MD6S is coloured by subdomain, bound Mg<sub>2+</sub>-AMPPNP coloured lilac and  $\alpha$   
1142 and  $\beta$ -tubulin are coloured light and dark grey respectively, along with their  
1143 corresponding cryo-EM densities. The same view as in panel b. (d) A view of the  
1144 KIF15\_MD6S-MT asymmetric unit showing a docked conformation of the neck-  
1145 linker (KNL). Model and density colouring as in panel c. (e) The tubulin binding  
1146 subdomain of KIF15\_MD alone from the crystal structure (PDB code:4BN2<sup>25</sup>). (f)  
1147 KIF15\_MD6S complexed with MTs with only the tubulin-binding subdomain and  $\alpha$   
1148 and  $\beta$ -tubulin shown, along with the tubulin binding subdomain cryo-EM density  
1149 (semi-transparent). K $\alpha$ 4 of KIF15\_MD6S is extended relative to the crystal structure  
1150 and KL12 adopts a new conformation on  $\alpha$ -tubulin, as indicated by the green dashed  
1151 lines. Panels d and e show the KIF15\_MD6S tubulin-binding subdomain from the  
1152 same viewpoint.

1153

1154 **Figure 3–figure supplement 2. Movement of K $\alpha$ 4 of the Kin TBsd upon KBP**  
1155 **binding.**

1156 (a) The KIF15\_MD alone crystal structure (PDB code:4BN2<sup>25</sup>) is shown coloured by  
1157 kinesin subdomain (as in Figure 3), fitted into the KBP-KIF15\_MD6S complex cryo-  
1158 EM map, with only density shown for the Mg<sub>2+</sub>-ADP as mesh. Density for Mg<sub>2+</sub>-ADP

1159 is found in the expected position between nucleotide binding elements KL9, KL11,  
1160 K $\alpha$ 2a and the P-loop. (b) As in Figure 3a, but with a clipped viewpoint zoomed on the  
1161 TBsd (in pale magenta to illustrate poor fit). Black arrows indicate unaccounted-for  
1162 density. (c) As in Figure 3b, with the clipped viewpoint as in panel b of this figure  
1163 (TBsd now opaque to illustrate good density fit). (d) The KBP-KIF15\_MD6S model  
1164 is shown as opaque ribbons, with kinesin subdomain colouring as in panel a and b and  
1165 as labelled. KBP is shown as a transparent light grey surface representation. The  
1166 boxed region indicates that shown in Figure 3c-e. (e) RMSD in Å corresponding to  
1167 the KIF15\_MD6S overlay in panel d, shown on the model of KIF15\_MD6S in  
1168 complex with KBP (grey transparent surface). Parts of the model coloured black are  
1169 disordered/missing in the KIF15\_MD alone crystal structure.

1170

1171 **Figure 3—figure supplement 3. Examples of TPR-containing  $\alpha$ -solenoid proteins**  
1172 **binding  $\alpha$ -helical SSE ligands.**

1173 (a-d) Comparison of (a) KBP- KIF15\_MD6S complex with other TPR-containing  $\alpha$ -  
1174 solenoids shown in blue, and binding peptide motifs shown in magenta or pink for  
1175 helical and random coil regions respectively; (b) the PINS-INSC complex<sup>30</sup>, (c) the  
1176 CDC16-CDC26 complex<sup>31</sup> and (d) the PscE/PscG-PscF complex<sup>32</sup>.

1177

1178 **Figure 4—figure supplement 1. Additional KBP  $\alpha$ -solenoid edge loops proximal to**  
1179 **KIF15\_MD6S.**

1180 Colouring and representation as in Figure 4. (a) A view showing  $\alpha$ -helical pairs  
1181  $\alpha$ HP1,  $\alpha$ HP2,  $\alpha$ HP3,  $\alpha$ HP8 and  $\alpha$ HP9 of KBP and the KIF15\_MD6S coloured by  
1182 subdomain as labelled. (b) Left zoomed region in panel a, with density removed,  
1183 showing KBP L1, L3, L5 and proximal kinesin elements KL9, KL11 and K $\alpha$ 6. ADP

1184 is coloured in light orchid. (c) Right zoomed region in panel a, with density removed,  
1185 showing KBP L16 and L18 and proximal KIF15\_MD6S elements KNT (kinesin N-  
1186 terminus), KNL (kinesin neck-linker) and KL12.

1187

1188 **Figure 5–figure supplement 1. KBP mutants show similar expression profiles in**  
1189 **COS-7 cells.**

1190 (a) Representative images of COS-7 cells expressing HA-KBP mutant constructs.  
1191 Scale bar, 10  $\mu$ m.

1192

1193 **Figure 5–figure supplement 2. Pull-down experiments demonstrate the effect of**  
1194 **KBP mutation on the interaction between KIF15 and KIF1A.**

1195 (a) Control pull-down experiment with bioGFP-EV, bioGFP-KIF1A\_MDC or  
1196 bioGFP-KIF15\_MDC and HA-KBP showing that KBP interacts with KIF1A\_MDC  
1197 and KIF15\_MDC, but not with bioGFP-EV. (b, c) Example of pull-down experiments  
1198 showing the interaction between (b) KIF15\_MDC or (c) KIF1A\_MDC and mutated  
1199 KBP constructs in HEK293T cell lysates. Graphs show the quantification of the  
1200 intensity of the mutated HA-KBP construct in the pull-down fraction over the input  
1201 fraction divided by the intensity of bioGFP\_MDC in the pull-down fraction and  
1202 normalized to HA-KBP. Data are displayed as mean  $\pm$  s.e.m. (data from two  
1203 independent experiments).

1204

1205 **Figure 5–figure supplement 3. Kinesin motors show different properties in the**  
1206 **peroxisome assay.**

1207 (a, b, c) Representative images of peroxisomes in COS-7 cells expressing  
1208 KIF1A\_MDC (a) or KIF15\_MDC-FRB (b, c), PEX-mRFP-FKBP and HA (left

1209 panels) with addition of rapalog. Images were thresholded at 10000 (a, b) or 7500 (c)  
1210 and peripheral areas of 5  $\mu\text{m}$  (a, b) or 7,5  $\mu\text{m}$  (c) are shown. Scale bars, 10  $\mu\text{m}$ .

1211

1212 **Video 1. KBP undergoes conformational change to relieve clashes when forming**  
1213 **a complex with KIF15\_MD6S.**

1214 The KBP-alone model was superimposed on the KBP- KIF15\_MD6S model using  
1215 UCSF Chimera's matchmaker<sup>57</sup>. A conformational morph movie was then generated  
1216 in Chimera between the KBP-alone and KIF15 motor domain bound states, with  
1217 KIF15\_MD6S shown throughout to illustrate the relief of clashes. The N-terminal and  
1218 C-terminal subdomains are coloured in olive and gold respectively, as in Figure 2a,b,  
1219 while KIF15\_MD6S is shown in pale magenta. Distances between identified clashing  
1220 atoms when KBP-alone is superimposed onto the KBP- KIF15\_MD6S model are  
1221 indicated by red linking lines and KBP clashing residues and side chains shown in  
1222 cyan. Atoms that were clashing remain coloured while the red lines gradually  
1223 disappear as the clashes are relieved by the conformational change. Clashes were  
1224 calculated in Chimera using default criteria.

1225

1226 **Video 2. Interaction of KBP with the KIF15 motor domain.**

1227 Model of the KBP- KIF15\_MD6S complex (ribbon representation) displayed in  
1228 experimental cryo-EM density. The N-terminal (olive) and C-terminal (gold)  
1229 subdomains and the linker region (black) are shown in KBP, while the KIF15\_MD6S  
1230 Switch 1/2 subdomain (Switch 1/2 subdomain) is coloured sienna, the P-loop  
1231 subdomain (Kin-PLsd) is coloured light pink and the Kif15\_MD tubulin-binding  
1232 subdomain (TBsd) is coloured magenta. Semi-transparent density is coloured



1233 regionally as per the fitted model and additional density for the linker loop is shown

1234 in semi-transparent black.

1235

1236

1237 **Table 1. Cryo-EM reconstruction information and model refinement statistics and**  
 1238 **model geometry.** Data collection, processing and model refinement information for the KBP,  
 1239 KBP- KIF15\_MD6S and KIF15\_MD6S-MT datasets.

	<b>KBP (EMDB: EMD- 11338, PDB: 6ZPG)</b>	<b>KBP- KIF15_MD6S (EMDB: EMD- 11339 , PDB: 6ZPH)</b>	<b>KIF15_MD6S- MT (EMDB: EMD- 11340, PDB: 6ZPI)</b>
<b>Data collection and processing</b>			
Pixel size (Å)*	1.055, 1.043 or 1.047	1.047	1.39
Number of micrographs (collected, final)*	9360, 7547	6497, 5138	214,202
Final particle number	258,049 (81,628 of which on graphene oxide)	7,513	12,674
Map resolution (Å)	4.6	6.9	4.5
FSC threshold†	Independent half-map FSC 0.143	Independent half-map FSC 0.143	Independent half-map FSC 0.143
<b>Refinement</b>			
Refinement resolution (Å)	<b>4.6</b>	<b>6.9</b>	<b>6</b>
CC_mask‡	<b>0.64</b>	<b>0.74</b>	<b>0.60</b>
Map sharpening <i>B</i> -factor (Å <sup>2</sup> )	<b>-200</b>	<b>-495</b>	<b>-134</b>
Model composition			
Nonhydrogen atoms	<b>3,808</b>	<b>6,232</b>	<b>9,420</b>
Protein residues	<b>610</b>	<b>948</b>	<b>1185</b>
Ligands	<b>0</b>	<b>1</b>	<b>4</b>
R.m.s. deviations§			
Bond lengths (Å)	<b>0.01</b>	<b>0.01</b>	<b>0.08</b>
Bond angles (°)	<b>0.96</b>	<b>1.07</b>	<b>0.17</b>
Validation#			
MolProbity score	<b>1.66</b>	<b>1.84</b>	<b>1.95</b>
Clashscore	<b>5.25</b>	<b>7.31</b>	<b>13.25</b>
Poor rotamers (%)	<b>0.5%</b>	<b>0.9%</b>	<b>0.1%</b>
Ramachandran plot#			
Favored (%)	<b>94.38</b>	<b>93.13</b>	<b>95.38</b>
Allowed (%)	<b>5.62</b>	<b>6.87</b>	<b>4.62</b>
Outliers (%)	<b>0</b>	<b>0</b>	<b>0</b>

1240 \*Inclusive of all data collection sessions

1241 †The resolution value at the gold-standard Fourier Shell Correlation (FSC) 0.143 criterion  
 1242 between independently refined half-maps.

1243 ‡Cross-correlation provided by Phenix real-space refine<sup>7</sup>.

1244 §Root-mean-square deviations of bond lengths or angles in the model.

1245 #As defined by the MolProbity validation server<sup>79</sup>.

1246 **Table 2. KBP mutants used in this study.** The original and mutated amino acid (top) and  
 1247 nucleotide sequences (bottom) are shown for each construct.  
 1248

Construct	Original sequence	Mutated to
L1	EKEPYK gagaaggaaccatacaag	AAAPAA gcagcagcaccagcagca
L3	TEE acggaggag	AGG gcaggagga
L5	REE agagaagaa	AGA gcaggagca
L10	KISATEDTPEAEGEVP aagatctcagccacagaagacactcctgaagctgaaggagaagtgccagagctt	AGAGAGAGPAGAGAGPGG gcaggagcaggagcaggagcaggaccagcaggagcaggagcaggaccaggagga
L12	DGY gatggttat	GGA ggcggcgcg
L14	DLNPQY gacctgaatccacagtat	AAGPAA gcagcaggaccagcagca
L16	NKVFPEHIGEDVL aataaagtattccctgagcatataggggaagatgttctt	AAGAPAGAGAGAA gcagcaggagcaccagcaggagcaggagcaggagcaggagcagca
L18	EKHPE gaaaagcatcctgag	AAGPG gcagcaggaccagga
$\alpha$ HP4a	YLAQ tacctagctcaa	ALAA gcactagctgca
$\alpha$ HP4b	Q cag	A gca
$\alpha$ HP5a	TLSQ acctgtcacag	ALSA gcgtgtcagcg

1249

1250

<b>Key Resources Table</b>				
<b>Reagent type (species) or resource</b>	<b>Designation</b>	<b>Source or reference</b>	<b>Identifiers</b>	<b>Additional information</b>
gene (Homo Sapiens)	KIAA1279	GenBank	HGNC:23419	
gene (Mus musculus)	KIF1A	GenBank	MGI:108391	
gene (Mus musculus)	KIF15	GenBank	MGI:109825 8	
strain, strain background ( <i>Escherichia coli</i> )	BL21(DE3)	NEB	Cat. #: C2527H	Competent cells
strain, strain background ( <i>Escherichia coli</i> )	BL21-Gold (DE3)	Agilent	Cat. #: 230130	Competent cells
strain, strain background ( <i>Escherichia coli</i> )	Rosetta2 (DE3)	Novagen	Cat. #: 71400	Competent cells
cell line (Homo Sapiens)	Human embryonic kidney 239T (HEK293T)	ATCC	CRL-3216 RRID:CVCL_0063	
cell line (Cercopithecus Aethiops)	Cercopithecus aethiops kidney (COS-7)	ATCC	CRL-1651 RRID:CVCL_0224	
peptide, recombinant protein	porcine tubulin (>99% pure)	Cytoskeleton Inc.	Cat. #: T240	
antibody	anti-HA (Mouse monoclonal)	Roche	Cat# 11666606001 ; RRID:AB_51	IF (1:500)

			4506	
antibody	anti-mouse IgG1, Alexa488 (Goat polyclonal)	Thermo Fisher Scientific	Cat# A-21121, RRID:AB_2535764	IF (1:400)
antibody	anti-HA (Mouse monoclonal)	Biolegend	Cat# 901533; RRID:AB_2801249	WB (1:2000)
antibody	anti-GFP (Rabbit polyclonal)	Abcam	Cat# ab290; RRID:AB_303395	WB (1:10000)
antibody	anti-Rabbit IgG Antibody, IRDye 680LT Conjugated (Goat polyclonal)	LI-COR Biosciences	Cat# 827-11081; RRID:AB_10795015	WB (1:20000)
antibody	anti-Mouse IgG Antibody, IRDye 800CW Conjugated (Goat polyclonal)	LI-COR Biosciences	Cat# 827-08364; RRID:AB_10793856	WB (1:15000)
recombinant DNA reagent	KBP (plasmid)	Kevenaer et al., 2016		Described in Materials and methods
recombinant DNA reagent	KIF1A_MD (plasmid)	Atherton et al., 2014		Described in Materials and methods
recombinant DNA reagent	KIF15_MD (plasmid)	This study		Described in Materials and methods

recombinant DNA reagent	pebioGFP (plasmid)	Van der Vaart et al., 2013	N/A	Described in Materials and methods
recombinant DNA reagent	BirA coding vector (plasmid)	Van der Vaart et al., 2013	N/A	Described in Materials and methods
recombinant DNA reagent	GW1-PEX3-mRFP-FKBP1 (plasmid)	Kevenaer et al., 2016	N/A	Described in Materials and methods
recombinant DNA reagent	$\beta$ actin-Kif1A_MDC-FRB (plasmid)	Kevenaer et al., 2016	N/A	Described in Materials and methods
recombinant DNA reagent	$\beta$ actin-Kif15_MDC-FRB (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pebioGFP-Kif1A_MDC (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pebioGFP-Kif15_MDC (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pGW1-HA-KBP (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pGW1-HA-KBP_L1 (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pGW1-HA-KBP_L3 (plasmid)	This study	N/A	Described in Materials and methods

recombinant DNA reagent	pGW1-HA-KBP_L5 (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pGW1-HA-KBP_L10 (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pGW1-HA-KBP_L12 (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pGW1-HA-KBP_L14 (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pGW1-HA-KBP_L16 (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pGW1-HA-KBP_L18 (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pGW1-HA-KBP_L10+L12 (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pGW1-HA-KBP_L10+L14 (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pGW1-HA-KBP_L12+L14 (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pGW1-HA-KBP_αHP4a (plasmid)	This study	N/A	Described in Materials and methods



recombinant DNA reagent	pGW1-HA-KBP_αHP4b (plasmid)	This study	N/A	Described in Materials and methods
recombinant DNA reagent	pGW1-HA-KBP_αHP5a (plasmid)	This study	N/A	Described in Materials and methods
sequence-based reagent	KBP_fwd	This study	PCR primer for KBP mutants	TATTATTA TGGCGCGC CAGGATCC CCGGAATT CGGCACGA GGGAGGC CGCTATGG CGAACGTT CCGTGGGC A
sequence-based reagent	KBP_rev	This study	PCR primer for KBP mutants	CTCGTCGA CTCCTAAT CCTTAAGT CAGGGCCA TCTT
sequence-based reagent	KBP_L1_fwd	This study	PCR primer for KBP_L1	CTGCATAA AAATCCGG CAGCAGCA CCAGCAGC ATCCAAAT ACAGCGCC
sequence-based reagent	KBP_L1_rev	This study	PCR primer for KBP_L1	GGCGCTGT ATTTGGAT GCTGCTGG TGCTGCTG CCGGATTT TTATGCAG
sequence-based reagent	KBP_L3_fwd	This study	PCR primer for KBP_L3	TGAACCAC ATCGACGC AGGAGGA CTGTCCGC GGGGGA
sequence-based reagent	KBP_L3_rev	This study	PCR primer for KBP_L3	TCCCCCGC CGACAGTC CTCCTGCG TCGATGTG

				GTTC A
sequence-based reagent	KBP_L5_fwd	This study	PCR primer for KBP_L5	ATCTTGTG GTCTGAAG CAGGAGC AATTGAAA CTGCACAG
sequence-based reagent	KBP_L5_rev	This study	PCR primer for KBP_L5	CTGTGCAG TTTCAATT GCTCCTGC TTCAGACC ACAAGAT
sequence-based reagent	KBP_L10_fwd	This study	PCR primer for KBP_L10	TTTGGTCA AACTGGAG CAGGAGC AGGAGCA GGAGCAG GACCAGCA GGAGCAG GAGCAGG ACCAGGA GGATATCA TCAAAGAA A
sequence-based reagent	KBP_L10_rev	This study	PCR primer for KBP_L10	TTTCTTTG ATGATATC CTCCTGGT CCTGCTCC TGCTCCTG CTGGTCCT GCTCCTGC TCCTGCTC CTGCTCCA GTTTGACC AAA
sequence-based reagent	KBP_L12_fwd	This study	PCR primer for KBP_L12	GAGTTCTT TCAGATTG GCGGCGCG GTC ACTGA CCATATT

sequence-based reagent	KBP_L12_rev	This study	PCR primer for KBP_L12	AATATGGT CAGTGACC GCGCCGCC AATCTGAA AGAACTC
sequence-based reagent	KBP_L14_fwd	This study	PCR primer for KBP_L14	TAGAGCCC CTAACTGT AGCAGCA GGACCAGC AGCATATC TGTTGGTC AAC
sequence-based reagent	KBP_L14_rev	This study	PCR primer for KBP_L14	GTTGACCA ACAGATAT GCTGCTGG TCCTGCTG CTACAGTT AGGGGCTC TA
sequence-based reagent	KBP_L16_fwd	This study	PCR primer for KBP_L16	TCCCTGAG AGACCCAG CAGCAGG AGCACCAG CAGGAGC AGGAGCA GGAGCAG CACGCCCT GCCATGTT A
sequence-based reagent	KBP_L16_rev	This study	PCR primer for KBP_L16	TAACATGG CAGGGCGT GCTGCTCC TGCTCCTG CTCCTGCT GGTGCTCC TGCTGCTG GGTCTCTC AGGGA
sequence-based reagent	KBP_L18_fwd	This study	PCR primer for KBP_L18	ATTGTTGA TTRACTGTG CAGCAGG ACCAGGA GCCGCCCA GGAAATA

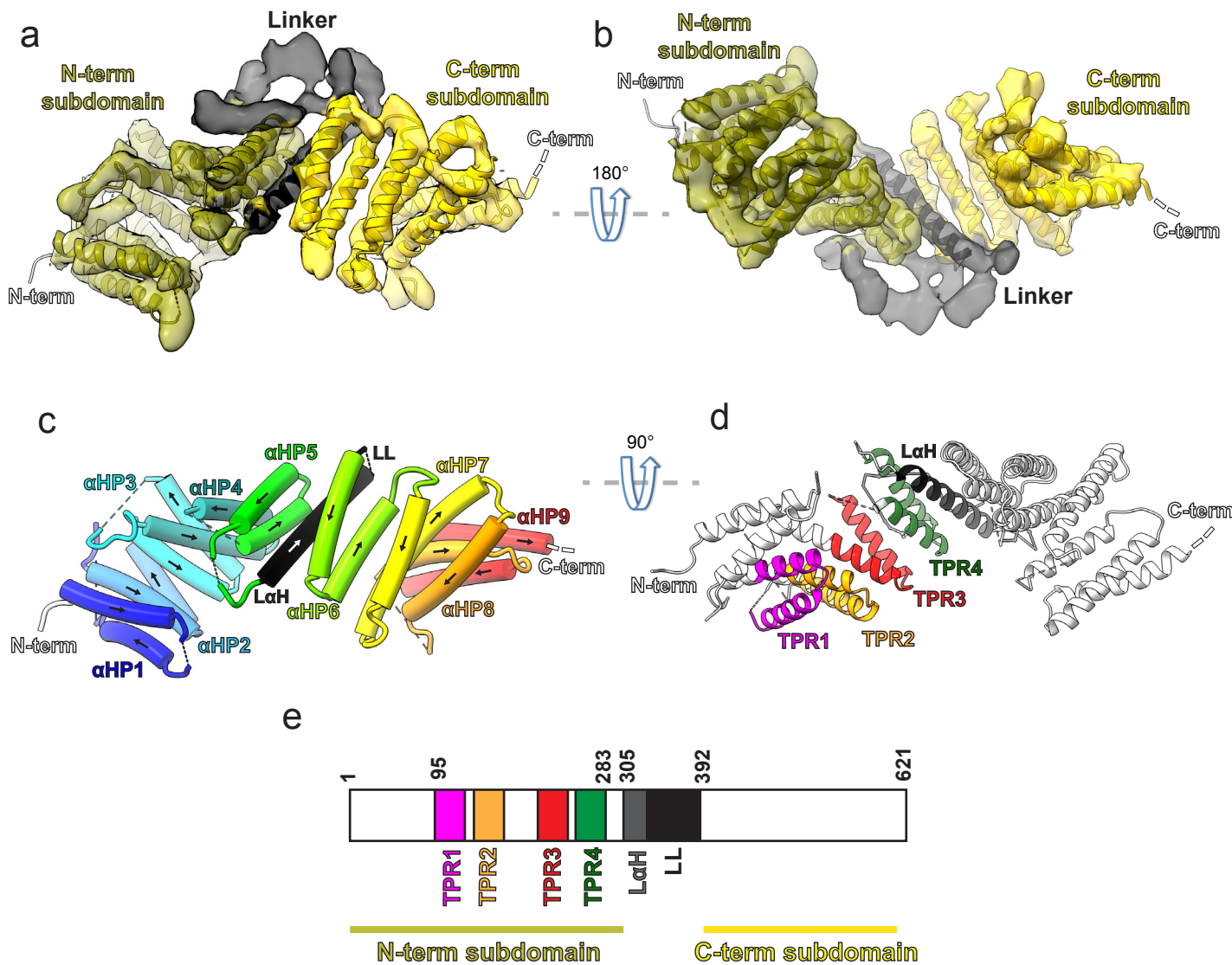
sequence-based reagent	KBP_L18_rev	This study	PCR primer for KBP_L18	TATTTCT GGGCGGCT CCTGGTCC TGCTGCAC AGTAATCA ACAAT
sequence-based reagent	KBP_HP4a_fwd	This study	PCR primer for KBP_L HP4a	ACTCATAA CCTATATG CACTAGCT GCAGTCTA CCAGCATC TG
sequence-based reagent	KBP_L HP4a_rev	This study	PCR primer for KBP_HP4a	CAGATGCT GGTAGACT GCAGCTAG TGCATATA GGTTATGA GT
sequence-based reagent	KBP_HP4b_fwd	This study	PCR primer for KBP_L HP4b	AGTACACT AAAACGC GCACTTGA GCACAATG CC
sequence-based reagent	KBP_L HP4b_rev	This study	PCR primer for KBP_HP4b	GGCATTGTG CTCAAGTGC GCGTTTTAGT GTA
sequence-based reagent	KBP_HP5a_fwd	This study	PCR primer for KBP_L HP5a	GCTATCAA TGCTGCTG CGTTGTCA GCGTTTAA CATCAATA AG
sequence-based reagent	KBP_HP5a_rev	This study	PCR primer for KBP_HP5a	CTTATTGA TGTA AAC GCTGACAA CGCAGCAG CATTGATA GC
sequence-based reagent	KIF15_FRB_fwd	This study	PCR primer for KIF15-FRB	AAGCTTGC CACCATGG GCGCGCCT GCCACCAT GGCTCCTG

				GCTGCAAA TCT
sequence- based reagent	KIF15_FRB_ rev	This study	PCR primer for KIF15- FRB	AGAGGATT CTAGAAGC AGGCGCGC CAGCGTAG TCTGGGAC GTCGTATG GGTAGAAT TCTCCTGG TGTCAGCT GCCAGA
sequence- based reagent	bioGFPKIF1 5_fwd	This study	PCR primer for bioGFPKIF1 5	AGCTCAAG CTTCGAAT TGGGCGCG CCAGCCAC CATGGCTC CTGGCTGC AAATCT
sequence- based reagent	bioGFPKIF1 5_rev	This study	PCR primer for bioGFPKIF1 5	GAATTCGA TATCCTGC AGGTCGAC TCCAGATC CTCATCCT GGTGTGCA CTGCCAG A
sequence- based reagent	bioGFPKIF1 A_fwd	This study	PCR primer for bioGFPKIF1 A	TATTATAA TGGCGCGC CAGCCACC GCCGGGGC CTCTGTGA AGGT
sequence- based reagent	bioGFPKIF1 A_rev	This study	PCR primer for bioGFPKIF1 A	CTCGTCGA CTCCTCCT CCTCATTT GGGAGAA AACACACC CAA
commercial assay or kit	EnzChek™ Phosphate Assay Kit	Invitrogen™	E6646	

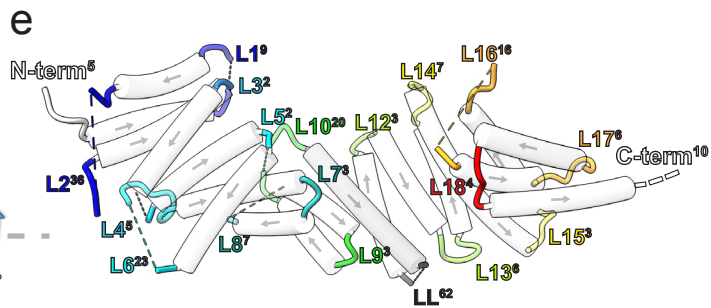
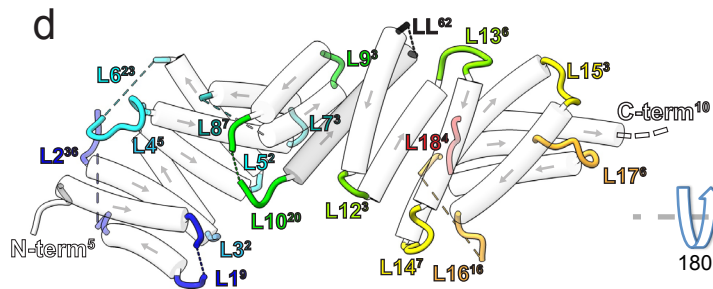
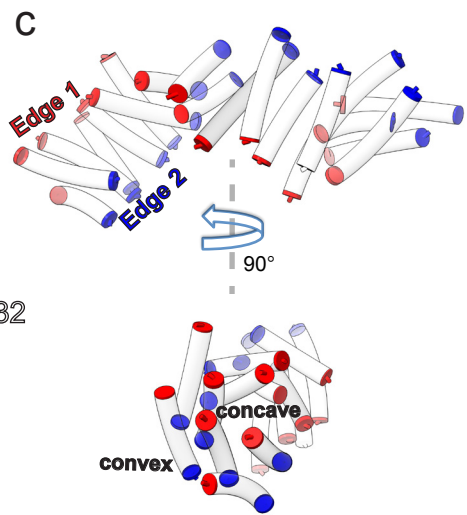
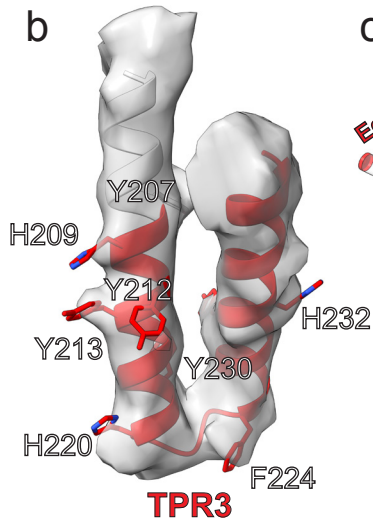
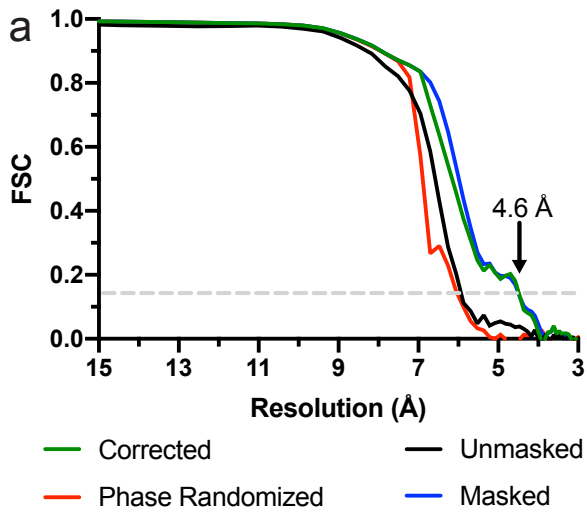
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chemical compound, drug	PEI	PolySciences	Cat# 24765-2	
chemical compound, drug	Fugene	Promega	Cat# E2692	
software, algorithm	ImageJ	NIH	<a href="https://imagej.nih.gov/ij/">https://imagej.nih.gov/ij/</a> ; RRID:SCR_003070	
software, algorithm	RELION	Zivanov et al., 2018	n/a	
software, algorithm	CryoSparc2	Punjani et al., 2017	n/a	
software, algorithm	CisTEM	Grant et al., 2018	n/a	
software, algorithm	MiRP	Cook et al., 2020	n/a	Protocol implemented in RELION

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# Figure 1

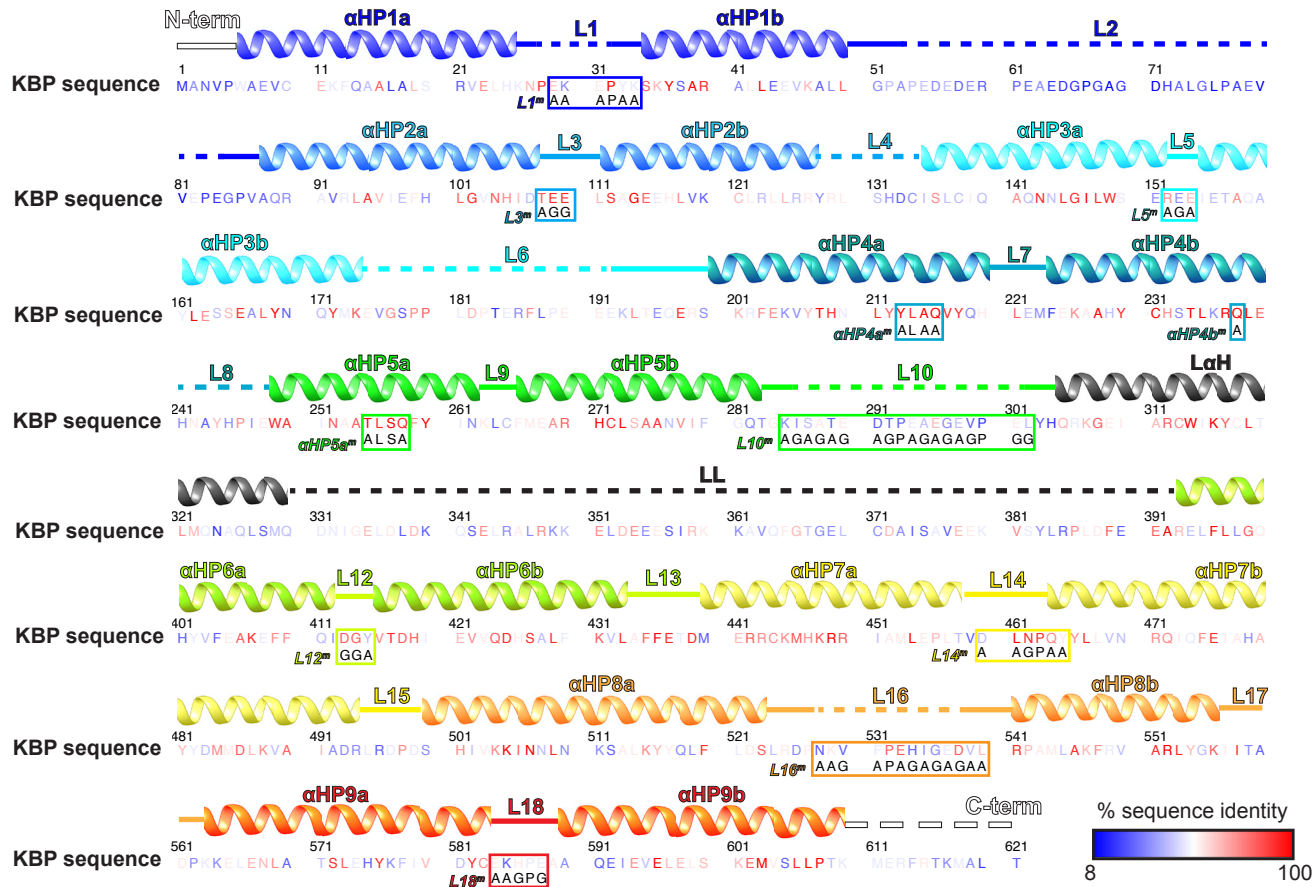


# Figure 1-figure supplement 1

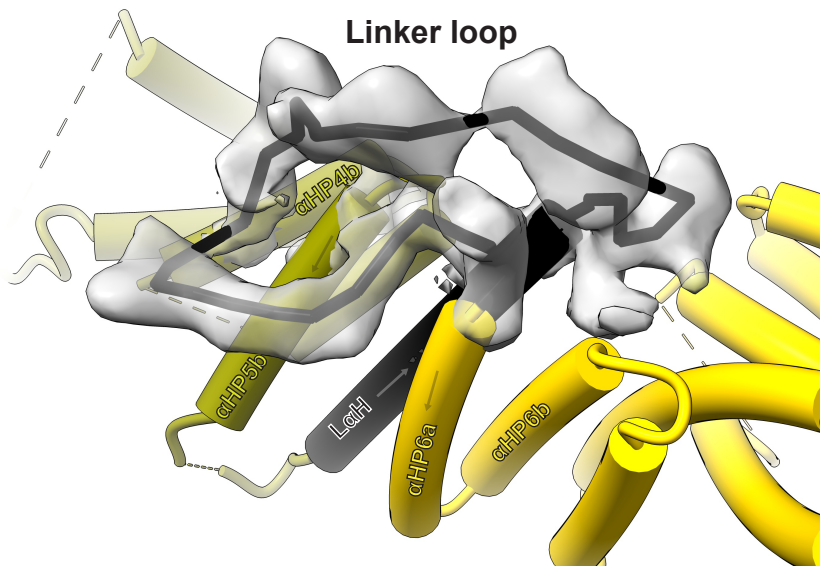




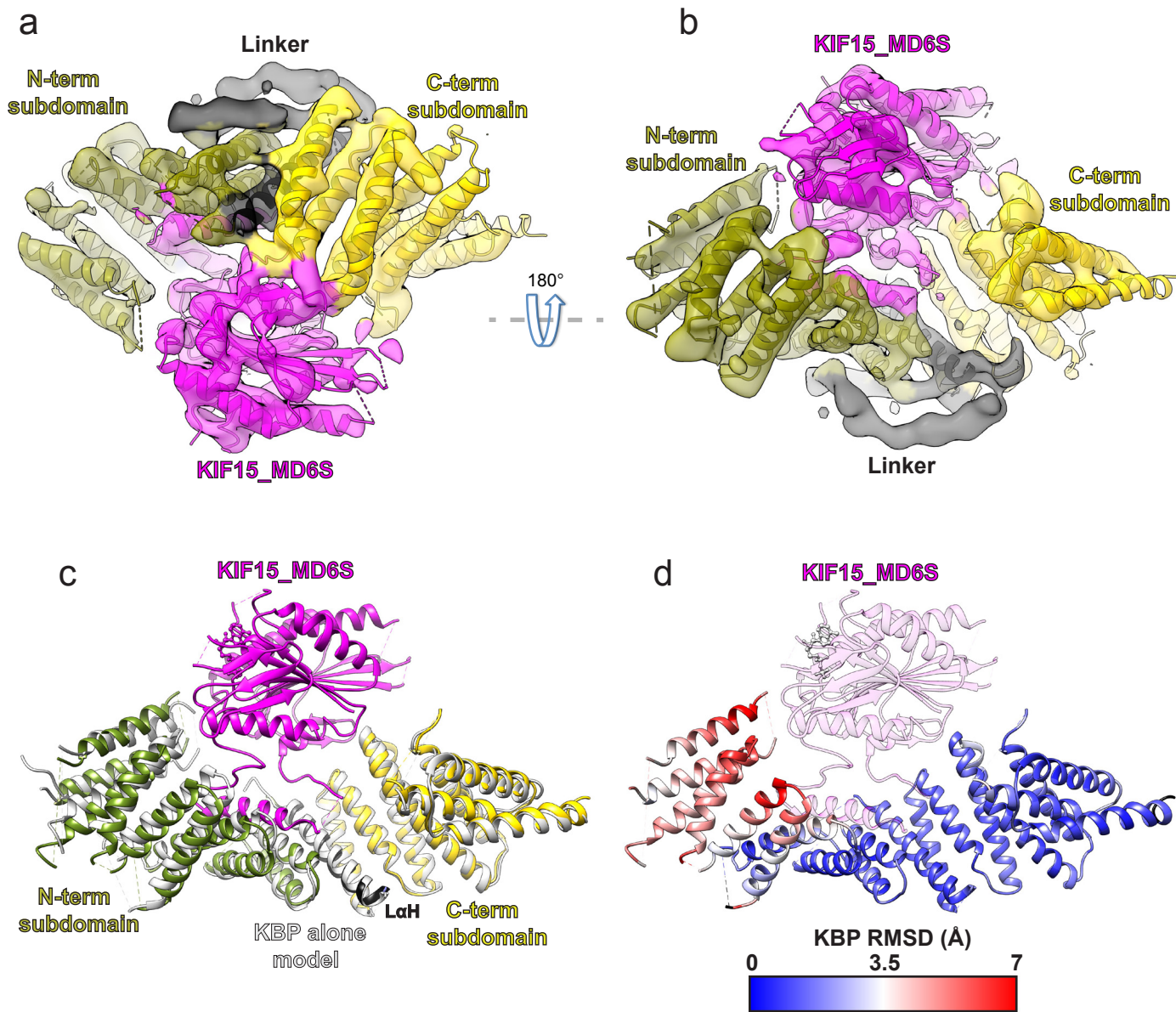
# Figure 1-figure supplement 2



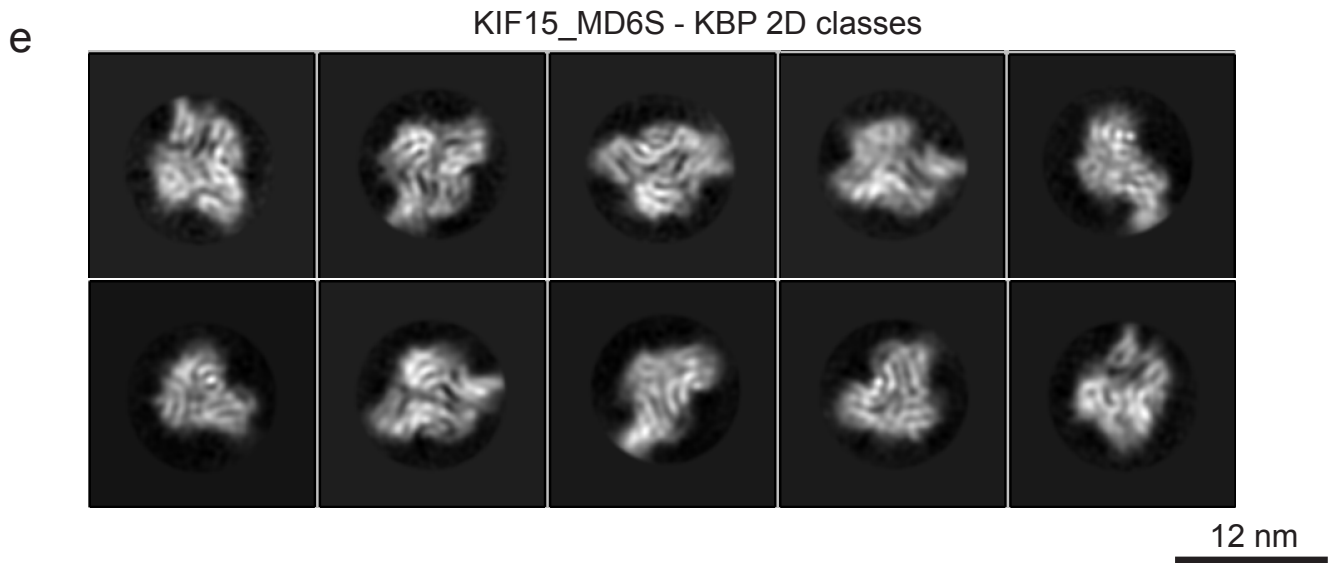
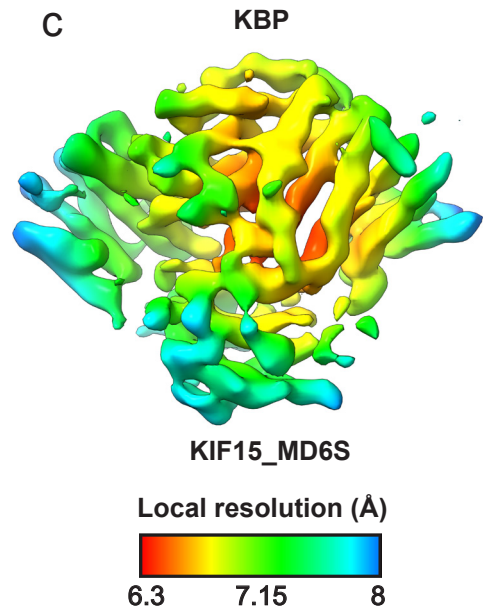
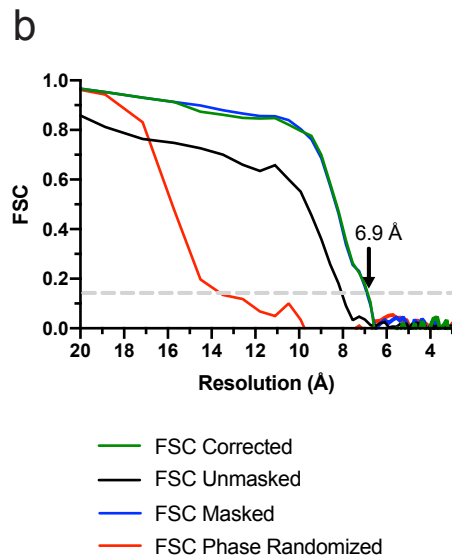
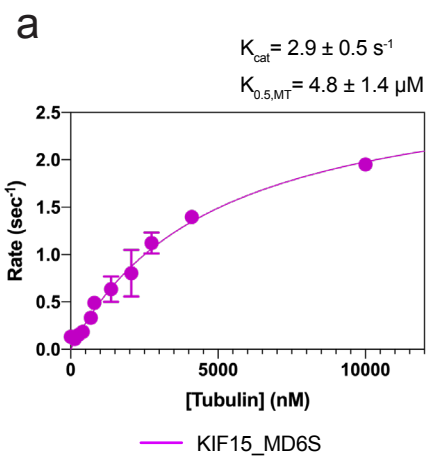
**Figure 1-figure supplement 3**



**Figure 2**

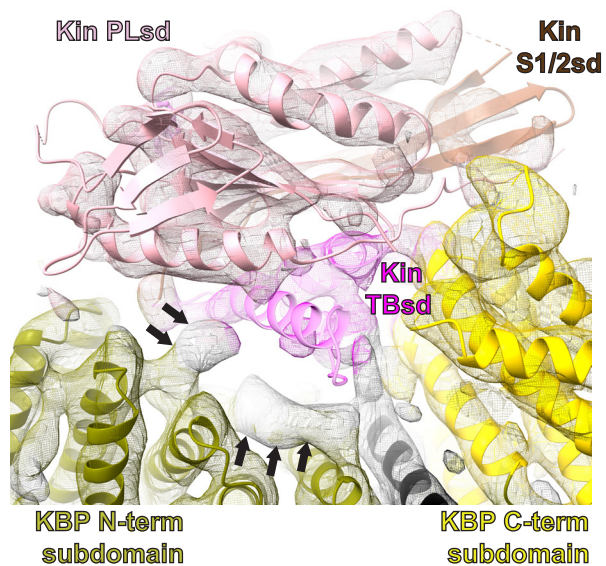


# Figure 2-figure supplement 1

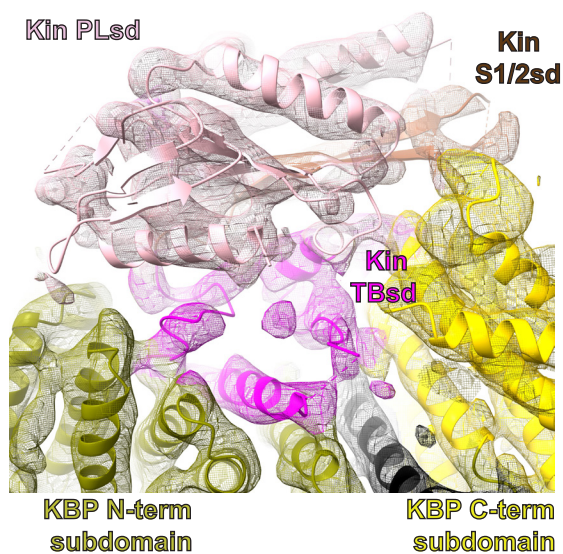


# Figure 3

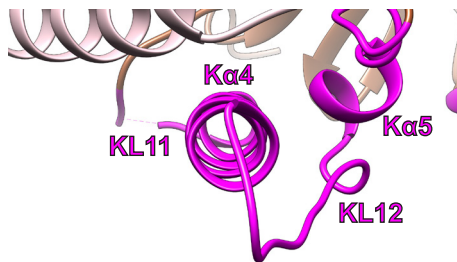
**a** KIF15\_MD x-ray (PDB:4BN2) + KBP



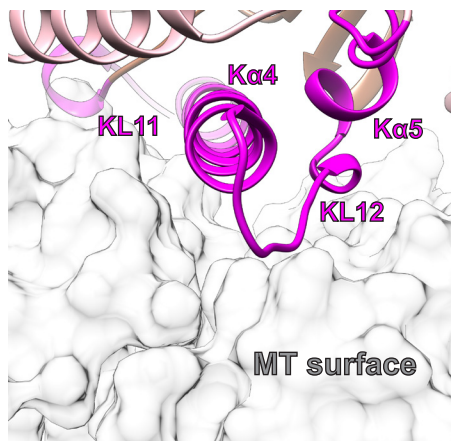
**b** KIF15\_MD6S-KBP



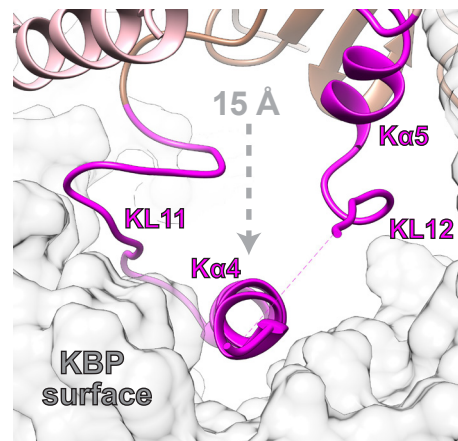
**c** KIF15\_MD x-ray



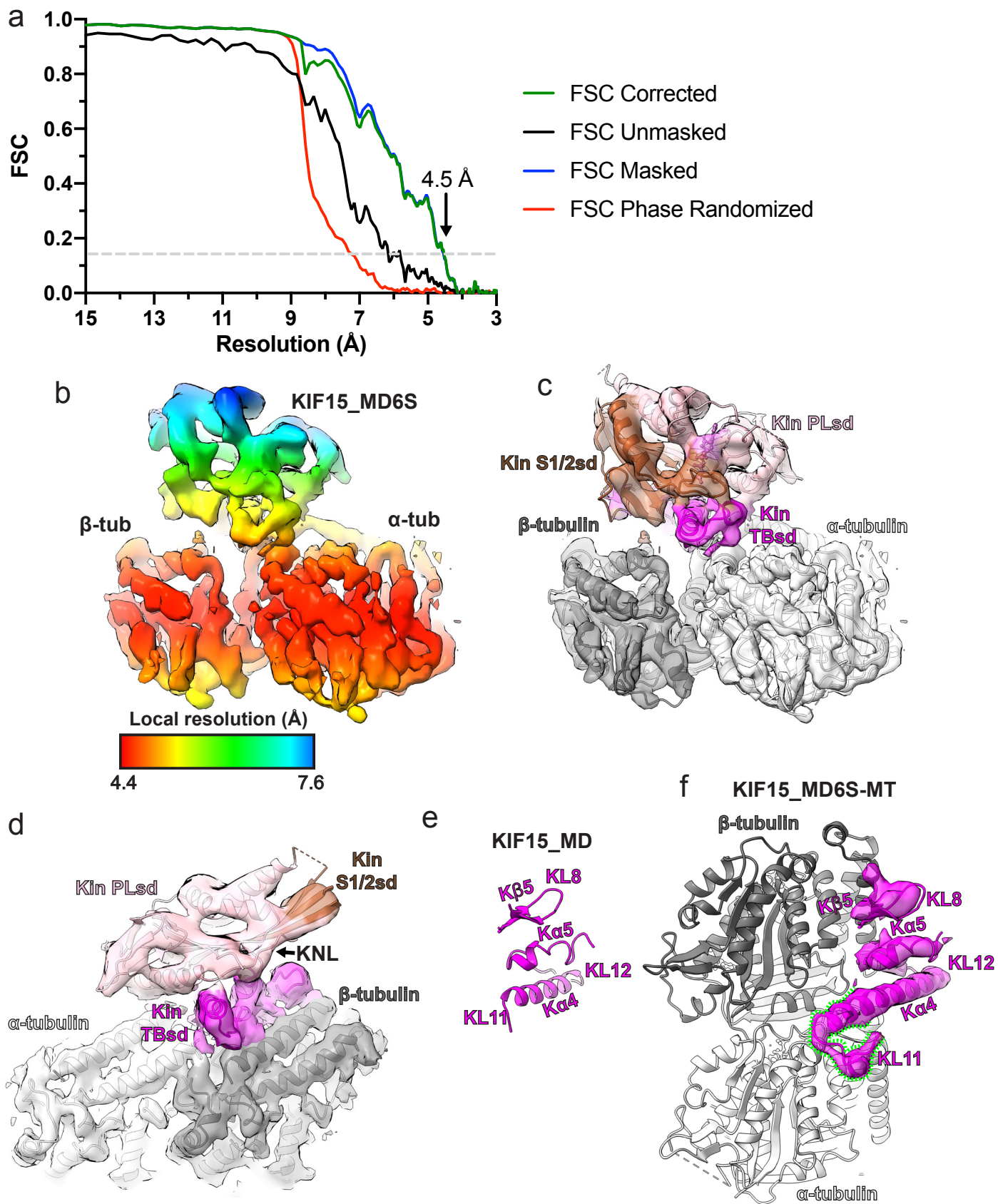
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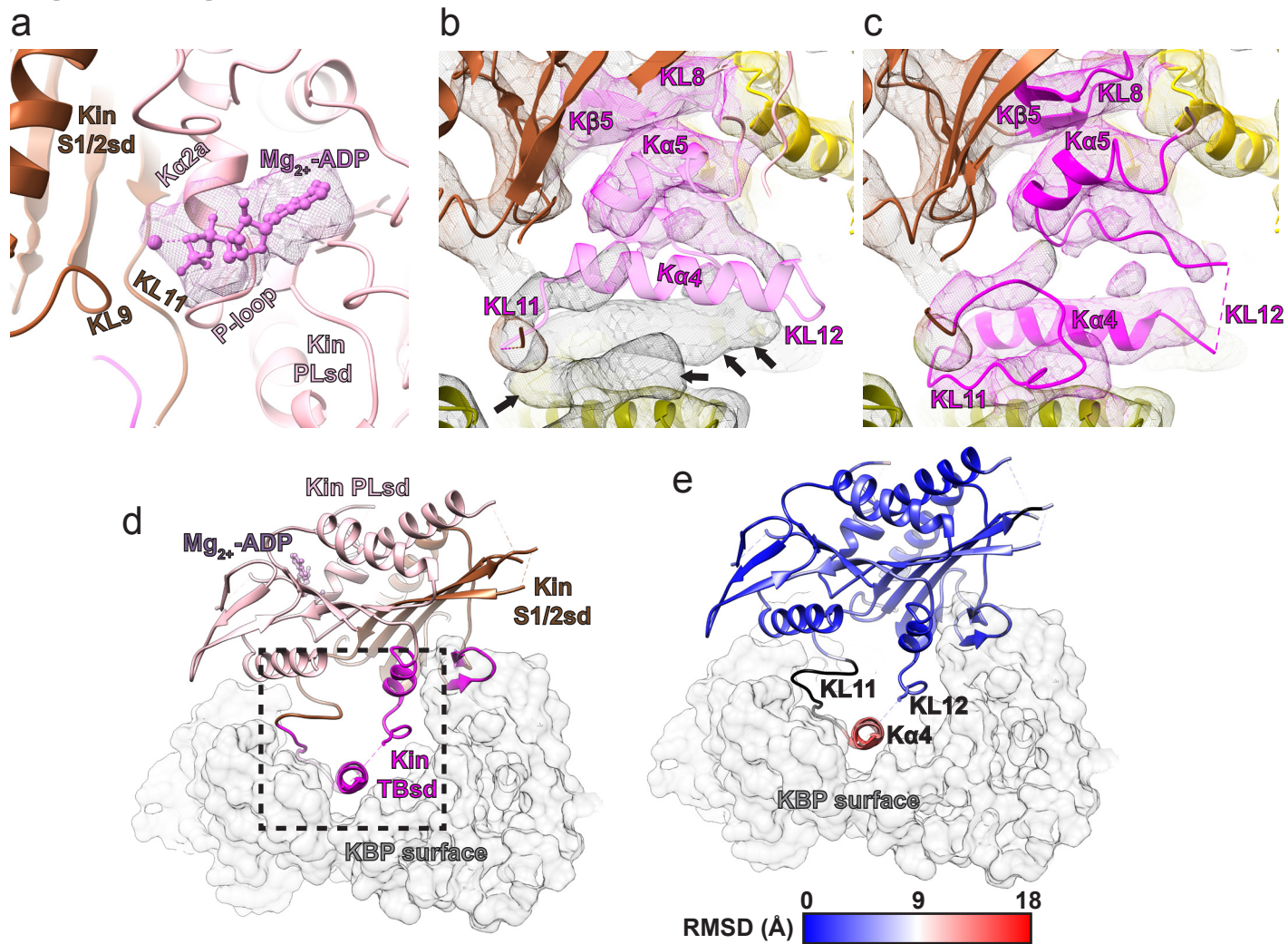
**e** KIF15\_MD6S-KBP



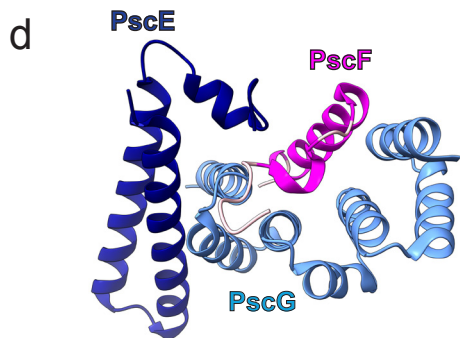
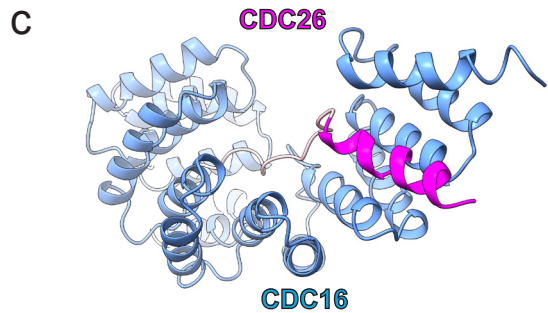
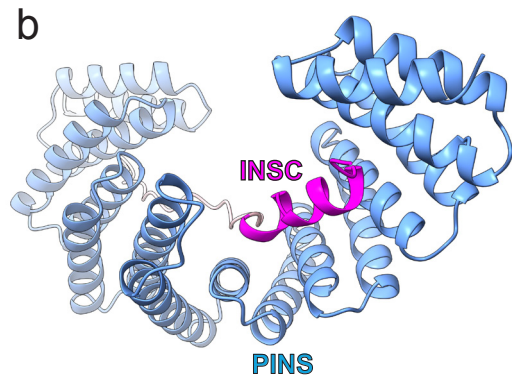
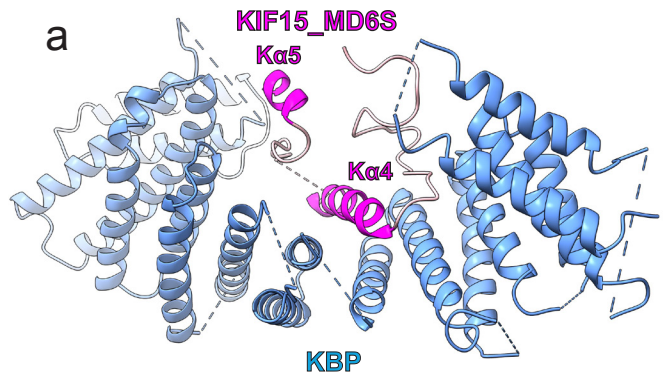
# Figure 3-figure supplement 1



**Figure 3-figure supplement 2**



# Figure 3-figure supplement 3





**Figure 4**

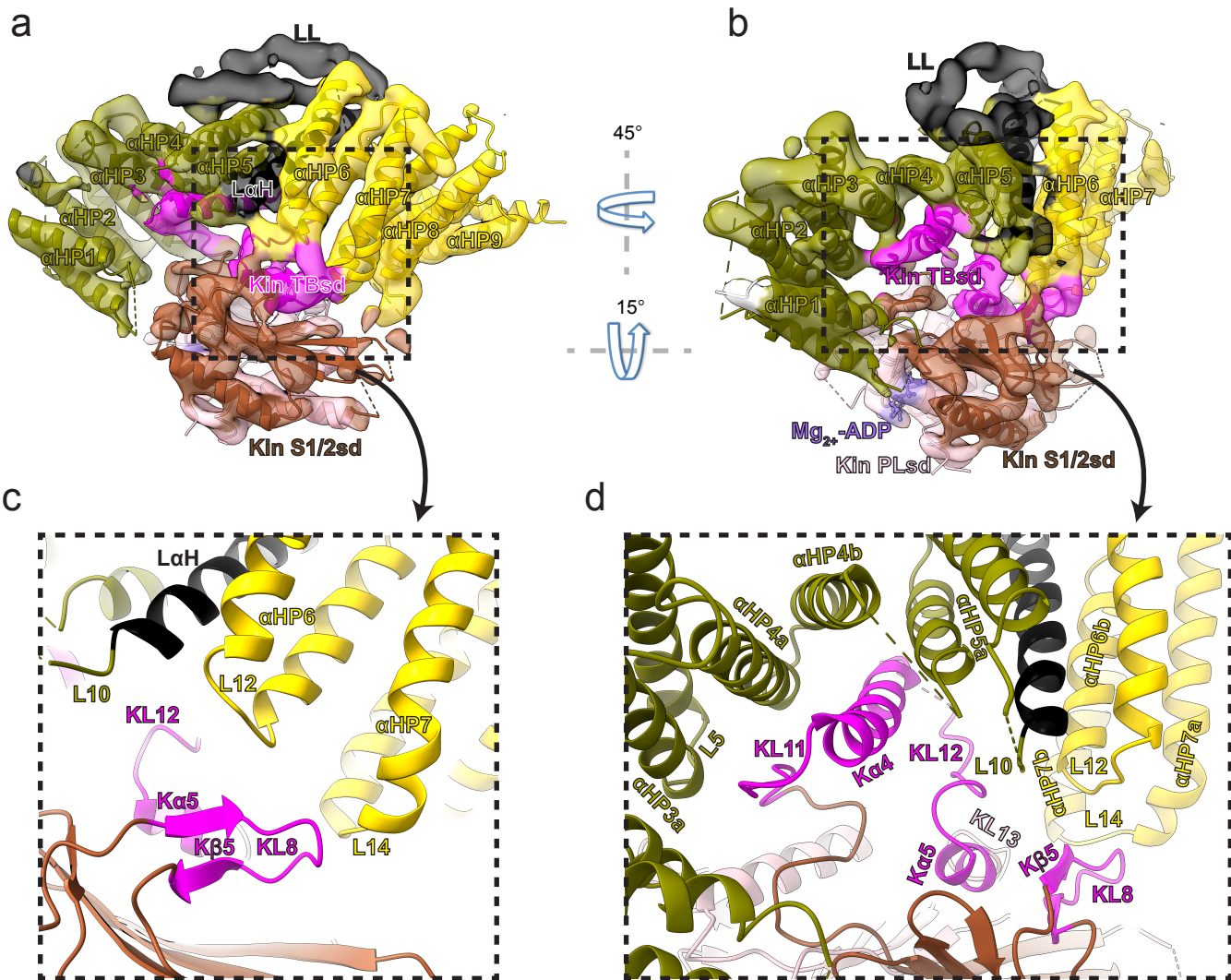
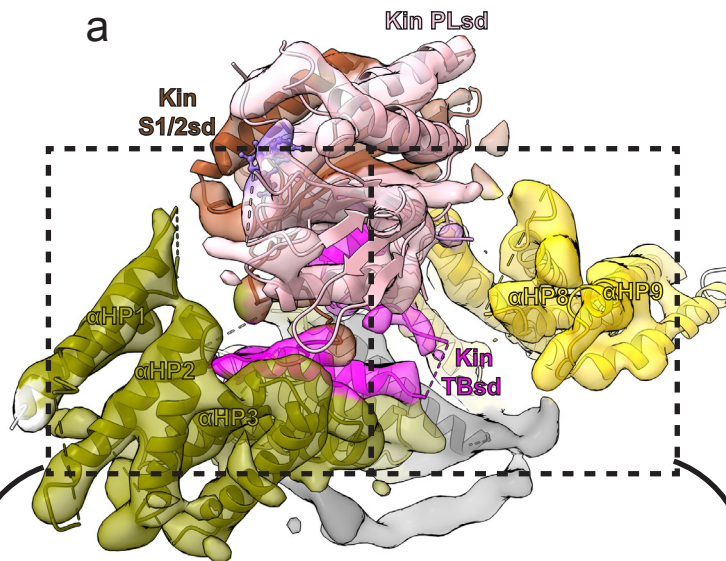
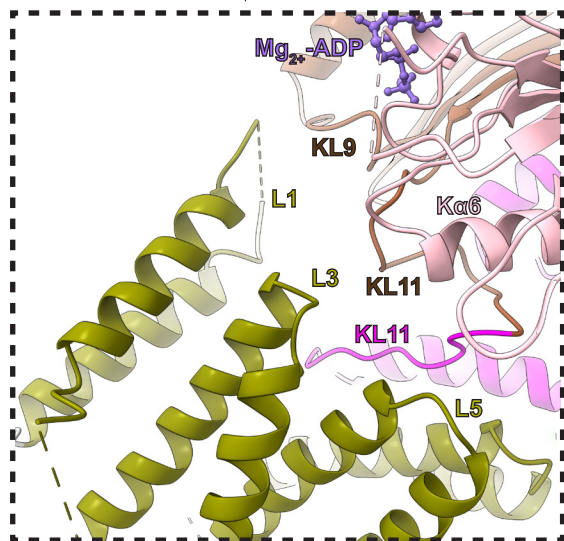


Figure 4-figure supplement 1

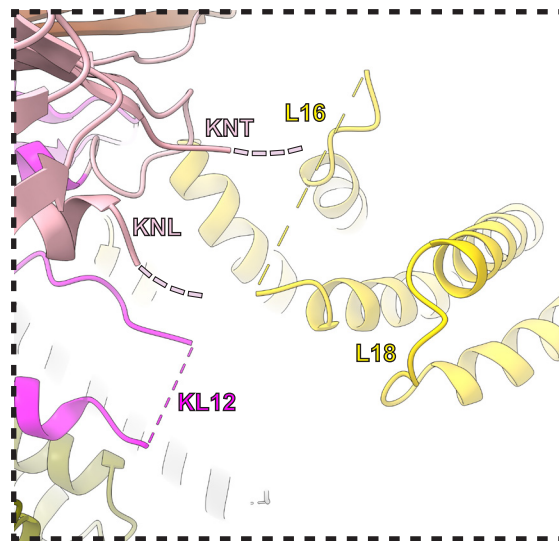
a



b

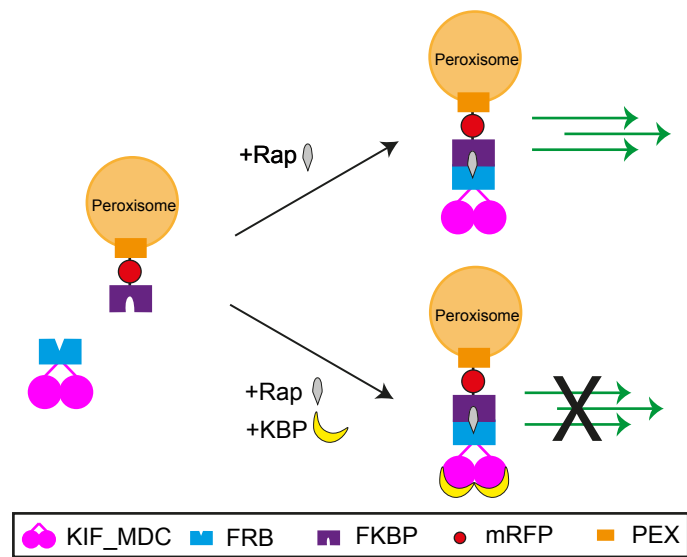


c

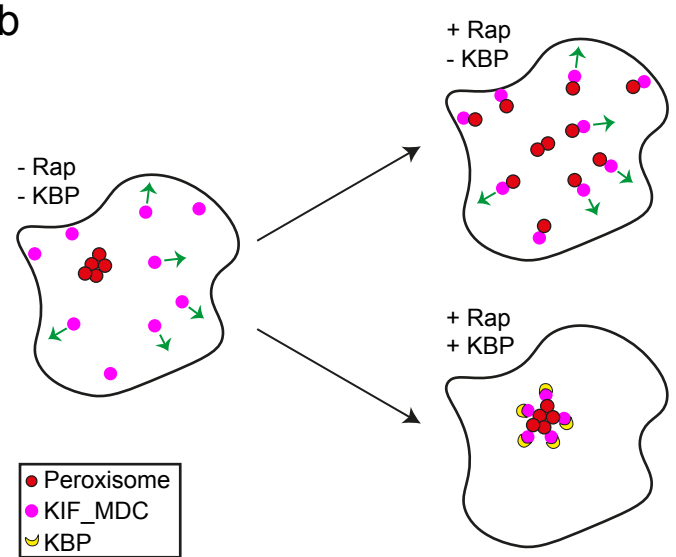


# Figure 5

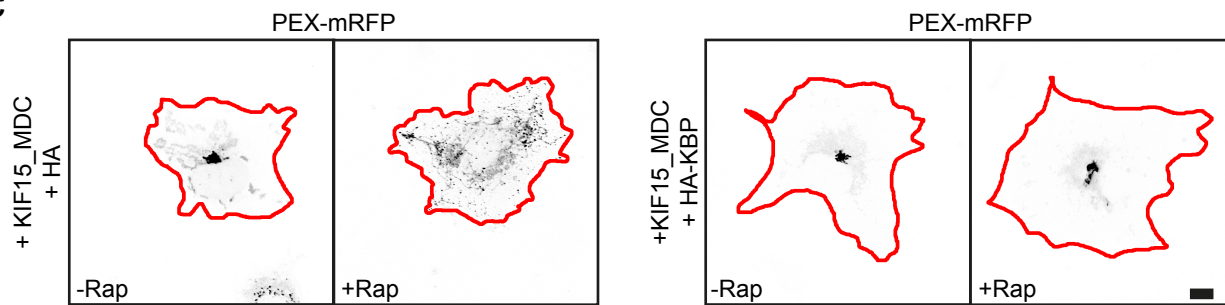
**a**



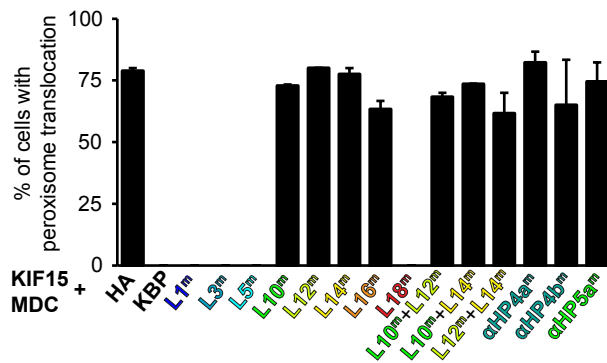
**b**



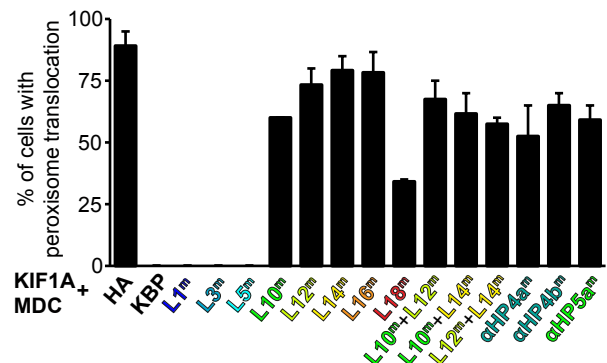
**c**



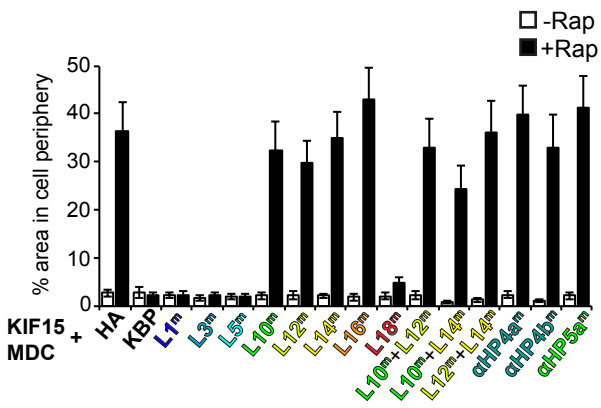
**d**



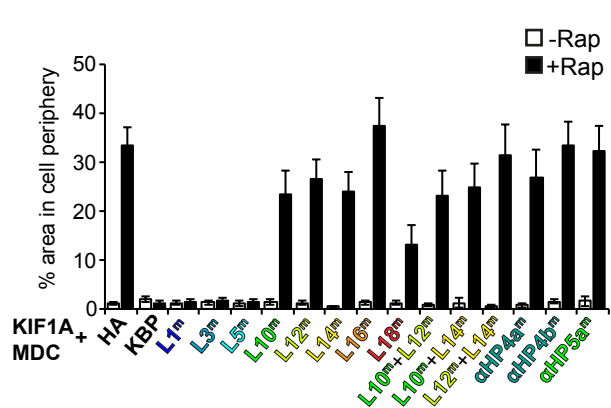
**e**



**f**

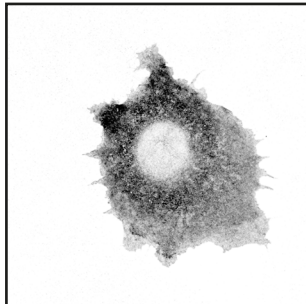


**g**

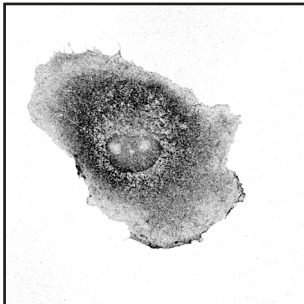


# Figure 5-figure supplement 1

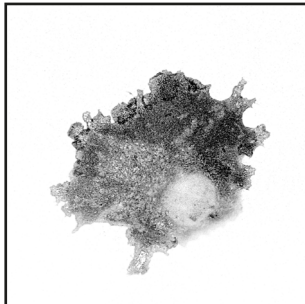
KBP



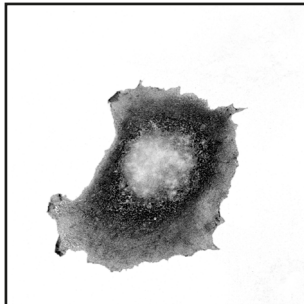
L1<sup>m</sup>



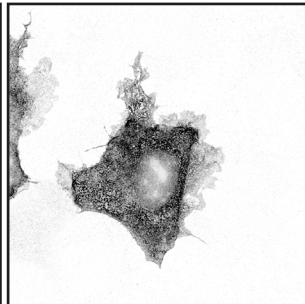
L3<sup>m</sup>



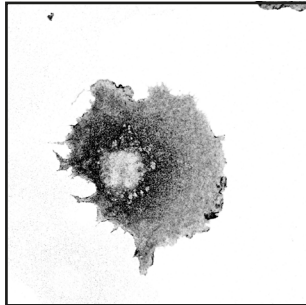
L5<sup>m</sup>



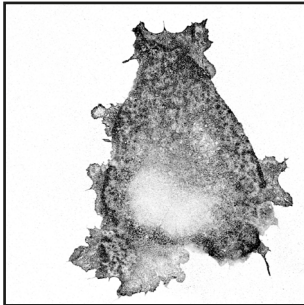
L10<sup>m</sup>



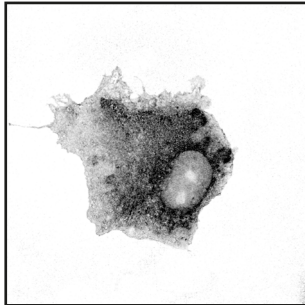
L12<sup>m</sup>



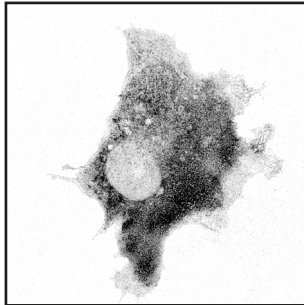
L14<sup>m</sup>



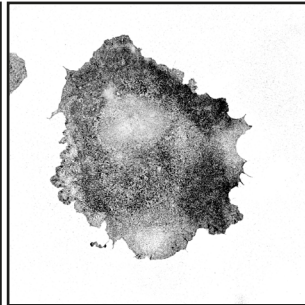
L16<sup>m</sup>



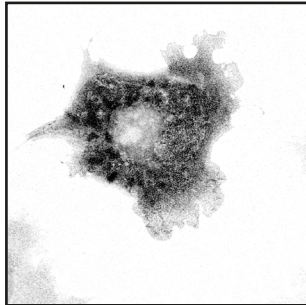
L18<sup>m</sup>



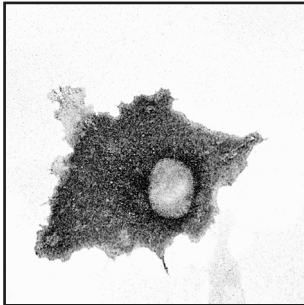
L10<sup>m</sup>+L12<sup>m</sup>



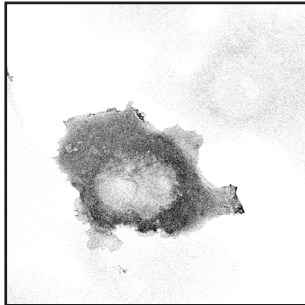
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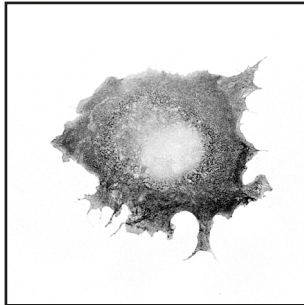
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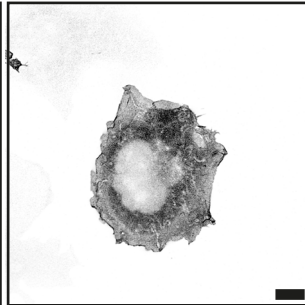
αHP4a<sup>m</sup>



αHP4b<sup>m</sup>

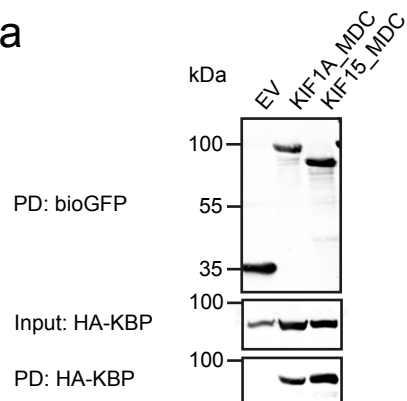


αHP5a<sup>m</sup>

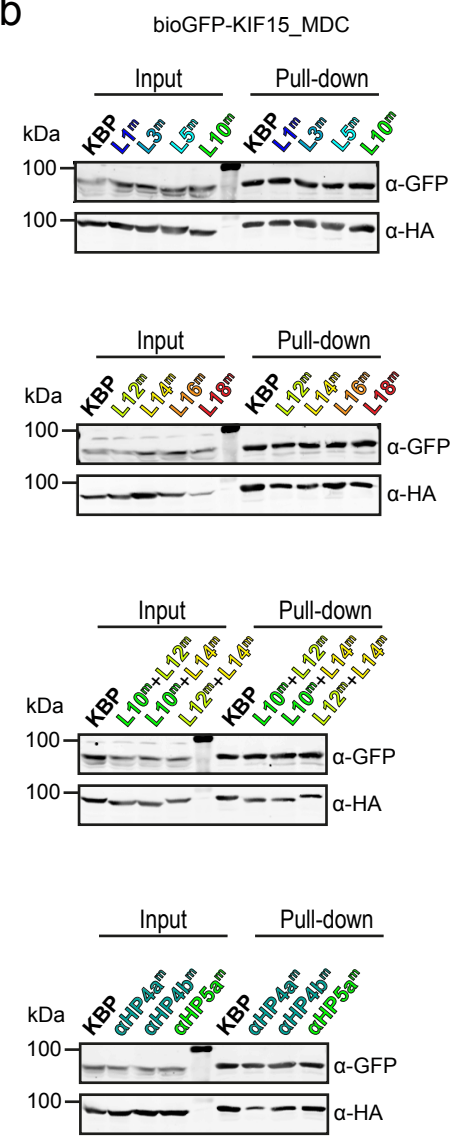


# Figure 5-figure supplement 2

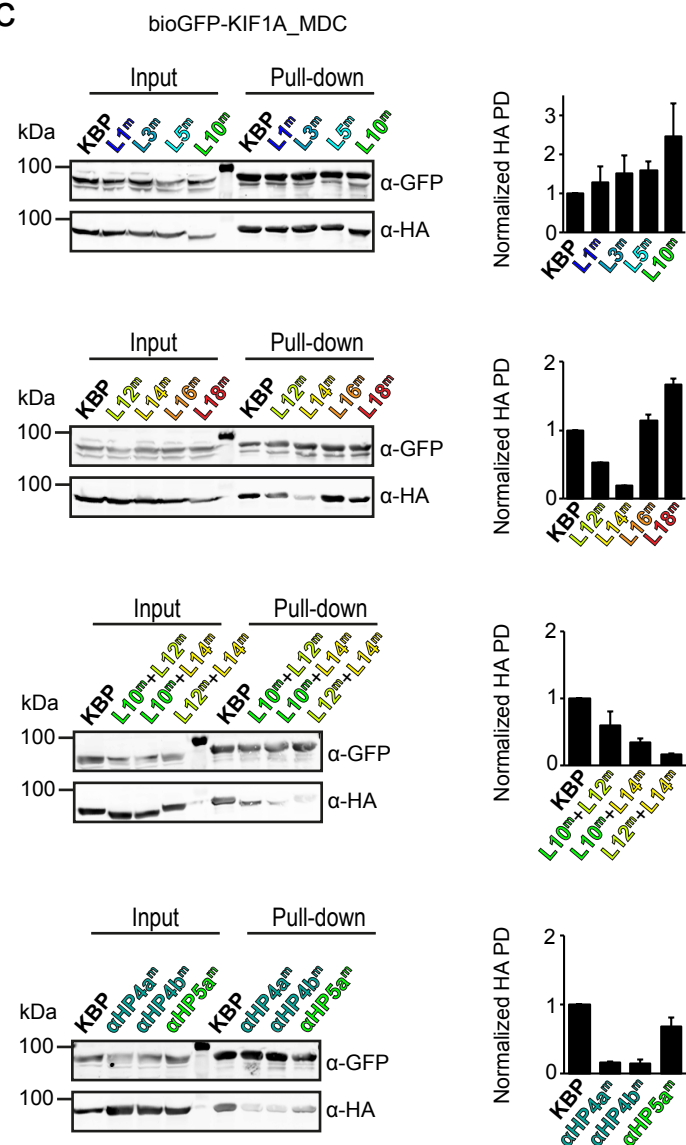
**a**



**b**



**c**



# Figure 5-figure supplement 3

