The mechanochemical origins of the microtubule sliding motility within the kinesin-5 domain organization

Stanley Nithianantham*, Malina K. Iwanski*[2], Ignas Gaska*, Himanshu Pandey, Tatyana Bodrug, Sayaka Inagaki[4], Jennifer Major[4], Gary J. Brouhard[2], Larissa Gheber, Steven S. Rosenfeld[4], Scott Forth[Ψ], Adam G. Hendricks[Ψ2], Jawdat Al-Bassam[Ψ]

[1] Dept. of Molecular and Cellular Biology, University of California, Davis, CA
[2] Dept. of Biology, Dept. of Bioengineering, McGill University, Montreal, QC
[3] Dept. of Biology, Rensselaer Polytechnic Institute, Troy
[3] Dept. of Chemistry, The Ben Gurion University, Ber Sheva, Israel
[4] Dept. of Pharmacology, Mayo Clinic, Jacksonville, FL

* These authors contributed equally to this work
[Ψ] These authors are co-corresponding

Abstract

The conserved kinesin-5 bipolar tetrameric motors slide apart microtubules during mitotic spindle assembly and elongation. Kinesin-5 bipolar organization originates from its conserved tetrameric helical minifilament, which position the C-terminal tail domains of two subunits near the N-terminal motor domains of two anti-parallel subunits (Scholey et al, 2014). This unique tetrameric structure enables kinesin-5 to simultaneously engage two microtubules and transmit forces between them, and for multiple kinesin-5 motors to organize via tail to motor interactions during microtubule sliding (Bodrug et al, 2020). Here, we show how these two structural adaptations, the kinesin-5 tail-motor domain interactions and the length of the tetrameric minifilament, determine critical aspects of kinesin-5 motility and sliding mechanisms. An x-ray structure of the 34-nm kinesin-5 minifilament reveals how the dual dimeric N-terminal coiled-coils emerge from the tetrameric central bundle. Using this structure, we generated active bipolar mini-tetrameric motors from *Drosophila* and human orthologs, which are half the length of native kinesin-5. Using single-molecule motility assays, we show that kinesin-5 tail domains promote mini-tetramers static pauses that punctuate processive motility. During such pauses, kinesin-5 mini-tetramers form multi-motor clusters mediated via tail to motor domain cross-interactions. These clusters undergo slow and highly processive motility and accumulate at microtubule plus-ends. In contrast to native kinesin-5, mini-tetramers require tail domains to initiate microtubule crosslinking. Although mini-tetramers are highly strained in initially aligning microtubules, they slide microtubules more efficiently than native kinesin-5, due to their decreased minifilament flexibility. Our studies reveal that the conserved kinesin-5 motor-tail mediated clustering and the length of the tetrameric minifilament are key features for sliding motility and are critical in organizing microtubules during mitotic spindle assembly and elongation.

Introduction

During cell division, microtubules (MTs) are rearranged into bipolar mitotic spindles from two overlapping MT asters during metaphase. Kinesin-5 motors are conserved across eukaryotes and are essential for the assembly and elongation of bipolar mitotic spindles. Kinesin-5 motors are 60-80 nm bipolar tetrameric proteins with a pair of N-terminal motor and C-terminal tail domains on either end of an α-helical central tetrameric minifilament (Kashina et al., 1996; Scholey et al., 2014). Although kinesin-5 motors are conserved across eukaryotes, there is a remarkable diversity in their mechanisms across species with the yeast kinesin-5 motors uniquely undergoing minus end directed motility as single motors, and switching direction upon clustering or during MT sliding toward plus-end directed motility (Pandey et al., 2021a; Pandey

53   et al., 2021b; Shapira et al., 2017; Singh et al., 2018). In contrast, metazoan Kinesin-5 motors
54   undergo motility towards MT plus ends both along single MTs and when sliding MTs (Bodrug et
55   al., 2020; Kapitein et al., 2005; Weinger et al., 2011).
56
57   Kinesin-5 motors slide apart MTs emanating from opposing spindle poles at the midzone region
58   by initially crosslinking, then aligning these MTs by undergoing motility with their bipolar ends
59   along two MTs using their unique tetrameric architecture (Kashina et al., 1996). This kinesin-5
60   MT sliding activity is conserved and critical for organizing bipolar mitotic spindles. The kinesin-5
61   tetrameric bipolar organization originates from the anti-parallel folding of the four α-helices from
62   the four subunits within the Bipolar assembly (BASS) domain (Scholey et al., 2014). The BASS
63   domain lies at the center of the kinesin-5 minifilament and forms the central force-bearing
64   structure that coordinates between the two motile motor ends, each of which supports hand
65   over-hand motility along MTs. While the kinesin-5 MT sliding motility is linked to the motor's
66   bipolar tetrameric organization, the functional relationship between the conserved kinesin-5
67   motor, tail or BASS domains and the unique MT crosslinking or MT sliding mechanisms remains
68   unknown (Kapitein et al., 2005).
69
70   The kinesin-5 C-terminal tail domains (termed tail from herein) emerge from bipolar tetrameric
71   minifilament near the motor domains of the antiparallel dimeric folded subunits (Acar et al.,
72   2013) and kinesin-5 tails are essential for MT sliding activity across species (Duselder et al.,
73   2015; Hildebrandt et al., 2006; Weinger et al., 2011). We recently discovered that the kinesin-5
74   tail regulates the MT-activated ATP hydrolysis in the motor domains and this regulation is
75   essential for kinesin-5 motors to transition from crosslinking to MT sliding motility (Bodrug et al.,
76   2020). This allosteric tail to motor domain interaction allow for trans interactions between
77   multiple kinesin-5 motors, resulting in multi-motor clustering (Bodrug et al., 2020). Tail-mediated
78   clustering is a critical for organizing the forces generated by multiple kinesin-5 motors promote
79   the alignment of MTs to crosslinked paired MTs then modulate their sliding (Bodrug et al., 2020).
80   However, it remains unknown how this tail- to motor regulation is impacted by the distinct
81   kinesin-5 central minifilament organization and how these two features regulate MT crosslinking,
82   alignment and sliding.
83
84   Here, we describe how the kinesin-5 tail-motor domain interaction and the length of the
85   tetrameric minifilament modulate kinesin-5 motor clustering, MT crosslinking, and MT sliding
86   activities. We determined an x-ray structure of a 34-nm extended BASS α-helical tetramer,
87   revealing rigid dimeric parallel N-terminal coiled-coil junctions that emerge from its central
88   tetrameric core. Using this structure as a platform, we engineered short human and *Drosophila*
89   bipolar 38-nm mini-tetrameric kinesin-5 motors, which are roughly half the length of native
90   kinesin-5 motors. Single-molecule motility assays reveal mini-tetramers without tail domains
91   undergo processive motility with infrequent pauses in which motors statically bind MTs without
92   diffusing. The tail domains enhance kinesin-5 mini-tetramer pausing and promote the assembly
93   of multiple motors into clusters. These multi-motor clusters assemble when motile motors
94   encounter paused motors along the MT. MT sliding assays reveal that unlike the native kinesin-5
95   motor, kinesin-5 mini-tetramers require the tail domain to crosslink, align and slide two MTs.
96   Kinesin-5 mini-tetramers are restricted in their ability to pair and align MTs. However, once MTs
97   are paired, these mini-tetramers slide these MTs more efficiently than native kinesin-5. Our data
98   demonstrate that the kinesin-5 tail and length of the minifilament are two critical structural
99   adaptations that allow it to effectively crosslink and slide apart MTs. The tail domain is required
100   to mediate motor pausing and clustering such that multiple motors can coordinate in MT
101   overlaps. The length of the kinesin-5 minifilament is critical to provide flexibility required for
102   efficient MT crosslinking and transmission of forces during MT alignment and sliding.
103
104   **Results**

105
106 **An extended BASS x-ray structure reveals rigid and dimeric coiled-coils emerging from**
107 **bipolar tetramer junctions**
108
109 To determine the organization of bipolar kinesin-5 minifilament and how the dimeric α-helical
110 coiled-coil junctions are formed, we solved the x-ray structure of an extended *Drosophila*
111 *melanogaster* KLP61F segment (residues 620-804) in which the kinesin-5 sequence is extended
112 by 40 residues N-terminally compared to the previous BASS x-ray structure (Scholey et al 2014;
113 termed BASS-XL from herein). The KLP61F BASS-XL was purified and crystallized in the space
114 group C2 (253.18-Å, 84.89-Å, 96.77-Å) (Figure 1 figure supplement 1). The X-ray diffraction data
115 were highly anisotropic, indicating translational pseudo-symmetry, and were thus elliptically
116 truncated to 4.4-Å resolution in reciprocal space axes (Figure 1 figure supplement 1; Table 1).
117 The BASS-XL structure was determined by molecular replacement using BASS structure as a
118 starting model and was refined to 4.4-Å resolution leading to a R_{work}/R_{free} (0.277/0.309). The 4.4-
119 Å BASS-XL x-ray crystal structure reveals a 34-nm α-helical minifilament, compared to the 27-
120 nm BASS minifilament (Figure 1, figure supplement 1A). The structure reveals the N-terminal
121 30-residues form parallel coiled-coils which emerge from both ends of the BASS tetrameric core
122 (Figure 1A)(Scholey et al., 2014). The N-terminal parallel coiled-coils form multiple heptad
123 repeat interactions with clear a and d contacts forming homotypic interfaces (residues 620-670)
124 (Figure 1B). These dimeric α-helical coiled-coil extend for 10-nm before they twist slightly out of
125 register into a swap junction (residues 693-697) to form a four α-helical anti-parallel bundle
126 within the BASS core tetramer (residues 697-760)(Figure 1A)(Scholey et al., 2014). The C-
127 terminus of the BASS-XL forms α-helices (residues 760-806), which stabilizes a junction of the
128 N-terminal coiled-coil dimer at each end of the BASS tetramer core for the subunit that emerges
129 from the opposite end (Figure 1A). This junction rigidly and tightly orients the BASS-XL N-
130 terminal coiled-coils onto the ends of the BASS core and positions the N-terminal ends of the
131 coiled-coil to be 180° with respect to those emerging from the opposite end of the BASS-XL
132 structure (Figure 1A).
133
134 Using the BASS-XL x-ray structure as template for a shortened kinesin-5 minifilament, we
135 engineered bipolar kinesin-5 motors with a 38-nm central minifilament, which we term kinesin-5
136 mini-tetramers from herein (Figure 1C). Using the pattern of heptad repeats observed in the
137 BASS-XL structure, a longer KLP61F BASS-XL sequence was utilized to generate mini-
138 tetrameric motors by including three coiled-coil heptad repeats (21 residues) on the coiled-coil
139 N-terminus observed in the BASS-XL x-ray structure, leading to a 38-nm long kinesin-5
140 minifilament (Figure 1 green; 21 residues 597-620). The N-terminal end of this revised 38 nm
141 minifilament was then fused to the C-terminal end of the kinesin-5 motor domain and neck linker
142 (residues 1-365) sequences. On its C-terminus, the BASS-XL mimi-tetramer sequence was
143 either terminated or was C-terminally fused to the N-terminal end of the kinesin-5 tail domain
144 (residues 903-1033) (Figure 1C; Figure 1 figure supplement 2). We generated a structural model
145 for the 38-nm kinesin-5 mini-tetramer, showing its short overall length, which are roughly half the
146 length of the native kinesin-5 (38 nm versus 80 nm)(Acar et al., 2013; Scholey et al., 2014)
147 (Figure 1 figure supplement 2C-D; Figure 1D). The kinesin-5 mini-tetramer model shows that
148 BASS-XL rigidly orients the two dimeric motor domain pairs on both of its ends, positioning them
149 180° rotated with respect to each other (Figure 1D; Figure 1 figure supplement 2C-D). Using this
150 strategy, we generated two mini-tetrameric constructs from two kinesin-5 orthologs and
151 compared their activities: *Drosophila* KLP61F mini-tetramers (- tail: kMBX, or + tail: kMBXt) or
152 human Eg5 kinesin-5 mini-tetramer motors (-tail: hMBX, or +tail: hMBXt) as described (Figure 1
153 figure supplement 1A-C; Figure 1C). The motor to coiled-coil junctions for the mini-tetramer
154 constructs are predicted to lack flexibility with the respect to BASS tetrameric core, and to exhibit
155 less torsional flexibility than the full-length native kinesin-5 (Figure 1D). The kinesin-5 mini-
156 tetramer constructs represent only 60% of the full-length kinesin-5 sequence, as they lack 280

157 residues coiled-coil region between the motor neck linker and the extended N-terminal end of
158 the BASS-XL structure, and 100 residues flexible region between the BASS-XL C-terminal
159 domain and the N-terminal end of the tail domain (Acar et al., 2013; Bodrug et al., 2020; Scholey
160 et al., 2014).
161
162 **Kinesin-5 mini-tetramers undergo processive plus end-directed motility along with**
163 **pausing.**
164
165 We reconstituted the motility of kMBX and kMBXt mini-tetramers fused to Neon Green (mNG)
166 along single MTs imaged them using total internal reflection fluorescence (TIRF) microscopy, as
167 previously described (Bodrug et al., 2020). Single kMBX mini-tetramer motors undergo slow
168 processive motility along MTs interspersed very seldom with brief pauses (Figure 2A-B). In
169 contrast, kMBXt motors undergo processive motility towards MT plus-ends and pause for
170 extended periods (Figure 2C-D). While paused along MTs, kMBXt motors often encounter other
171 motile motors and merge to form brighter multi-motor clusters. These clusters of kMBXt motors
172 undergo motility together as singular entities until reaching MT plus-ends, where they
173 concentrate for extensive time periods. Thus, kMBXt motors pause for extended periods and
174 form multi-motor clusters, which are not observed in the case of the kMBX motors, suggesting
175 that the kinesin-5 tail-motor domain interaction promotes pausing and multi-motor clustering,
176 recapitulating activities observed for full-length Eg5 (Bodrug et al., 2020).
177
178 To understand how the *Drosophila* KLP61F tail domain regulates the mini-tetramer motor
179 properties, we studied how motility of the kMBX and kMBXt are affected by changes in ionic
180 strength (25, 50, 100 mM KCl). At 25 mM KCl, kMBXt motors undergo very slow motility, which
181 is 30% slower than kMBX motors (Table 2: 78 vs 115 nm/s). At 50 mM KCl, both kMBX and
182 kMBXt undergo slightly faster motility, with velocity of kMBX motor being about 10% faster than
183 kMBXt motors (Table 2: 127 nm/s vs 144 nm/s). At 100 mM KCl, the average motility velocity of
184 kMBX is 45% higher compared to that for kMBXt motors (Table 2:156 nm/s vs 114 nm/s). At
185 higher ionic strengths, kMBXt velocities progressively decreased (Table 2). In contrast, the
186 kMBX motors bound and dissociated rapidly from MTs at the higher salt concentrations and did
187 not undergo processive motility. At 25 mM KCl, kMBX motors undergo extremely long run
188 lengths. At 50 mM KCl, kMBX motors undergo motility at 66% shorter average run lengths than
189 the kMBXt motors (Table 2:1014 vs 3135 nm) and 66% shorter run time (Table 2:9 vs 29 s). At
190 100 mM KCl, the kMBX motor undergo motility with a 60% shorter average run length compared
191 to KMBXt (Table 2: 2137 nm vs 5060 nm), and 60% shorter average run time of kMBXt (Table
192 2:16 s vs 51 s). These data suggest that the kinesin-5 tail domain down-regulates the motor
193 domain by decreasing its MT activated ATPase leading to slower motility but increasing run
194 lengths and run times during each processive motility event.
195
196 To explore whether the tail-motor interaction and their regulatory mechanism observed for
197 *Drosophila* KLP61F are conserved in human Eg5, we generated human Eg5 motor mini-
198 tetramers hMBX (-tail) and hMBXt (+tail) (Figure 2 2E-H). The hMBX and hMBXt motors were
199 engineered with reactive cysteines for labeling with Tetra-methyl Rhodamine (TMR) fluorophores
200 (see materials and methods). The bright, stable TMR fluorophores on hMBX and hMBXt motors
201 allowed robust, high-resolution single-molecule tracking analyses. The hMBX motors undergo
202 processive motility towards MT plus-ends over long distances with infrequent pauses (Figure 2E-
203 F), while pauses are more frequent for the hMBXt motors containing the tail, similar to our
204 observation with the kMBXt motors (Figure 2G-H). The hMBXt motors exhibited multiple
205 intensities with bright and dim spots suggesting multi-motor clustering similar to the properties of
206 kMBXt motors. Thus, for both *Drosophila* and human kinesin-5 mini-tetramer motors, the
207 presence of the kinesin-5 C-terminal tail results in slower motors that undergo pauses, form
208 clusters, and are more processive (Figure 2I, J).

209
210
211 **The kinesin-5 tail domain promotes static pausing**
212
213 To examine kinesin-5 mini-tetramer pausing behavior at the single molecule level, we analyzed
214 the hMBX motility tracks by sub-pixel localization and linked these into trajectories (Arcizet et al.,
215 2008; Tinevez et al., 2017)(Hafner et al., 2016; Zajac et al., 2013). The mean-squared
216 displacements (MSDs) were compared as a function of time on a log-log plot, where a slope of
217 α=1 indicates a purely diffusive process, α<1 in the case of confined diffusion, and α=2 in the
218 case of processive transport (Arcizet et al., 2008); (Hafner et al., 2016; Ruthardt et al., 2011;
219 Zajac et al., 2013). We repeated this fitting process in a sliding window along the trajectory to
220 calculate a local slope or α-value for each point in the trajectory (Figure 3A, Figure 3 figure
221 supplement 1), and then used change-point analysis to identify segments of processive and
222 paused motility (Beausang et al., 2011). The α-value fluctuated between ~0 and ~2, suggesting
223 hMBX motors are either undergoing processive motility or remain tightly bound to single sites
224 along MTs, without diffusing. We observe a bimodal α-value distribution with peaks at 0.627+/-
225 0.041 and 1.760 +/- 0.041 (Figure 3C). Our analysis suggests that hMBX motility changed
226 significantly within a given trajectory between either paused or processive motile segments. The
227 change point analysis likely underestimates of the number of short pauses. To account for
228 pausing more effectively, we also fit the data for pauses ≥5 frames to a single exponential
229 distribution, yielding a mean pause time of ~13.5 s (Figure 3 figure supplement 1). The pause
230 time distribution is described well by a single exponential. Quantifying the number of pauses per
231 trajectory revealed that ~41%, ~27%, and ~12% of long trajectories contained zero, one, or two
232 pauses, respectively (Figure 3 figure supplement 1). To allow for more averaging and minimize
233 the effect of statistical fluctuations, we next calculated the MSD for all of the hMBX motility
234 segments identified as paused or processive. As the localization error is most prominent at short
235 timescales (Michalet, 2011) and is expected to be of a magnitude similar to the displacement of
236 the motor due to directed motion in our experimental conditions, we focused our analysis on
237 timescales of ~3-7 seconds (Duselder et al., 2015), also avoiding errors due to limited averaging
238 at longer timescales (Michalet, 2011). The hMBX motor MSD curves exhibit two unique α-values
239 of 0.627 and 1.760 indicating either paused and processive motility trajectories, respectively
240 (Figure 3C).
241
242 To determine the effect of the human Eg5 tail on motility properties of mini-tetramers, we
243 compared hMBXt to hMBX motility independent of the change point analysis, we also calculated
244 the MSD for all of the hMBXt motility events (paused and processive segments) and fit it to the
245 function $<r(\tau)^2> = (\phi v \tau)^2 + \phi 2D\tau + 2\epsilon^2$ where ϕ is the fraction of time in the processive state
246 (Chugh et al., 2018). From this global analysis, the fraction of time paused $(1 - \phi)$ is 0.47 for
247 hMBXt compared to 0.03 for hMBX motors (Figure 3B). These results indicate that pauses are
248 due to a statically bound state of the hMBX motor. For processive sections, the hMBX motors α-
249 value near 2 indicates that the motor's motility is dominated by active transport. Thus, our
250 analysis suggests that hMBX motor paused states represent a single state that is tightly bound
251 to MTs without diffusion. The hMBX motor motility velocity during processive motility segments
252 displayed a single distribution (Figure 3D) (54.2 ± 1.4 nm/s). The velocity distribution for the
253 paused fraction peaked near 0 nm/s (μ = 3.9 ± 1.8nm/s and σ = 52.3 ± 1.3nm/s) suggesting
254 clearly static motors that are strongly attached to MTs (Figure 3D).
255
256   Next, we analyzed the duration of hMBX processive motility events between paused
257 states. We determined the distance travelled by a motor without pausing as the total run length.
258 Since not all motile hMBX motors exhibited pauses, we observed two sub-populations: motors in
259 which there was a pause, and motors in which the motility was uninterrupted. Correspondingly,
260 distributions for total run lengths for hMBX motors were fit to a two-term exponential distribution,

261 with run lengths of 554 ± 60nm and 2942 ± 1106nm, with 90 ± 5% of spots in the shorter run
262 length population (Figure 3E). Plotting the inter-pause run lengths revealed a single exponential
263 distribution with a mean run length of 1263 ± 167nm (Figure 3E, Figure 3 figure supplement 1).
264 Thus, the shorter of the two estimates for total run lengths likely corresponds to the inter-pause
265 run length or that of motors, which do not pause. This suggests that hMBXt motors may string
266 together multiple shorter runs with pauses, allowing for a longer total run length. Both human
267 and *Drosophila* mini-tetramers exhibit very similar pausing behavior (Figure 2C-D, G-H).
268
269 To confirm the static nature of the hMBX pauses, we performed motility assays with the
270 slowly hydrolysable ATP analog, AMPPNP, which is expected to trap motors in a strongly bound
271 state (Chen et al., 2016). As expected, hMBX exhibited only static binding in the presence of
272 AMPPNP (Figure 3 figure supplement 1) with normally distributed α-value with a maximum near
273 0.103 and a standard deviation 0.316, indicative of static binding (Figure 3 figure supplement 1).
274 AMPPNP trapped hMBX motors show static binding similar to pauses observed in the presence
275 of ATP. Thus, we propose that hMBX and kMBX motors switch between processive and static
276 bound states, while moving along MTs toward plus-ends. We propose these paused states
277 represent the nucleotide-free state rather than the ATP bound state, in both of which the motor
278 domain has high affinity for MT lattice sites (Cross and McAinsh, 2014).
279
280 **The kinesin-5 tail domain promotes motor clustering**
281
282 Both kMBXt and hMBXt mini-tetramers formed multi-motor clusters along MTs during pauses
283 (Figure 4A, B). Thus, we next analyzed the properties of these multi-motor clusters and how they
284 form on MTs. First, we studied the role of electrostatic interactions in motor clustering and the
285 impact of clustering on the motile properties of the motors. We compared kMBXt motor behavior
286 across five ionic strength conditions (25, 50, 100,125,150 mM KCl) with the properties for kMBX
287 motors in two ionic conditions (50 and 100 mM KCl) (Figure 4C). For each condition, we
288 normalized the intensity of spots corresponding to motile motors along MTs visible in each field
289 of view and analyzed the corresponding intensity distribution to determine the average intensity
290 of individual mini-tetramer (Pandey et al., 2021)(Figure 4 figure supplement 1). The analysis
291 revealed a major peak of spot intensity likely representing individual kMBXt mini-tetramers, but
292 higher intensity spots were also observed, representing larger multi-motor clusters. We also
293 determined the rate of kMBXt clustering formed during motility by measuring and quantifying the
294 events in which motor intensities merged to form brighter spots, and quantified these events in
295 the pools of motile motors in the different ionic strength conditions (Figure 4C). At 25-50 mM
296 KCl, the majority of kMBXt motors formed clusters with either 2 (1+1) or 3(1+2), denoting the
297 mini-tetramer ratio of motile motor to static motor (motile + static) components(Figure 4, figure
298 supplement 2). However, at 50-150 mM KCl, a wider range of larger clusters was observed,
299 representing 5-10 motors per cluster (1+4 to 2+10). We then calculated the total frequency for
300 kMBXt cluster merging per unit MT length at 25-150 mM KCl, revealing the highest frequency
301 peaks around 100 mM KCl, but this frequency is substantially lower at 25 mM or 150 mM KCl. In
302 contrast few clustering events were observed for the KMBX motor in the absence of the tail
303 domain at 50 and 100 mM KCl (Figure 4C). Thus, the kinesin-5 tail is responsible for kMBXt
304 multi-motor clustering. At 25-50 mM KCl, clustering is likely low because the lower ionic
305 strengths promote an intra-molecular tail-motor domain interaction due to their proximity. In
306 contrast, at intermediate ionic strengths (100 mM KCl), the tail promotes interactions between
307 multiple mini-tetramers and form clusters. At high ionic strengths (150 mM KCl), neither
308 interaction is favored, again limiting the occurrence of interactions between mini-tetramers and
309 thus diminishing the formation of clusters.
310
311 Next, we studied the relationship between kMBXt motor cluster size and average velocity, run
312 length and run time. At 25 and 50 mM KCl, run length and run time are shorter and correlated

313 with fewer motors per cluster. In contrast, at 100 and 125 mM KCl we observe an 80-100%
314 increase in run length and run time and these features correlate with the larger motor-clusters.
315 However, we found no direct correlation between cluster size and an increase in run length or
316 run time within a single pool of motile kMBXt motors. Thus, our data demonstrate a critical role
317 for the kinesin-5 tail mediated clustering in promoting slower motility and tight association with
318 MT-plus ends. At lower ionic strength kMBXt motors behave more as individual tetrameric
319 motors with shorter run lengths. At 100 mM KCl, kMBXt motor clustering becomes dramatically
320 enhanced leading to motors that undergo motility more slowly with longer run lengths, run times.
321
322 The hMBXt motile motors similarly formed clusters with paused hMBXt motor along MTs (Figure
323 4A-B, Figure 4 figure supplement 1-3). To estimate more precisely the number of hMBXt motors
324 in these clusters, we examined their spot intensities using multi-modal distribution analysis,
325 revealing with a broad peak at ~6500au and peaks at 2 and 3 times this value (~13000au,
326 ~19500au) (Figure 3D). The major peak likely represents individual hMBXt mini-tetramers, with
327 the other peaks representing approximately 2 or 3 mini-tetramers. We tested the number of
328 hMBXt motors in these clusters in the presence of AMPPNP, which led to non-motile attachment
329 events. The spot intensity distribution for hMBXt in the presence of AMPPNP was predominantly
330 unimodal (major peak at ~6100au and minor peak at ~13000au), suggesting that motility is
331 required for hMBXt clusters to form along MTs (Figure 4E). In the presence of AMPPNP (i.e.
332 without motor stepping), the major and minor peaks were clearly segregated; however, in the
333 presence of ATP, these peaks were far less distinct (Figure 4D). This is likely because of the
334 quenching and unquenching of the fluorophore pairs occurring with each step of the motor
335 (multiple times during each frame) (Toprak et al., 2009). The multi-modal spot intensity
336 distribution of hMBX motors was similar to that of the hMBXt motors in the presence of
337 AMPPNP, with a major peak at ~5300 au (Figure 4F). The small shift in intensity is likely due to
338 a difference in TMR labeling ratios for the two constructs.
339
340   Next, we measured the run length of hMBXt motor clusters moving along MTs (Figure
341 4G). Quantifying the total run length of "clusters" (spots with an intensity ≥1.5-fold higher than
342 the mean intensity for single hMBXt tetramers) revealed run lengths of 752 ± 385 nm and 2443 ±
343 1649 nm, similar to the run lengths of 530 ± 58 nm and 3112 ± 1468 nm for spots likely
344 representing single hMBXt motors (Figure 4A). However, only 7 ± 4% of single hMBXt motors
345 were in the longer lifetime population, whereas 36 ± 40% of hMBXt clusters were in this
346 population. Additionally, many clusters reached the plus-ends of the MT on which they were
347 moving, remaining bound at the MT plus-end. For very processive motors, the MT length has an
348 effect on the measured run length distribution because many motors reach MT ends such as
349 their runs are artificially truncated (McHugh et al., 2018; Thompson et al., 2013). Thus, we
350 accounted for this censoring when fitting the run length distribution. Notably, satisfactory fits to
351 either of the run length distributions, for either single motors or clusters could not be obtained
352 using single exponential distributions, indicating that there is likely an inter-pause and total run
353 length. Thus both single motors and clusters are able to string together runs with pauses.
354
355   We subsequently analyzed the accumulation of hMBXt and kMBXt motors at MT plus
356 ends. The hMBXt and kMBXt motors are highly processive, with run lengths that depend on the
357 lengths of MTs, since many motors reach MT ends (McHugh et al., 2018). We observe an
358 accumulation of both hMBXt and kMBXt motors at MT plus-ends, indicative of the stable
359 association of tail-containing motors with MTs (Figure 4A). We quantified the dwell times of
360 hMBXt motors at MT plus ends by measuring the intensity in a region centered at the plus-end of
361 each MT and defining the period of time for which the intensity was >3 standard deviations
362 above the background signal as the cluster lifetime. The tail domain enables mini-tetramers to
363 maintain association at MT plus ends, where hMBXt motors pause for ~10x as long as hMBX
364 motors (Figure 4H, I). For hMBXt, we could not obtain a satisfactory fit to cluster lifetimes using a

365 single Weibull distribution, prompting us to use a mixed Weibull distribution with dwell-times of
366 16.4 ± 4.9 seconds and 483.7 ± 153.2 seconds (Figure 4I, Figure 4 figure supplement 5). This
367 suggests the presence of two populations of plus-end clusters with different dwell times, with 49
368 ± 15% of motors in the shorter lifetime population. Next, we analyzed cluster lifetimes as a
369 function of the intensity of the first 5 frames of that end cluster, revealing that motors that arrived
370 in a cluster that had "pre-formed" along the MT were likely to remain associated with the plus-
371 end (Figure 4J). We used this measure of the starting spot intensity rather than the mean spot
372 intensity to distinguish between motors arriving in "pre-formed" clusters and motors arriving
373 individually and gradually forming a cluster at MT plus-end. The arrival of "pre-formed" clusters
374 was supported by the observation that intensity traces in the region near MT plus-ends generally
375 increased above baseline or decreased to baseline in one or a few frames, regardless of the
376 plateau intensity (Figure 4J, Figure 4 figure supplement 6). Thus, the shorter lifetime likely
377 represents the dwell time of single hMBXt motors at the ends of MTs, while the longer lifetime
378 likely represents that of clusters.
379
380   The hMBX motors did not accumulate as robustly at MT plus-ends (Figure 4I). The dwell
381 times of hMBX motors at the plus-ends of MTs were 14.6 ± 3.2 seconds and 139.9 ± 137.5
382 seconds, with 74 ± 18% of motors in the shorter lifetime population (Figure 4I, Figure 4 figure
383 supplement 4). Thus, the shorter dwell times for hMBX and hMXBt clusters are similar, in
384 agreement with the idea that this represents the lifetime of single motors (present for both
385 constructs) at MT plus-ends. In contrast, the longer lifetimes are shorter for hMBX motors, and a
386 smaller fraction of motors is likely to be in this longer dwell-time subpopulation, indicating that
387 the inability of hMBX motors to cluster may prevent them from remaining bound at MT plus-ends
388 for extended periods (Figure 4H, I, J). To determine the possibility of hMBXt clusters reaching
389 MT plus-ends, we next compared the distribution of frame-to-frame velocities for single motors
390 and clusters in the paused and processive states (Figure 4). Our analysis agrees with the visual
391 observations, that hMBXt clusters move more slowly than single hMBXt motors: whereas the
392 mean paused velocities of single motors (4.6 ± 3.1nm/s and σ = 64.8 ± 2.2nm/s) and clusters
393 (3.6 ± 1.9nm/s and σ = 37.6 ± 1.3nm/s) were similar, the processive velocity of single motors
394 (63.3 ± 2.3nm/s) was higher than that of clusters (43.2 ± 1.6nm/s). Additionally, the velocity of
395 single motors was more variable than that of clusters. During the processive sections of
396 trajectories, the standard deviation of single motor velocities was 65.3 ± 1.6nm/s, whereas that
397 of clusters was 39.5 ± 1.2nm/s. This might contribute to the observation that the velocity of
398 hMBXt motors (54.2 ± 1.0nm/s) is slower than that of hMBX motors (80.4 ± 4.4nm/s).
399
400   We also measured the average dwell time for ensembles of kMBX and kMBXt motors at
401 MT plus ends in different ionic strength conditions. We observe that kMBX motors dwell at MT
402 ends for 78 ± 19 s at 50 mM KCl and for 25 ± 18 s at 100 mM KCl, showing a three-fold
403 decrease upon ionic strength increase (Figure 4K). In contrast kMBXt motors show consistently
404 longer dwell time at MT plus-ends, which is two-three folds higher than kMBX motors (125 to
405 250 s), and are not influenced by changes in ionic strength (25- 150 mM KCl) (Figure 4K). The
406 majority of kMBXt clustering events also correlate with the motors arriving at MT plus-ends
407 particularly at 100 mM KCl condition. This is likely due to the enhanced multi-motor clustering
408 coupled with the enhanced dwell time at MT plus-ends at 100 mM KCl. Our data suggests that
409 the MT plus-end dwell time is likely related to the motors pausing at MT plus-ends, and that the
410 tail enhances this property by down-regulating the ATP hydrolysis activity of the motor domain.
411
412 **Short kinesin-5 mini-tetramers show unique MT crosslinking and MT sliding features**
413
414 We next sought to determine whether kinesin-5 mini-tetramers are capable of crosslinking,
415 aligning, and sliding pairs of MTs. To do this, we immobilized taxol-stabilized HiLyte 646 and
416 biotin labeled MTs on a coverslip via a streptavidin-biotin linkage. We found that to achieve

417 efficient MT bundling, particularly for the mini-tetramer motors, we required high velocity flow of
418 MTs in flow cells such that they were well nearly aligned with the direction of flow. Next, motors
419 were introduced into the sample chamber at a specified concentration and allowed to decorate
420 the coverslip-attached MTs. Finally, Rhodamine-labeled MTs and ATP were introduced with
421 rapid flow rates, and kinesin-5 mediated crosslinked MT bundles were allowed to form and
422 motors to initiate relative MT sliding. We then imaged both the coverslip attached MT, paired
423 MTs and kinesin-5 motors using the three different channels via TIRF microscopy at 2-5 sec
424 frame rates.
425
426 We first set out to determine how efficiently four different kinesin-5 motor constructs can
427 crosslink and pair MTs, by comparison: full-length Eg5 with tail (hFLt), full-length Eg5 without the
428 tail (hFL), previously prepared as described (Bodrug et al., 2020), kinesin-5 mini-tetramer with
429 tail (kMBXt), and kinesin-5 mini-tetramer without tail (kMBX). We then imaged multiple fields-of-
430 view immediately after flowing in ATP and Rhodamine-MTs and measured how many MT
431 bundles had formed relative to the population of surface-bound MTs (Figure 6a-b). We found
432 that both hFLt and hFL motor constructs recruited free MTs from solution, forming MT pairs that
433 underwent sliding at relatively high rates, with 63±5% and 54±16% in the hFLt and hFL motor
434 conditions, respectively. This consistent with observations we made previously at similar
435 concentrations (Bodrug et al., 2020). In contrast, the kMBXt motor formed MT bundles 3-fold
436 less frequently (24±14%) compared to hFLT, while the kMBX almost never recruited a free MT
437 from solution and rarely formed MT pairs, with only 2% of all surface MTs observed to bundle a
438 second MT (Figure 5A-B). Together, these data suggest that the kinesin-5 mini-tetramer motors
439 are less efficient than the full-length kinesin-5 motors at spontaneously forming antiparallel MT
440 bundles and that the tail domain is critical for establishing the MT crosslinked geometries,
441 especially in the mini-tetramer constructs.
442
443 We next sought to determine how kinesin-5 mini-tetramers compared to full-length kinesin-5 in
444 MT crosslinking, pairing or alignment and then MT sliding. We therefore monitored the positions
445 of the mobile MT and the full length or mini-tetramer kinesin-5 motors during these sliding events
446 across a range of conditions. As we were unable to form bundles using kMBX motor in our
447 assays, we focused on comparing the mechanics of the full-length (hFLt) motor and mini-
448 tetramer motor (kMBXt) both of which contain kinesin-5 tail domains. Both motors were able to
449 slide MTs apart efficiently (Figure 5C). Interestingly, kMBXt motor appeared less mobile and
450 more clustered relative to the hFLt motor along the MTs throughout MT sliding events (Figure
451 6c). We also observed that the mobile free-MT occasionally paused or exhibited reduced
452 velocity for brief stretches when undergoing sliding by the full-length kinesin-5 (hFLt). In contrast,
453 MT sliding generated by kMBXt motor tended to exhibit more consistently continuous MT sliding
454 motility with faster velocities throughout (Figure 5C). To examine this difference, we determined
455 the frequency of observed pauses or velocity decreases across many MT sliding examples and
456 found that MT bundles in the full length kinesin-5 (hFLt) condition exhibited pauses 0.21+/-0.06
457 times per micron (μm) of distance travelled, while kinesin-5 mini-tetramer (kMBXt) paused at a
458 lower rate of 0.08+/-0.04 times per micron (μm) which is three-folds lower than the pausing
459 exhibited by full length (hFLt) motor MT sliding (Figure 5D). Finally, we calculated the MT sliding
460 velocity across a range of ATP concentrations for both constructs (Figure 5E). We found that the
461 MT sliding velocity was approximately twice the speed of single motor stepping motor velocity,
462 consistent with the previously reported finding that both pairs of motor domains at each of the
463 bipolar end of kinesin-5 motors are stepping along their respective MT in the opposite direction,
464 leading to a two-fold MT sliding motility compared to the motility generated along single MTs
465 (Kapitein et al., 2005). Importantly, we observed that the average velocity of kinesin-5 mini-
466 tetramer (kMBXt)-driven MT sliding at 100 and 250 μM ATP was about 10% higher than that of
467 full-length kinesin (hFLt)(table 3). Together, these results indicate that, while the kinesin-5 mini-
468 tetramers are not as efficient at initially crosslinking aligning two MTs as native kinesin-5, they

469   are capable at sliding MTs, doing so within clusters, with less three-fold less pausing and 10%
470   higher velocities compared to those generated by full-length kinesin-5.
471
472
473   **Discussion**
474
475   Human cells encode 45 kinesin motors, each of which is adapted to perform a unique set of
476   cellular functions. This functional diversity is demonstrated beautifully in the mitotic spindle,
477   where kinesins perform diverse tasks ranging from regulating dynamics to crosslinking and
478   sliding MTs. To fulfill their functional niches in mitosis, kinesins have evolved biophysical and
479   biochemical differences that render them uniquely well-suited for these roles. Through
480   comparing the motility and structure of full-length and minimal tetrameric kinesin-5 motors, our
481   studies reveal the role of the C-terminal tail and bipolar tetrameric minifilament domains in
482   governing kinesin-5 motor clustering and MT sliding motility. The structural and biochemical
483   features of these domains are highly conserved across all the kinesin-5 motors. Our structure of
484   the extended kinesin-5 BASS domain reveals that it possesses the unique capacity to form a
485   force-bearing junction for two pairs of motors positioned at opposite ends of its bipolar structure.
486   We designed and studied Human and *Drosophila* kinesin-5 mini-tetramer motors based on the
487   BASS-XL x-ray structure (Figure 1), which recapitulate critical aspects but reveal unique
488   differences compared to full-length kinesin-5 motor motility (Bodrug et al., 2020). These
489   structural adaptations are critical for kinesin-5 function in bipolar mitotic spindle assembly,
490   organization, and elongation.
491
492   **The kinesin-5 tail domain binding regulates the motor domain mechanochemical cycle**
493   **and promotes slower motility and increased processivity.**
494
495   The kinesin-5 tail domains down-regulate MT activated motor domain ATP hydrolysis by
496   stabilizing the MT bound nucleotide free state. This interaction leads to slower stepping during
497   processive motility, longer run lengths, and frequent pauses (Figure 2-3). As with full length
498   kinesin-5 (Bodrug et al., 2020), the presence of the tail domains in both *Drosophila* and human
499   mini-tetramers leads to slow motility with multiple pauses in which motors are statically bound to
500   MTs (Fig. 2). The pauses represent strongly-bound states with extended lifetimes in which the
501   leading motor domain is in the no nucleotide state, while and the trailing motor domain is either
502   in the ADP-P_i or ADP state, similar to the so-called ATP gate or stepping gate (Andreasson et
503   al., 2012; Bodrug et al., 2020; Cross and McAinsh, 2014). This is similar to motor motility in the
504   presence of a mixture of ATP and AMP-PNP, in which comparable switches in motility are
505   observed (Subramanian and Gelles, 2007; Vugmeyster et al., 1998). We suggest that the
506   kinesin-5 tail domain docking onto the motor domain serves as an externally imposed gate to
507   organize multiple kinesin-5 motors in clustered assemblies. The kinesin-5 tail domain enhances
508   the pausing and promotes motor assembly into clusters via encounters between motile and
509   paused motors along MTs. The tail to motor trans-interactions between the tails of one kinesin-5
510   tetramer and the motor domains of other tetramers. Interestingly, motor clustering has also been
511   described for the budding yeast, *Saccharomyces cerevisiae,* ortholog Cin8 and has been
512   proposed to mediate its minus-end to plus-end MT motility directionality reversal (Pandey et al.,
513   2021a; Pandey et al., 2021b; Shapira et al., 2017; Singh et al., 2018).
514
515   **The kinesin-5 tail domain drives the formation of multi-motor clusters with different**
516   **motile properties than single motors**
517
518   Our studies of *Drosophila* and human kinesin-5 mini-tetramers reveal that tail to motor domain
519   interaction is highly conserved, in which the tail domains induce pausing and drive kinesin-5
520   motors to assemble into multi-motor clusters. By increasing the pause frequency, the tail domain

521 increases the frequency of encounters between motile and paused motors, allowing for the
522 formation of multi-motor clusters mediated by trans-interactions between the tails of one
523 tetramer and the motor domains of other tetramers (Bodrug et al., 2020). The tail to motor
524 interaction leads to a decrease in overall motility velocities. Within clusters, multiple motors
525 move together as assemblies (Figure 4). The tail may stochastically dissociate from the front
526 motor domain, allowing the motor domains to continue stepping. The increase in the number of
527 motors in these clusters likely leads to longer run lengths likely due to the increase in the
528 numbers of stepping motors domains. Indeed, we observed an increased total run lengths in
529 kMBXt and hMBXt motors compared to kMBX and hMBX motors, supporting the effect of the tail
530 on increasing processive run lengths. The lifetime of motors at MT plus-ends is related to the
531 number of motors in the clusters that arrive. The kMBX or hMBX motors generally dissociate
532 from the MT plus-end more quickly than clusters of kMBXt or hMBXt motors (Figure 4).
533 Additionally, kMBXt or hMBXt motor clusters were found to move at slower and less variable
534 speeds than single motors (Figure 4). Clusters and single motors may be fulfilling different roles
535 during spindle assembly. Clusters of motors may be present between parallel MTs, such as near
536 the spindle poles, where, because of their increased association time with MTs, they could assist
537 in MT capture early in spindle assembly. Kinesin-5 clusters may be selectively retained in this
538 region because they move slower than single motors (Fig. 4). In contrast, faster moving single
539 motors may "escape" and travel towards MT plus-ends, localizing them in the region of
540 antiparallel MT overlap in the spindle midzone. Here, they could facilitate spindle pole separation
541 and/or regulate the rate at which this occurs
542
543 **The kinesin-5 tail-motor interaction is likely tuned by mitotic phosphorylation of the**
544 **conserved BimC box in the tail domain.**
545
546 The kinesin-5 tail domain contains a conserved BimC box or CDK1 phosphorylation site
547 (Threonine 926 in human kinesin-5) and the more distal regulatory regions of the tail domain (i.e.
548 the KEN box, D box, and Nek6 phosphorylation site (Blangy et al., 1995)(Bertran et al., 2011;
549 Drosopoulos et al., 2014; Rapley et al., 2008). In particular, the phosphorylation of the kinesin-5
550 tail by Cdk1 kinase has been shown to increase the affinity of the motor for MTs *in vitro* (Cahu et
551 al., 2008). Based on our observations, we hypothesize that such phosphorylation may enhance
552 the tail-motor interaction by adding a negative charge to the tail, potentially increasing its affinity
553 for the motor domain, which is positively charged near the ATP binding site. The CDK1
554 phosphorylation of the tail domain could act to increase the affinity of the motor for MTs by
555 promoting tail docking, hereby increasing the time that the motor spends in its strongly-bound
556 state. The enhancement of the tail regulation may lead to changes in the MT sliding mechanism
557 or enhancing the brake function for kinesin-5 during anaphase. The impact of phosphorylation of
558 the kinesin-5 tail warrants further study.
559
560 **The tail to motor interaction supports kinesin-5's function as a brake during mitosis**
561 Kinesin-5 motors have been suggested to act as a brake to slow the rate of MT sliding by other
562 motors, both during anaphase (Rozelle et al., 2011; Saunders et al., 2007) and in non-mitotic
563 cells (Falnikar et al., 2011; Lin et al., 2011; Myers and Baas, 2007; Nadar et al., 2008; Nadar et
564 al., 2012). Moreover, the ability of kinesin-5 motors to act as a brake between parallel MT or
565 quickly sliding antiparallel MTs has been demonstrated *in vitro* (Shimamoto et al., 2015). The tail
566 docking onto the motor domain to induce pausing and motor clustering may contribute to the
567 capacity of kinesin-5's brake-like behavior by increasing the time these motors spend in their
568 strongly bound state on the MT lattice. This would allow the motor to prevent rapid sliding of two
569 MTs in a parallel orientation. The tail's regulation is critical for optimal force transmission through
570 kinesin-5 minifilaments to promote efficient crosslinking and sliding. We expect that a motor is
571 less likely to detach when subject to loads while in tail-induced clustered and paused states than
572 while moving processively as individual tetramers. The paused state of clustered motors would

573 be particularly strongly attached to MTs, allowing them to withstand substantial loads when
574 acting as a brake.
575
576 **Clusters of Kinesin-5 remain stably associated with the plus-ends of stabilized MTs**
577 Kinesin-5 clusters often reached MT plus ends and they remained bound for extended periods of
578 time (Figure 4). The tail to motor interaction strongly enhances the kinesin-5 the MT plus-end
579 association. The accumulation of kinesin-5 at MT plus-ends has been described previously
580 (Chen & Hancock, 2015; Kapitein et al., 2005). We interpret this MT plus-end accumulation to be
581 due to the extended lifetime of kinesin-5 strongly-bound motor states, compounded by tail to
582 motor interaction enhancing the binding affinities of the motors and enhancing their clustering
583 (Furuta et al., 2013; Vershinin et al., 2007). Indeed, we observed that the motors that arrived at
584 MT ends in clusters remained bound for longer periods of time (Figure 6c). In contrast to
585 previous reports employing dimeric kinesin-5 constructs, it has proposed that this MT plus-end
586 accumulation reveals a role for kinesin-5 motor domain in modulating MT dynamics (Chen et al.,
587 2019; Chen and Hancock, 2015). However, we find that kinesin-5 mini-tetramers lacking the tail
588 domain have short life times at MT plus ends, likely due to tail to motor interaction stabilizing the
589 high affinity motor states and inducing multi-motor clusters. Furthermore kinesin-5 cluster also
590 move with a slow velocity of ~43nm/s, which may be too slow to allow them to accumulate at the
591 ends of dynamic MTs within the spindle.
592
593 **The length of the Kinesin-5 central minifilament directly regulates force transmission**
594 **during MT sliding motility**
595 Our studies help to elucidate the crucial role of the 60-80 nm Kinesin-5 minifilament in the
596 kinesin-5 MT sliding mechanism. We compared kinesin-5 mini-tetramers with full-length kinesin-
597 5 motors to determine the relationship between the length of the kinesin-5 minifilament to the
598 efficiency of MT pair formation, and its impact on MT sliding motility activities. The kinesin-5
599 mini-tetramers are half the length of native kinesin-5 motors. The short minifilaments in kinesin-5
600 mini-tetramers are stiffer due to the shorter dimeric coiled-coils on either side of the BASS
601 domain. This leads to low torsional flexibility and therefore enhance the force coupling between
602 the bipolar ends of kinesin-5 as they engage the two MTs they crosslink. However, the
603 decreased flexibility either end of the kinesin-5 mini-tetramers appears to impede efficient MT
604 alignment into bundles. In particular, this decreased flexibility leads to defects in the initial
605 crosslinking of MT pairs prior to MT sliding. Once crosslinked and aligned, however, the shorter
606 minifilament leads to a higher maximal MT sliding velocity compared to native kinesin-5 due to
607 the increased stiffness of kinesin-5 minitetramers.
608
609 Our studies also show the relationship between the kinesin-5 minifilament length and the motor
610 to tail regulation in modulating MT sliding motility. The decreased kinesin-5 minifilament torsional
611 flexibility in the mini-tetramer motors leads to dependence on the tail-motor regulation during MT
612 crosslinking, alignment and sliding in contrast to full-length kinesin-5, where the tail was not
613 strictly required *in vitro*. The kinesin-5 mini-tetramers show a very poor capacity to assemble
614 paired sliding MTs in contrast to the full-length kinesin-5 motors such as the hFL (Δtail kinesin-
615 5), which assembles MT sliding pairs, but with decreased efficiency relative to the hFLt (full
616 length kinesin-5)(Bodrug et al., 2020). These data also show that the tail-motor regulation
617 enhances force transmission properties of kinesin-5 engaged motor ends, and that the motility
618 resulting forces are transmitted between kinesin-5 bipolar ends via their central tetrameric
619 minifilaments.
620
621 Taken together, our studies identify how the unique structural adaptations of the kinesin-5 tail
622 and tetramerization domains enable its role in organizing the mitotic spindle. The tail domain
623 promotes motor pausing and clustering. Single motors and clusters behave differently,

624   suggesting that they may fulfill different roles in spindle assembly. The length of the minifilament
625   contributes flexibility to enable kinesin-5 to initiate MT crosslinking efficiently.
626
627

628   **Acknowledgement**
629

Materials and Methods

Protein Production, X-ray crystallography and Model building.

The KLP61F minifilament extended region (residues 597-833) was expressed and purified in BL21 *E. coli* as described in Scholey et al 2014. Bacterial pellets were lysed using a micro-fluidizer in (300 mM KCl, 50 mM HEPES, 1 mM MgCl2, 3 mM β-mercaptoethanol with protease inhibitors). The bacterial lysate was clarified by centrifugation at 18k rpm for 30 min at 4°C. Ni-NTA affinity was used to purify BASS-XL, and passage over HiTrap Q HP cation exchange in low salt (70 mM KCl, 50 mM HEPES, 1 mM MgCl2) was used to remove contaminants where BASS-XL eluted in the flow through. A second Ni-NTA affinity step was used in conjunction with 10K Amicon Filters to concentrate the BASS-XL. The concentrated BASS-XL tetramer was applied on a HiLoad 16/600 Superdex 200 gel filtration column using an AKTA Purifier (GE Healthcare). Crystallization conditions were screened using a Mosquito Robot (TTP Labtech) by mixing a 100 nL of protein with 100 nL precipitant conditions. Crystals were obtained and refined in 0.01 M FeCl3, 0.1M sodium citrate pH 5.6, 12% Jeffamine M-600 at 18°C and cryoprotected with 20% glycerol. Crystals were diffracted at the SSRL 11-1 beamline and showed highly anisotropic x-ray diffraction. Crystals adopt space group C2 with four molecules in the asymmetric unit. We used 4.4 Å as the high-resolution cut-off to avoid excessive loss of completeness. The diffraction data was truncated using boundaries determined via the Anisotropic server (Strong et al., 2006). The BASS-XL structure was determined using molecular replacement using the previously determined BASS model (Scholey et al 2014). Data from each monomer were combined using non-crystallographic symmetry and were averaged and refined using PHENIX with cycles of model building using coot program (Emsley et al., 2010; Liebschner et al., 2019).   The individual positional coordinates and anisotropic B-factor were refined with automatic weight optimization in the final stage. The final model includes BASS core domain with extended parallel helices at the N-terminal end.

Engineering *Drosophila* and human mini-tetramer kinesin-5 motors

Human and *Drosophila* kinesin-5 mini-tetramers were designed using the BASS-XL x-ray structure as a template. For the KLP61F KMBX and KMBXt mini-tetramer constructs, the BASS XL was extended by 20 residues based on the heptad pattern observed in BASS-XL the structure (residues 597-833) and fused at its N-terminal end to the KLP61F motor and neck linker domain (residues 1-369) and were either contained or lacked a C-terminal extension of the KLP61F tail domain (residues 910-1066) with a C-terminal his tag. For the Eg5 mini-tetramer hMBX and hMBXt constructs the *Homo sapiens* Eg5 motor domain and neck linker (1-374) were fused to the N-terminal end of the *Drosophila melanogaster* BASS-XL domain (597-799), either contained or lacked the *Homo sapiens* Eg5 C-terminal tail domain (913-1056), and a C-terminal 6x-His tag with mutations (C25V, C43S, C87A, C99A, N358C, C964S, and C1003S) to allow for the specific labeling of the motor at a single reactive cysteine residue in the neck linker (N358C). Tubulin was purified from Pork or Bovine brains (Castoldi and Popov, 2003). After purification, tubulin was cycled or labeled (with Alexa-546 or Alexa 647 (Thermo Fisher Scientific; Waltham MA), HiLyte 488 (AnaSpec; Fremont CA) or Biotin-LC-NHS (Thermo Fisher Scientific)) and then cycled prior to use. Unless otherwise stated, all chemicals and proteins were purchased from MilliporeSigma (Burlington, MA).

Motility assays for Drosophila Kinesin-5 (kMBX and kMBXt) minitetramers

Flow chambers were assembled from N 1.5 glass coverslips (0.16 mm thick; Ted Pella) that were cleaned with the Piranha protocol and functionalized with 2 mg/mL PEG-2000-silane containing 2 μg/mL biotin-PEG-3400-silane (Laysan Bio) suspended in 80% at pH 1 (Henty-

696 Ridilla et al., 2016). After the flow chamber was assembled, 0.1 mg/mL NeutrAvidin
697 (Thermofisher) was used to functionalize surfaces. Biotin and Alexa-Fluor-633-labeled porcine
698 tubulin were generated in the laboratory as described (Al-Bassam, 2014) and were polymerized
699 using the non-hydrolysable GTP analog guanosine-5'-[(α,β)-methyleno] triphosphate (termed
700 GMPCPP; Jena Biosciences) or using the MT stabilizing drug, Paclitaxel (sigma). These MTs
701 (100–200 µg/mL in BRB-80: 80 mM PIPES, 1 mM MgCl2 and 1 mM ETGA; pH 6.8, 1% glycerol,
702 0.5% pluronic-F127, 0.3 mg/ml casein, 3 mM BME, 4 mM ATP-MgCl2) were flowed into
703 chambers and attached to glass via biotin-neutravidin linkage. Flow chambers were then
704 extensively washed with imaging buffer (25 mM HEPES, 25–150 mM KCl, pH 7.5, 10 mM beta-
705 mercatopethanol; 1% glycerol, 0.5% Pluronic-F127, 0.3 mg/ml casein, 3 mM BME, 4 mM ATP-
706 MgCl2). Kinesin-5 MT-stimulated motility was reconstituted at 25°C by injecting 1–20 nM FL-
707 Eg5-GFP combined with a photo-bleach-correction mixture into flow chambers (Telley et al.,
708 2011). Movies were captured in TIRF mode using a Nikon Eclipse Ti microscope using 1.5 Na
709 objective and an Andor IXon3 EM-CCD operating with three (488 nm, 560 nm and 640 nm)
710 emission filters using alternating filter wheel in 2 s increments operated using elements software
711 (Nikon).
712
713 **Motility assays for human Kinesin-5 (hMBX and hMBXt) mini-tetramers**
714
715 Coverslips (22x30mm; Thermo Fisher Scientific) were cleaned by soaking in acetone for 10
716 minutes, sonicating in 50% methanol for 20 minutes, sonicating in 0.5M KOH for 20 minutes,
717 and then rinsing in MilliQ water three times before drying using nitrogen gas. Cleaned coverslips
718 were stored covered at room temperature. Immediately prior to silanization, coverslips were
719 plasma treated on "high" (~18W; 200mTorr) for 45 seconds after evacuating the chamber.
720 Subsequently, they were soaked in PlusOne Repel-Silane ES (GE Healthcare; Chicago IL) for
721 20 minutes, transferred to 95% ethanol for 5 minutes, and then sonicated in fresh 95% ethanol
722 for 10 minutes. Finally, they were dried again using nitrogen gas and stored covered at room
723 temperature for up to 2 months.
724 Taxol-stabilized MTs were prepared as follows: purified unlabeled, Alexa 647-labelled, and
725 biotinylated tubulin were combined at 50µM in BRB80 in a 50:2:1.5 ratio and supplemented with
726 2mM GTP. After incubating at 37°C for 40 minutes, 40µM Taxol (Cytoskeleton; Denver CO) was
727 added, and MTs were incubated for an additional 30 minutes. Subsequently, MTs were pelleted
728 by centrifugation (8000 rcf, 10 minutes, 25°C), and, after washing the pellet, resuspended in
729 fresh BRB80 supplemented with 40µM Taxol. The pelleting, washing, and resuspension were
730 repeated twice sequentially.
731 Single molecule motility assays were carried out as follows: Flow chambers with a volume of
732 ~20µL were prepared from a silanized coverslip, a glass slide, and double-sided tape.
733 NeutraAvidin was introduced at 20µg/mL and incubated for 5 minutes, before blocking the
734 remaining surface using 50mg/mL Pluronic F-127 for 30 minutes. Stabilized MTs containing
735 biotinylated tubulin were then flowed in at a concentration of ~0.14mg/mL and allowed to bind for
736 5 minutes, at which point any unbound MTs were removed by washing the chamber with BRB80
737 supplemented with 20µM Taxol. Just prior to imaging, the assay buffer consisting of BRB80
738 supplemented with 20µM Taxol, 0.5mg/mL BSA, 10mM DTT, 2mM ATP, 0.5mg/mL glucose
739 oxidase, 7mg/mL glucose, 0.2mg/mL catalase, 0.2%w/v PEG, 40mM potassium acetate, and
740 ~22nM kinesin-5 (with or without the tail) was introduced. The flow chamber was then sealed at
741 either end using vacuum grease and imaged immediately for no more than 30 minutes. Assays
742 with AMP-PNP were performed identically, except that motors were diluted 36x more and the
743 ATP was substituted with 2mM AMP-PNP (Roche Diagnostics; Indianapolis IN).
744 TIRF microscopy imaging was performed using an Eclipse Ti-E inverted microscope (Nikon;
745 Melville NY) equipped with diode lasers (100mW; 405nm, 488nm, 561nm, and 640nm)
746 (Coherent; Santa Clara CA), custom optics for TIRF, a 1.49 NA 100x objective, and an additional
747 1.5X lens to increase the magnification. Two-color image series were acquired by capturing

748 images of the MTs (640nm, 100ms exposure, 1mW) and the motors (561nm, 200ms exposure,
749 2mW) in an alternating fashion by alternating the laser illumination in synchrony with the rotating
750 filter wheel using a custom-written LabView program, resulting in a frame rate of ~1.2s^{-1}. Images
751 were captured on an iXon U897 EMCCD camera (Andor Technology; South Windsor CT).
752
753 **Single Molecule Analyses for *Drosophila* kinesin-5 (kMBX and kMBXt)**
754
755 Motility, run length and run time analyses were carried out as follows: Image movie stacks were
756 pre-processed with photobleach correction and image stabilization plugins using the program
757 FIJI (Schindelin et al., 2012). For motility along individual MTs, individual kMBX and kMBXt
758 motor motility events were identified along anchored MTs based on kymographs in generated for
759 multiple channels. The FIJI kymograph TrackMate plugins (Schindelin et al., 2012) were used to
760 measure particle motility rates, identify their run lengths as and run time. Large collections of
761 motile events along fields of MTs were collected for kMBXt motors at 25, 50, and 100, 125 and
762 150 mM KCl conditions and collected for kMBX motors at 50 and 100 mM KCl conditions (Table
763 2). Average MT parameters were determined by frequency binning the motility events in a range
764 conditions and then fitting these events using Gaussian distributions using the program Prism
765 (Table 2). In general, all parameters fit single Gaussian distributions. Run lengths were fitted
766 using exponential decay to identify the half-length for each motor condition. T-tests were
767 performed to determine significance of the differences observed.
768 The stoichiometry of kMBX and kMBXt motors per multi-motor cluster were determined as
769 previously described (Pandey et al., 2021). Briefly, following correction for the uneven
770 illumination of images and background subtraction, intensities of all NG-labeled kMBX or kMBXt
771 in the first frame of a time-lapse sequence were measured using the TrackMate plugin of the
772 ImageJ-Fiji software(Schindelin et al., 2012; Tinevez et al., 2017). Since kMBX and kMBXt are
773 homo-tetrameric, each motor contains at least four NGs per tetrameric motor. The major peak of
774 the intensity distribution histogram of these mNG-labeled mini-tetramers was fitted to a Gaussian
775 distribution. The center of the Gaussian peak lay at ~600 a.u., which corresponds to the average
776 intensity of single kMBX or kMBXt motor containing one, two, three, or four fluorescent mNG,
777 with each fluorescent mNG molecule contributing ~240 a.u. to the total intensity. Thus, the neon
778 green labeled motor population within this Gaussian peak likely represents single kMBX or
779 kMBXt motors. By this method, we assigned intensity ranges for kMBX or kMBXt molecules
780 fluorescence as <960, 960-1920 and >1920 for single mini-tetramer, pairs mini-tetramers, and
781 higher order oligomers of mini-tetramers, respectively. All the fluorescence intensity
782 measurements to assign cluster size to an kMBX or kMBXt molecule were performed only in the
783 first frame for each data set, thereby significantly reducing the possibility of photobleaching
784 effects.
785
786 **Single molecule analyses of Human kinesin-5 (hMBX and hMBXt) mini-tetramers:**
787
788 Image series were drift corrected using the MT images with the Image Stabilizer plug-in for
789 ImageJ. The output coefficients were then applied to the motor images using the Image
790 Stabilizer Log Applier plug-in. These transformation coefficients were saved and used for later
791 steps in the analysis. For images presented in the text, the MT channel was bleach corrected
792 using the Histogram Matching algorithm in ImageJ.
793 Single molecule tracking was performed on the original, unprocessed images using the
794 TrackMate (Schindelin et al., 2012; Tinevez et al., 2017) plug-in for ImageJ. Briefly, sub-pixel
795 localization was performed using a Laplacian of Gaussian (LoG) filter with an estimated spot
796 diameter of 0.4μm to detect motors. Spots detected in sequential frames were then linked using
797 the Linear Assignment Problem (LAP) tracker using a maximum displacement of 0.6μm,
798 allowing for a two-frame absence of spots, and not permitting track merging or splitting. No
799 filtering of the detected spots or tracks was done in ImageJ.

800 Quantitative analysis of motor motility activities was performed as follows: MTs were manually
801 traced out in ImageJ, and the x, y coordinates of these ROIs were saved and imported into
802 MATLAB. Detected trajectories >3 frames were then projected along the axis of their respective
803 MT for run length and velocity analysis. Trajectories with a total run length <150nm were not
804 included the analysis to filter out statically-bound motors (e.g., those adsorbed on the coverslip
805 near a MT). All trajectories within 600nm of another MT, or within 600nm of either end of the MT,
806 were ignored in the analysis of pausing/motility. The local α-value analysis was performed on all
807 trajectories with >10 frames using drift-corrected (but not projected) x, y localizations. These
808 were linearly interpolated to account for the slightly varying frame rate. α-values were calculated
809 in sequential 8-frame windows using delays of 2, 3, and 4 frames. Change points were detected
810 in the resulting α-values using the findchangepts function in MATLAB (The MathWorks; Natick
811 MA) with a minimum improvement in the residual error of 0.3 and a minimum distance between
812 consecutive change points of 3 frames. The means between detected change points were then
813 evaluated and sections with a mean α-value >1.1 were deemed processive. The localizations in
814 the original trajectory were then assigned as processive or paused based on the classification of
815 the interpolated frame that was nearest to them in time. Subsequent analysis of intensities,
816 velocities, and inter-pause run lengths was performed using these classifications. Fitting was
817 done using maximum likelihood estimation (MLE). For the velocity histograms, normal
818 distributions were fit using the central 96% of the data. For pause time estimates, distributions
819 were fit using the lower 95% of the data because of the presence of a small number of inactive
820 motors that are classified to have long "pauses". For analysis of trajectories in the presence of
821 AMP-PNP, no minimum run length was used, and the α-value histogram was fit to the upper
822 95% of the data to ignore the skew.
823 MT plus-End motor localization intensity analyses were performed as follows: The duration of
824 time motors/clusters were present at the plus-ends of MTs was quantified using ImageJ. Briefly,
825 all unobstructed MT plus-ends (identified based on the direction of motor motility) were marked
826 using point regions of interest (ROIs), which were added to the ROI Manager. These were then
827 converted to circular ROIs with a diameter of 7 pixels centered on the point using a custom-
828 written script. The intensity in these ROIs was traced over time using Multi Measure, ensuring
829 that the recorded measurements included standard deviation, min & max gray value, center of
830 mass, integrated density, and mean gray value. This process was also repeated for 9
831 background spots spread throughout the field of view. The output from each movie was saved,
832 and all data were imported into MATLAB for further analysis. A moving average was applied to
833 the raw integrated density signal (IDS). In every ROI, a cluster/motor was deemed present in a
834 given frame of the movie if this filtered IDS in the ROI exceeded that of background ROIs by 3
835 standard deviations. Only cluster lifetimes >9 frames were considered based on the approximate
836 time taken for a motor to walk through the ROI. The lifetimes of any clusters present in the first
837 or last frame of the movie were marked as censored for the subsequent lifetime analysis.
838 Lifetime analysis was performed by fitting the empirical cumulative distribution function (CDF) to
839 a mixture of two Weibull CDFs using MLE, taking censoring into account.
840
841 **Reconstitution of full length and mini-tetramer Kinesin-5 MT crosslinking and sliding**
842
843 MTs for surface-immobilization were generated via mixture of HiLyte 647 tubulin (TL670M),
844 biotinylated tubulin (T333P), and unmodified tubulin (T240) at a ratio of 1:1:20 along with 1mM
845 GMPCPP. MTs were polymerized at 37°C for 1 hour before clarification and stabilization in
846 30uM Taxol following published protocols (Shimamoto et al, 2015). 'Free' MTs for gliding were
847 generated via mixture of rhodamine tubulin (TL590M) and unmodified tubulin at a ratio of 1:20
848 along with 1mM GMPCPP, and were polymerized, clarified, and stabilized in 30uM Taxol
849 following similar protocols.
850 The flow chamber design and assay preparation were modified from a previously described
851 protocol (Shimamoto et al., 2015). Anti-parallel MT bundles were constructed using passivated

852   glass coverslips coated with SVA-PEG at a ratio of 50 PEG:1 biotin-PEG. All reagents were
853   prepared with 1X BRB80 buffer. Following each reagent flow-in and incubation, a flush with ~3
854   chamber volumes of BRB80 was performed. Reagents were introduced stepwise with the
855   following order and incubation times: (1) 0.5 mg/mL neutravidin, 2 minutes; (2) 0.5 mg/mL alpha
856   casein surface block, 3 minutes; (3) HiLyte-647 biotinylated MTs with 0.2mg/mL alpha casein
857   with no additional incubation and immediate flush; (4) kinesin-5 construct at desired final
858   concentration as reported with 0.2 mg/mL alpha casein, 2 minutes; (5) Rhodamine 561 MTs with
859   0.2 mg/mL alpha casein, 5 minutes, and the corresponding chamber flush included 1 mM TCEP
860   bond breaker solution in BRB80; (6) imaging buffer (25 mM HEPES, 25–150 mM KCl, pH 7.5, 10
861   mM beta-mercatopethanol; 1% glycerol, 0.5% Pluronic-F127, 0.3 mg/ml casein, 3 mM BME, 4
862   mM ATP-MgCl2) with Oxygen Scavenging System (4.5 mg/ml glucose, 350 U/ml glucose
863   oxidase, 34 U/ml catalase, 1 mM DTT) was then used to flush any unattached MTs. The
864   chamber was then sealed with clear nail polish prior to experiments.
865   MT bundles were imaged using three-channel TIRF microscopy using the following exposure
866   times and laser lines from a Nikon LUNA four-channel laser launch module: HiLyte-647 MTs:
867   640 nm laser (60% power, 200 ms exposure); GFP-tagged kinesin-5: 488 nm laser (30% power,
868   100 ms exposure); and rhodamine MTs: 561 nm laser (30% power, 100 ms exposure). Imaging
869   was performed on a Nikon Ti-E inverted microscope with a CFI Apo 100X/1.49NA oil immersion
870   TIRF objective. Images were acquired using a Photometric Prime 95B camera controlled with
871   Nikon NIS Elements software. Analysis of fluorescent data and generation of intensity linescan
872   data sets were performed using FIJI (ImageJ) tools(Schindelin et al., 2012).
873
874
875

Figure Legends

Figure 1: **The x-ray structure of the kinesin-5 BASS-XL minifilament reveals rigid dimeric coiled-coils emerge from a tetramer core and allow designing kinesin-5 mini-tetramers**

A) Top, a side views of 34 nm long KLP61F BASS-XL minifilament crystal structure reveals the formation of dimeric coiled-coils (cyan: 40-nm long) that are rigidly attached to the BASS tetrameric core (red) and stabilized by the C-terminal extension (dark Blue). Bottom panel, 90°rotated view compared to top panel.

B) Top sequence alignment reveals the conservation, and the heptad repeat pattern of the dimeric coiled-coil (marked in a and d residues), mapped based on the x-ray BASS-XL structure.
   Bottom, view of the dimeric coiled-coil heptad interactions marked in B on the structural interface shown in A

C) Domain organization of the designer Kinesin-5 mini-tetramers. The Motor and neck linker (residues 1-369; Blue) are fused to 20 residue N-terminal coiled-coils (green) and then fused to the N-terminal dimeric-coiled coil junctions (cyan) of the BASS-XL and the tetramer core (red) followed by the C-terminal extension (deep blue). The mini-tetramers may either include or lack the Kinesin-5 C-terminal tail domain (orange).

D) The structural organization of the designed 38-nm long kinesin-5 mini-tetrameric motor based on the fusion of domains based on matching the heptad repeats with neck helical coiled-coil. The tail domains extend near the motor domains of the subunits folded in anti-parallel orientations.

E) Scaled-comparison of the mini-tetramer kinesin-5 motors (38 nm), shown on the top, reveals that they are half the length of the native kinesin-5 tetrameric motors (80 nm), shown on the bottom.

Figure 2: Kinesin-5 mini-tetramers undergo processive motility interrupted by static pauses along MTs *in vitro*.

A) Top, kMBX domain organization. Motor and neck linker domains (1-365, blue) extended coiled-coil (green), BASS-XL minifilament with its dimerized zone (orange), tetrameric zone (red) and C-terminal zone (cyan). Middle, structural model for the kMBX mini-tetramer as shown in Figure 1D.

B) Top, TIRF microscopy reconstitution setup to examine kMBX motors motility along MTs. Middle panel, Image of individual MT (red) with kMBX motors moving along toward Mt plus ends (right side). Bottom panel, Kymograph of above image with kMBX motor motility along MTs revealing their processive motility with extended pauses (arrows) and lack of accumulation at MT plus ends.

C) Top, kMBX domain organization. Motor and neck linker domains (1-365, orange) extended coiled-coil (green), BASS-XL minifilament with its dimerized zone (cyan), tetrameric zone(red), C-terminal zone (blue) and C-terminal tail domain (pink). Middle, structural model for the kMBX mini-tetramer as shown in Figure 1D.

D) Top, TIRF microscopy setup for kMBXt MT motility assays. Middle panel, Images of individual MTs (red) with KMBXt motors (green) moving along with MT plus end to the right. Bottom panel, kymograph of above image with KMBXt motor motility along MTs revealing their extended pausing and clustering (marked by arrows) and accumulating kMBXt motor at MT plus ends.

E) The hMBX-TMR mini-tetramer consists of the human Eg5 motor-neck linker domain (1-374, orange), the Drosophila BASS domain (597-799) with its extended coiled-coil (green), with its dimerized zone (cyan), tetrameric zone (red), C-terminal zone (blue).

F) Kymographs of hMBX mini-tetramer motor show that undergo processive motility towards MT plus-ends and pause infrequently.

G) The hMBXt-TMR mini-tetramer consists of the human Eg5 motor-neck linker domain (1-374, orange), the *Drosophila* BASS-XL minifilament (597-799) with its extended coiled-coil (green), with its dimerized zone (cyan), tetrameric zone(red), C-terminal zone (blue) and the human Eg5 C-terminal tail domain (913-1056, pink).

H) An example of a very long run observed for hMBXt motors are shown. 20 frames between time points. Kymographs for motors showing the presence of pauses between periods of processive motility. The start (red) and end (blue) points of pauses are identified.

I) Histogram distributions for motility velocity (μm/s) of the kMBX (green), kMBXt (pink), hMBx (green) and hMBXt (pink) motors along MTs showing that the kMBXt or hMBXt undergo slower motility than the kMBX and hMBX motors.

J) Histogram distributions for motility run lengths (μm) the kMBX (green), kMBXt (pink), hMBx (green) and hMBXt (pink) motors along MTs revealing that the kMBXt or hMBXt motors are generally more processive than the hMBX and kMBX motors.

Figure 3. The Kinesin-5 tail domain induces pauses, increases run lengths and decreases motility velocities of the mini-tetramer kinesin-5.

A) Pauses (red) and periods of processive motility for hMBX motor (blue) were identified in trajectories based on the slope of the mean-squared displacement (α) calculated within a sliding window along the trajectory. α fluctuates between ~2 and ~0 indicating processive and stationary motility (bottom).

B) The MSD for the entire trajectories of hMBX motors were fit to an expression that describes periods of stationary pauses and motile events consisting of processive and diffusive movements, $MSD=(\phi vt)^2+\phi 2Dt+2\epsilon^2$ where ϕ is the fraction of time in a motile state. HMBX motors pause more frequently.

C) Plotting all local α-values reveals primarily processive motility (green), while hMBXt exhibits a bimodal distribution with peaks consistent with static binding ($\mu 1$) and processive motility ($\mu 2$) (purple).

D) Histograms showing the frame-to-frame velocity of hMBXt motors (purple; solid line) and hMBX motors (green; dotted line). hMBX motors move faster than hMBXt motors during the processive sections of trajectories. Velocities are similar during paused sections. (E) The total run length for hMBX motors was fit with a single exponential distribution with mean μ. All trajectories >3 frames were included (n = 2056 trajectories).   Unless otherwise specified, n = 111 trajectories from 3 independent experiments for hMBX constructs and n = 143 trajectories from 9 independent experiments for hMBXt constructs.

Figure 4: Kinesin-5 mini-tetramers with the tail domain, but not those without the tail, form multi-motor clusters while undergoing motility along a MT.

A) Mini-tetramers with the tail form clusters while moving along the MT. Two such examples are shown. Often, one of the motors involved is paused (yellow arrow), a processively moving motor joins it (orange arrow), and the two continue moving together (orange) arrow). 6 (left) or 10 (right) frames between time points. Scale bars 1µm wide.

B) Kymographs showing the formation of clusters. Note that many of these cluster's form while one of the motors involved is paused (red triangles). Scale bars 10s wide and 1µm tall.

C) The cumulative frequencies and their average for kMBX (green) and kMBXt (pink) motors to merge into clusters in relationship to the salt concentration (mM) conditions. The extended data for kMBX and kMBXt motor clustering are shown in figure supplement.

979   D, E, F) Intensity distribution for constructs (D) with the tail domain in the presence of ATP,
980   (E) with the tail domain in the presence of AMP-PNP, and (F) without the tail domain in
981   the presence of ATP, suggesting that the mini-tetramers require the tail domain and
982   motility in order to form clusters. Clusters likely correspond to 2-3 motors, and the
983   blurring of the intensity distribution for motors in the presence of ATP is likely due to the
984   quenching and unquenching of the TMR sensors used as labels during motor stepping.
985   Lines are ~6500au apart. For D, n = 1406 trajectories from 9 independent experiments.
986   For E, n = 1298 trajectories from 2 independent experiments. For F, n = 2056 trajectories
987   from 3 independent experiments.
988   G) Total run length distributions for single motors (light purple; dotted line) and clusters (dark
989   purple; solid line), both with the tail. Distributions were fit with a double exponential
990   distribution, and both groups have a short ($\mu1$) and long ($\mu2$) run length population.
991   However, the fraction of motors in the long run length population (p2) is larger for
992   clusters.
993   H, I) Motors often reach the plus-ends of stabilized MTs and remain bound there for some
994   time. Lifetimes were fit to a Weibull distribution. The hMBXt motors were best fit by a
995   double Weibull distribution, suggesting the presence of populations with a short and long
996   lifetime. The short lifetime ($\tau1$) was similar for both constructs, whereas the long lifetime
997   ($\tau2$) was longer for hMBXt than hMBX motors. The fraction of the population with a short
998   lifetime (p1) was also higher for hMBX motors, agreeing with the observation that hMBX
999   motors do not form clusters along the length of the MT.
1000   J) Distribution of lifetimes of motors/clusters at the ends of MTs as a function of their starting
1001   intensity (average intensity of the first 5 frames they are detected). hMBX motors (green)
1002   generally arrive as single motors, whereas hMBXt motors (purple) often arrive as
1003   clusters. Open circles denote censored lifetimes (present in first or last frame of movie,
1004   indicating they are at least as long or longer than the point plotted).
1005   K) The cumulative MT plus-end association times for kMBX and kMBXt motors and their
1006   average values in relationship to the salt concentration (mM). The extended data for
1007   kMBX and kMBXt motor clustering are shown in Figure 4 figure supplement 2-3.
1008
1009 **Figure 5: Kinesin-5 mini-tetramers show defects in crosslinking MTs but enhanced MT**
1010 **sliding once aligned, compared to native kinesin-5.**
1011
1012   A) Representative TIRF images of surface-immobilized MTs (magenta) crosslinked via
1013   kinesin-5 (green) constructs to free MTs (red). Four different kinesin-5 constructs were
1014   examined: hFLt, hFL, kMBXt, and kMBX. Scale bar = 10 microns.
1015   B) Percentage of surface-immobilized MTs that engaged in kinesin-5 mediated crosslinking
1016   with free MTs for each kinesin-5 construct. N=8 fields of view analyzed per condition.
1017   C) Sample MT pairs (top) and kymographs (bottom) depicting MT sliding driven by either
1018   hFLt (left) or kMBXt (right). Free MT (red) and kinesin-5-GFP or nNG (green) are shown.
1019   Pauses are identified by vertical yellow bar. Scale bar = 4 microns; frame rate for hFLT =
1020   10 seconds per frame; for kMBXt = 5 seconds per frame.
1021   D) Number of pauses or velocity reduction events observed per micron for hFLt and kMBXt
1022   driven MT sliding. N = 11 events for hFLt, N = 9 events for kMBXt.
1023   E) Average MT sliding rate calculated for bundles at different ATP concentrations. N = 6
1024   events for each condition. Error bars are S.D. Values are reported in Table 3.
1025
1026 **Figure 6: Kinesin-5 tail to motor regulation and minifilament length modulate motor**
1027 **clustering and features of MT-sliding motility.**
1028

A) The Kinesin-5 mini-tetramers are 38 nm in length leading to a decrease in torsional flexibility compared to native kinesin-5, which are 80 nm in length and have higher torsional flexibility.

B) Top panel, kinesin-5 mini-tetramers without the tail domain show processive motility punctuated by pauses and little residence at MT-plus ends. Bottom panel, kinesin-5 mini-tetramers with the tail domain show increased pausing, coupled with motor clustering mediated by cross motor-tail interactions between mini-tetramers.

C) MT sliding motility mediated by native kinesin-5 leads to 80-nm separation between paired MTs, which are slide apart with normal sliding velocity, which is punctuated by pauses and is lower than twice motility velocity of each motor end along each MT.

D) MT sliding motility by the kinesin-5 mini-tetramers leads to 38-nm separation between the paired MTs and a more efficient MT sliding motility that approaches closely to twice the motility velocity of each motor end.

E) The native kinesin-5 motor MT pair alignment is efficient due to torsional flexibility of the minifilament

F) Kinesin-5 mini-tetramer MT pair alignment is poor due to decreased torsional flexibility of its shortened minifilament.

1051   Table 1. X-ray crystallographic Data collection and refinement statistics.

1052

	BASS-XL
Data collection	
Resolution range	126.55- 4.40 (4.64 - 4.40)
Wavelength (Å)	0.979
Space group	*C* 2
Unit cell (Å): *a*, *b*, *c*	253.18, 84.89, 96.77
(˚): β	91.44
Total number of observed reflections	55597 (7964)
Unique reflections	13060 {10508}†
Average mosaicity	0.33
Multiplicity	4.3 (4.3)
Completeness (%)	98.7 (98.8) {79.76}†
Mean I/sigma(I)	4.6 (1.8)
Wilson B-factor	38.14
$R_{merge}{}^{a}$	0.10 (0.47)
Structure refinement	
R_{work}	0.274 (0.251)
R_{free}	0.310 (0.368)
Molecules per asymmetric unit	4
Number of non-hydrogen atoms	5037
macromolecules	5037
Solvent	0
Protein residues	702
RMS bond lengths (Å)	0.002
RMS bond angles (°)	0.56
Ramachandran favored (%)	96.2
Ramachandran allowed (%)	3.6
Ramachandran outliers (%)	0.43
Rotamer outliers (%)	0.41
Clashscore	11.42
Mean *B* values (Å^2)	
Overall	113.93
macromolecules	113.93
Number of TLS groups	11

1053   Parentheses numbers represent the highest-resolution shell.
1054   †Numbers represent the truncated data after treated with ellipsoidal truncation and anisotropic scaling.
1055 $^{a}R_{merge} = \Sigma_{hkl}\Sigma_{i}|I_{i}(hkl)-I_{av}(hkl)|/\Sigma_{hkl}\Sigma_{i}I_{i}(hkl)$.

1056

1057 Table 2: *Drosophila* Kinesin-5 mini-tetramer motor motility properties
1058

kMBX	Velocity (nm/s)	Run length (nm)	Run time (s)	Merging freq ($\mu m^{-1} min^{-1}$)	MT plus-end dwell time(s)
25 mM KCl	115 ± 3 n=340	N/A	N/A	N/A	N/A
50 mM KCl	144 ± 6 n=136	1014±222	9±1	0.006±0.001	78±19
100 mM KCl	156 ± 8 n =66	2137±428	16±4	0.001±0.0007	25±18
kMBXt					
25 mM KCl	78 ± 4 n=52	3832±433	56 ±8	0.007 ± 0.002	125±51
50 mM KCl	127 ± 4 n=136	3135±222	29 ±1	0.011 ± 0.002	117±15
100 mM KCl	114 ± 5 n=88	5060±605	51 ± 7	0.050 ± 0.008	267±45
125 mM KCl	136 ± 4 n=149	5440±362	45 ± 4	0.023 ± 0.006	147±34
150 mM KCl	119 ± 6 n=19	3347±538	29 ± 5	0.001 ± 0.0008	25±17

1059
1060
1061 Table 3: MT sliding velocities for Native and mini-tetramer kinesin-5 motors
1062

ATP (μM)	hFLt	kMBXt
10	N/A	23 +/- 11 nm/s (n=9)
25	N/A	35 +/- 15 nm/s (n=6)
50	N/A	51 +/- 12 nm/s (n=11)
100	69 +/- 11 nm/s (n=13)	82 +/- 17 nm/s (n=15)
250	91 +/- 14 nm/s (n=17)	103 +/-21 nm/s (n=14)

1063
1064
1065

Figure Legends for figure supplements

Figure 1 figure supplement 1. Details of the BASS-XL x-ray crystal structure determination.

A) Purification and Crystallization of the BASS-XL protein: left panel, Size Exclusion Chromatography (SEC) trace showing the purified BASS-XL. Middle panel, SDS-PAGE of purified BASS-XL. Right panel, images of BASS-XL C2 crystals used for x-ray structure determination.

B) Alignment of the BASS (grey) and BASS-XL (red) N-terminal structure ends reveal the extension of the BASS-XL and formation of the heptad repeats at the N-terminal end.

C) 2Fo-Fc map revealing the final model for BASS-XL built using the BASS-XL crystallographic data at 4.4-Å resolution. The model reveals the detailed helical coiled-coil dimers at the BASS-XL N-terminal end.

D) Crystallographic packing organization of BASS-XL minifilaments within the C2 space group for crystals used for structure determination.

Figure 1 figure supplement 2. Designing kinesin-5 mini-tetramers using the BASS-XL x-ray structure as a short mini-filament template.

A) Domain organization of designer Drosophila kinesin-5 mini-tetramers. KLP61F motor and neck linker domain (orange: residues 1-369) were fused to an extended BASS-XL minifilament, with its coiled-coil extension (green), dimerized region (cyan), tetrameric region (red) and C-terminal stabilizing zone (blue). The kMBXt construct included fusing the C-terminal kinesin-5 tail domain (residues 910-1036) to the C-terminal domain of the BASS-XL minifilament.

B) SEC-trace and SDS-PAGE for a peak fraction of the bacterially expressed of the C-terminally neon green (mNG) tagged KMBX and kMBXt motors.

C) Top, a side view of a structural model revealing the organization of the designed bipolar mini-tetrameric kinesin-5 motor with various regions (colored as described A) shown. The model reveals the motor neck-linker domains are oriented to face in different directions due to their positioning on the BASS-XL minifilament and the mini-tetrameric motor is 38-nm in length, which is roughly half the length of native kinesin-5. Below, a 90° rotated view of the view shown on top. D) a subunit colored view of the kinesin-5 mini-tetrameric motor design revealing the subunit organization of the motor and minifilament domains. Below, a 90° rotated view of the view shown on top.

Figure 3-figure supplement 1.

A) Additional traces of parsing analysis for trajectories of hMBXt, similar to Figure 1d. Pauses (red) and periods of processive motility (blue) were identified in trajectories (top plots) based on the local α-value calculated within a sliding window along the trajectory, which fluctuated between ~2 and ~0 (bottom plots).

B) Histograms showing the number of distinct sections identified as paused for hMBXt motors (purple) and hMBX motors (green) in a given trajectory as a fraction of the number of long trajectories (n = 143 for hMBXt and n = 111 for hMBX).

C) the number of all trajectories >3 frames (n = 1406 for hMBXt motors and n = 2056 for hMBX). This shows that fewer of the trajectories for hMBX motors were long enough to use in our parsing analysis and, as short trajectories were less likely to contain pauses (see, for example Figure 2C), shows that a small fraction of total trajectories contained pauses for hMBX motors.

D, E) MSD curves for paused (red) and processive (blue) sections for hMBXt **(D)** and hMBX **(E)** motors plotted on a linear scale, revealing the parabolic shape characteristic of super-

1118 diffusive motility for processive sections, and a horizontal line for paused sections. Lines were
1119 fit to $<x^2>=(v^2+\sigma_v^2)\tau^2+2\varepsilon^2$ and $<x^2>=2\varepsilon^2$, respectively. Error bars represent SEM. For a, n =
1120 143 trajectories from 9 independent experiments. For b, n = 111 trajectories from 3
1121 independent experiments.
1122 **F)** Kymograph showing the static binding of mini-tetramers in the presence of AMP-PNP. Scale
1123 bar 10s wide and 1μm tall.
1124 **G)** Repeating the local α-value analysis for mini-tetramers with the tail domain in the presence of
1125 AMP-PNP (black; dotted line) reveals a uni-modal distribution, similar to the mean α-value of
1126 the paused sections (red; solid line) in the presence of ATP. In contrast, the mean α-value of
1127 processive sections (blue; solid line) in the presence of ATP is closer to 2. For AMP-PNP, n =
1128 442 trajectories from 2 independent experiments. For ATP, n = 143 trajectories from 9
1129 independent experiments.
1130
1131 **Figure 4-figure supplement 1**. Measuring kMBXt clustering using tracking and intensity
1132 analysis.
1133
1134 A) Low magnification view of the MT (left) and kMBXt (right field) prior to analysis.
1135 B) The intensity analysis (right) for all spots detected (left) identified via TrackMate plugin using
1136 FIJI/imageJ. The kMBXt intensity histogram is presented was converted into cluster values
1137 based on the fluorescence intensity of NG per motor subunit within each mini-tetramer.
1138 Details are described in materials and methods.
1139
1140 **Figure 4 figure supplement 2: Example kymographs for kMBXt clustering at increasing**
1141 **ionic strengths**
1142
1143 Example kymographs of kMBXt clustering along MTs at (A) 25 mM, (B) 50 mM, (C)100 mM,
1144 (D) 125 mM KCl and (E) 150 mM KCl conditions. The kymographs show that clustering
1145 increases dramatically from 25 to 100 mM KCl but it decreases at 125 and 150 mM KCl. The
1146 MT plus-end accumulation seems to concentrate the largest kMBX clusters (left side of each
1147 kymograph). (F) Description for types of cluster and motor merging events described in Figure
1148 4 figure supplement 4B.
1149
1150 **Figure 4-figure supplement 3:** Characterizing kMBXt motility properties in relation cluster
1151 formation. Distribution for motility velocity A), Run length. B) and Run time. C) for kMBX
1152 (green) and kMBXt (purple) at 25-150 mM KCl.   The types of clusters formed for kMBXt at
1153 different conditions. For each type a smaller intensity spot merges with larger intensity spot
1154 (i.e., 1+2). These intensities were delineated using the approach presented in Figure 4 figure
1155 supplement 1 and described in materials and methods. D) Relationship of kMBXt motility
1156 velocity (left), Run length (middle) and run time(right) to cluster size for the 25 mM (green), 50
1157 mM (pink), 100 mM (Gray), 125 mM (Cyan) and 150 mM KCl (orange). These data show that
1158 cluster size does not correlate to any of the properties described.
1159
1160 **Figure 4 figure supplement 4: Analyses of MT plus-end accumulation of kinesin-5 mini-**
1161 **tetramers**
1162
1163 **A)** Clusters, which generally arrive at plus-ends as assemblies and dissociate as assemblies,
1164 were detected based on the intensity in a ROI over time. Blue line denotes intensity trace in
1165 ROI at the plus-end of a MT. Magenta spots identify frames in which no motor is identified as
1166 present. Green spots identify frames in which motor is identified as present. Other thin lines
1167 denote intensity traces of background ROIs used as a reference to identify the presence of a
1168 motor. Inset: An example of a motor arriving and falling off a plus-end is shown. 20 frames
1169 between time points. Scale bar 1μm wide.

1170 **B)** Histograms of the frame-to-frame velocities for single motors (light purple; dotted line) and
1171   clusters (dark purple; solid line). During the processive sections of trajectories, clusters move
1172   more slowly than single motors. Their velocities during paused sections are similar. For a and
1173   e, n = 143 trajectories from 9 independent experiments for hMBXt and n = 111 trajectories
1174   from 3 independent experiments for hMBX. For b and c, n = 351 plus-end clusters detected
1175   from 8 independent experiments for hMBXt motors and n = 211 plus-end clusters detected
1176   from 3 independent experiments for hMBX motors.
1177 C) Additional traces of cluster detection at the plus-ends of MTs, similar to Figure 4C. Clusters
1178   generally arrive at plus-ends as a whole and dissociate as a whole, regardless of the plateau
1179   intensity, but we did observe single motors appearing to join a pre-existing cluster, as well as
1180   some clusters forming at the plus-end (e.g., top left). Blue line denotes intensity trace in ROI
1181   at the plus-end of a MT. Magenta spots identify frames in which no motor is identified as
1182   present. Green spots identify frames in which motor is identified as present. Other thin lines
1183   denote intensity traces of background ROIs used as a reference to identify the presence of a
1184   motor. Top four traces for hMBXt motors and lower two for hMBX motors.
1185 D) The cumulative distribution function for the mixed Weibull distribution used to fit the empirical
1186   cumulative distribution of lifetimes for hMBX and hMBXt motors, as well as the obtained
1187   estimates of the parameters. Note that the short lifetimes and shape parameters are similar,
1188   while the longer lifetime is longer for hMBXt motors. Notably, the weighting of the shorter
1189   lifetime is also lower for hMBXt motors, suggesting that a larger fraction of motors is in the
1190   longer lifetime population.
1191 E) An example of a cluster of human kinesin-5 HMBXt motors dragging the plus-end at which the
1192   motors accumulated along another MT, acting to align the two MTs. This demonstrates that
1193   the plus-end clusters are still motile and are able to withstand load (the MTs are quite bent and
1194   still anchored to the coverslip at some sites by an avidin-biotin interaction). Note that to
1195   observe this, a higher density of MTs was immobilized using a lower density of Neutravidin. 50
1196   frames between time points. Scale bar 2μm wide.
1197
1198 **Figure 5 figure supplement 1:** Analyses of **Velocity of MT sliding for native and mini-**
1199 **tetramer kiensin-5:** Individual frames from time-lapse image series were processed via linescan
1200 analysis to determine MT minus-end positions. These values are then plotted as a function of
1201 time for (a) hFLt and (b) kMBXt proteins. Velocities are calculated by applying a sliding window
1202 function of width 5 frames to the position data and a linear slope is calculated for the
1203 corresponding data sets (b,d). Velocity values below 15 nm/s for more than 3 consecutive
1204 frames were then classified as pausing events (red dashed line in b,d).
1205
1206

Video Legends

Video 1: Large-scale view of hMBXt motors (green) undergoing motility along MTs (magenta) revealing kinesin-5 mini-tetramer motor pausing, clustering, and MT plus end association.

Video 2: Close-view of hMBX motors (green) undergoing motility, forming clusters, with pausing and while dwelling at MT (magenta) plus-ends.

Video 3: motility of kMBXt motors at four different ionic strength conditions (25-150 mM KCl) revealing the impact of ionic strength on motor clustering, processive motility and plus-end attachment. Clustering is improved at 50 mM KCl and decreases at 100-150 mM KCl. MT plus end accumulation occurs at 25-100 mM KCl and is diminished at 150 mM KCl.

Video 4: An example of a cluster of kinesin-5 hMBXt motors dragging the plus-end at which the motors accumulated along another MT, acting to align the two MTs. This demonstrates that the plus-end clusters are still motile and are able to withstand load (the MTs are quite bent and still anchored to the coverslip at some sites by an avidin-biotin interaction). Note that to observe this, a higher density of MTs was immobilized using a lower density of Neutravidin. 50 frames between time points. Scale bar 2μm wide.

Video 5: Three examples of MT sliding by native kinesin-5 (hFLt). The hFLt motors (green) slide a paired MT (red) along the anchored MT (magenta).

Video 6: Three examples of MT sliding by mini-tetramer-kinesin-5 (kMBXt). The kMBXt motors (green) slide a paired MT (red) along the anchored MT (magenta).

References:

Acar, S., Carlson, D.B., Budamagunta, M.S., Yarov-Yarovoy, V., Correia, J.J., Ninonuevo, M.R., Jia, W., Tao, L., Leary, J.A., Voss, J.C.*, et al.* (2013). The bipolar assembly domain of the mitotic motor kinesin-5. Nat Commun *4*, 1343.

Al-Bassam, J. (2014). Reconstituting Dynamic Microtubule Polymerization Regulation by TOG Domain Proteins. Reconstituting the Cytoskeleton *540*, 131-148.

Andreasson, J.O.L., Clancy, B.E., Behnke-Parks, W., Rosenfeld, S.S., and Block, S.M. (2012). A Universal Pathway for Kinesin Stepping. Biophys J *102*, 369a-369a.

Arcizet, D., Meier, B., Sackmann, E., Radler, J.O., and Heinrich, D. (2008). Temporal Analysis of Active and Passive Transport in Living Cells. Phys Rev Lett *101*.

Beausang, J.F., Goldman, Y.E., and Nelson, P.C. (2011). Changepoint Analysis for Single-Molecule Polarized Total Internal Reflection Fluorescence Microscopy Experiments. Method Enzymol, 431-463.

Bertran, M.T., Sdelci, S., Regue, L., Avruch, J., Caelles, C., and Roig, J. (2011). Nek9 is a Plk1-activated kinase that controls early centrosome separation through Nek6/7 and Eg5. Embo Journal *30*, 2634-2647.

Blangy, A., Lane, H.A., d'Herin, P., Harper, M., Kress, M., and Nigg, E.A. (1995). Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. Cell *83*, 1159-1169.

Bodrug, T., Wilson-Kubalek, E.M., Nithianantham, S., Thompson, A.F., Alfieri, A., Gaska, I., Major, J., Debs, G., Inagaki, S., Gutierrez, P.*, et al.* (2020). The kinesin-5 tail domain directly modulates the mechanochemical cycle of the motor domain for anti-parallel microtubule sliding. Elife *9*.

Cahu, J., Olichon, A., Hentrich, C., Schek, H., Drinjakovic, J., Zhang, C.J., Doherty-Kirby, A., Lajoie, G., and Surrey, T. (2008). Phosphorylation by Cdk1 Increases the Binding of Eg5 to Microtubules In Vitro and in Xenopus Egg Extract Spindles. Plos One *3*.

Castoldi, M., and Popov, A.V. (2003). Purification of brain tubulin through two cycles of polymerization-depolymerization in a high-molarity buffer. Protein Expr Purif *32*, 83-88.

Chen, G.Y., Cleary, J.M., Asenjo, A.B., Chen, Y., Mascaro, J.A., Arginteanu, D.F.J., Sosa, H., and Hancock, W.O. (2019). Kinesin-5 Promotes Microtubule Nucleation and Assembly by Stabilizing a Lattice-Competent Conformation of Tubulin. Current Biology *29*, 2259-+.

Chen, G.Y., Mickolajczyk, K.J., and Hancock, W.O. (2016). The Kinesin-5 Chemomechanical Cycle Is Dominated by a Two-heads-bound State. Journal of Biological Chemistry *291*, 20283-20294.

Chen, Y.L., and Hancock, W.O. (2015). Kinesin-5 is a microtubule polymerase. Nature Communications *6*.

Chugh, M., Reissner, M., Bugiel, M., Lipka, E., Herrmann, A., Roy, B., Muller, S., and Schaffer, E. (2018). Phragmoplast Orienting Kinesin 2 Is a Weak Motor Switching between Processive and Diffusive Modes. Biophys J *115*, 375-385.

Cross, R.A., and McAinsh, A. (2014). Prime movers: the mechanochemistry of mitotic kinesins. Nat Rev Mol Cell Bio *15*, 257-271.

Drosopoulos, K., Tang, C., Chao, W.C.H., and Linardopoulos, S. (2014). APC/C is an essential regulator of centrosome clustering. Nature Communications *5*.

Duselder, A., Fridman, V., Thiede, C., Wiesbaum, A., Goldstein, A., Klopfenstein, D.R., Zaitseva, O., Janson, M.E., Gheber, L., and Schmidt, C.F. (2015). Deletion of the Tail Domain of the Kinesin-5 Cin8 Affects Its Directionality. J Biol Chem *290*, 16841-16850.

Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and development of Coot. Acta Crystallogr D Biol Crystallogr *66*, 486-501.

Falnikar, A., Tole, S., and Baas, P.W. (2011). Kinesin-5, a mitotic microtubule-associated motor protein, modulates neuronal migration. Molecular Biology of the Cell *22*, 1561-1574.

Furuta, K., Furuta, A., Toyoshima, Y.Y., Amino, M., Oiwa, K., and Kojima, H. (2013). Measuring collective transport by defined numbers of processive and nonprocessive kinesin motors. P Natl Acad Sci USA *110*, 501-506.

Hafner, A.E., Santen, L., Rieger, H., and Shaebani, M.R. (2016). Run-and-pause dynamics of cytoskeletal motor proteins. Sci Rep-Uk *6*.

Henty-Ridilla, J.L., Rankova, A., Eskin, J.A., Kenny, K., and Goode, B.L. (2016). Accelerated actin filament polymerization from microtubule plus ends. Science *352*, 1004-1009.

Hildebrandt, E.R., Gheber, L., Kingsbury, T., and Hoyt, M.A. (2006). Homotetrameric form of Cin8p, a Saccharomyces cerevisiae kinesin-5 motor, is essential for its in vivo function. J Biol Chem *281*, 26004-26013.

Kapitein, L.C., Peterman, E.J., Kwok, B.H., Kim, J.H., Kapoor, T.M., and Schmidt, C.F. (2005). The bipolar mitotic kinesin Eg5 moves on both microtubules that it crosslinks. Nature *435*, 114-118.

Kashina, A.S., Scholey, J.M., Leszyk, J.D., and Saxton, W.M. (1996). An essential bipolar mitotic motor. Nature *384*, 225.

Liebschner, D., Afonine, P.V., Baker, M.L., Bunkoczi, G., Chen, V.B., Croll, T.I., Hintze, B., Hung, L.W., Jain, S., McCoy, A.J.*, et al.* (2019). Macromolecular structure determination using X-rays, neutrons and electrons: recent developments in Phenix. Acta Crystallogr D *75*, 861-877.

Lin, S., Liu, M., Son, Y.J., Himes, B.T., Snow, D.M., Yu, W.Q., and Baas, P.W. (2011). Inhibition of Kinesin-5, a Microtubule-Based Motor Protein, As a Strategy for Enhancing Regeneration of Adult Axons. Traffic *12*, 269-286.

McHugh, T., Gluszek, A.A., and Welburn, J.P.I. (2018). Microtubule end tethering of a processive kinesin-8 motor Kif18b is required for spindle positioning. Journal of Cell Biology *217*, 2403-2416.

Michalet, X. (2011). Mean Square Displacement Analysis of Single-Particle Trajectories with Localization Error. Biophys J *100*, 252-252.

Myers, K.A., and Baas, P.W. (2007). Kinesin-5 regulates the growth of the axon by acting as a brake on its microtubule array. Journal of Cell Biology *178*, 1081-1091.

Nadar, V.C., Ketschek, A., Myers, K.A., Gallo, G., and Baas, P.W. (2008). Kinesin-5 Is Essential for Growth-Cone Turning. Current Biology *18*, 1972-1977.

Nadar, V.C., Lin, S., and Baas, P.W. (2012). Microtubule Redistribution in Growth Cones Elicited by Focal Inactivation of Kinesin-5. J Neurosci *32*, 5783-5794.

Pandey, H., Reithmann, E., Goldstein-Levitin, A., Al-Bassam, J., Frey, E., and Gheber, L. (2021). Drag-induced directionality switching of kinesin-5 Cin8 revealed by cluster-motility analysis. Sci Adv *7*.

Rapley, J., Nicolas, M., Groen, A., Regue, L., Bertran, M.T., Caelles, C., Avruch, J., and Roig, J. (2008). The NIMA-family kinase Nek6 phosphorylates the kinesin Eg5 at a novel site necessary for mitotic spindle formation. Journal of Cell Science *121*, 3912-3921.

Rozelle, D.K., Hansen, S.D., and Kaplan, K.B. (2011). Chromosome passenger complexes control anaphase duration and spindle elongation via a kinesin-5 brake. Journal of Cell Biology *193*, 285-294.

Ruthardt, N., Lamb, D.C., and Brauchle, C. (2011). Single-particle Tracking as a Quantitative Microscopy-based Approach to Unravel Cell Entry Mechanisms of Viruses and Pharmaceutical Nanoparticles. Mol Ther *19*, 1199-1211.

Saunders, A.M., Powers, J., Strome, S., and Saxton, W.M. (2007). Kinesin-5 acts as a brake in anaphase spindle elongation. Current Biology *17*, R453-R454.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B.*, et al.* (2012). Fiji: an open-source platform for biological-image analysis. Nature Methods *9*, 676-682.

Scholey, J.E., Nithianantham, S., Scholey, J.M., and Al-Bassam, J. (2014). Structural basis for the assembly of the mitotic motor Kinesin-5 into bipolar tetramers. Elife *3*, e02217.

1335 Shapira, O., Goldstein, A., Al-Bassam, J., and Gheber, L. (2017). A potential physiological role
1336 for bi-directional motility and motor clustering of mitotic kinesin-5 Cin8 in yeast mitosis. J Cell Sci
1337 *130*, 725-734.
1338 Shimamoto, Y., Forth, S., and Kapoor, T.M. (2015). Measuring Pushing and Braking Forces
1339 Generated by Ensembles of Kinesin-5 Crosslinking Two Microtubules. Dev Cell *34*, 669-681.
1340 Strong, M., Sawaya, M.R., Wang, S.S., Phillips, M., Cascio, D., and Eisenberg, D. (2006).
1341 Toward the structural genomics of complexes: Crystal structure of a PE/PPE protein complex
1342 from Mycobacterium tuberculosis. P Natl Acad Sci USA *103*, 8060-8065.
1343 Subramanian, R., and Gelles, J. (2007). Two distinct modes of processive kinesin movement in
1344 mixtures of ATP and AMP-PNP. J Gen Physiol *130*, 445-455.
1345 Telley, I.A., Bieling, P., and Surrey, T. (2011). Reconstitution and Quantification of Dynamic
1346 Microtubule End Tracking In Vitro Using TIRF Microscopy. Methods Mol Biol *777*, 127-145.
1347 Tinevez, J.Y., Perry, N., Schindelin, J., Hoopes, G.M., Reynolds, G.D., Laplantine, E., Bednarek,
1348 S.Y., Shorte, S.L., and Eliceiri, K.W. (2017). TrackMate: An open and extensible platform for
1349 single-particle tracking. Methods *115*, 80-90.
1350 Toprak, E., Yildiz, A., Hoffman, M.T., Rosenfeld, S.S., and Selvin, P.R. (2009). Why kinesin is so
1351 processive. P Natl Acad Sci USA *106*, 12717-12722.
1352 Valentine, M.T., and Block, S.M. (2009). Force and Premature Binding of ADP Can Regulate the
1353 Processivity of Individual Eg5 Dimers. Biophys J *97*, 1671-1677.
1354 Vershinin, M., Carter, B.C., Razafsky, D.S., King, S.J., and Gross, S.P. (2007). Multiple-motor
1355 based transport and its regulation by Tau. P Natl Acad Sci USA *104*, 87-92.
1356 Vugmeyster, Y., Berliner, E., and Gelles, J. (1998). Release of isolated single kinesin molecules
1357 from microtubules. Biochemistry-Us *37*, 747-757.
1358 Weinger, J.S., Qiu, M., Yang, G., and Kapoor, T.M. (2011). A nonmotor microtubule binding site
1359 in kinesin-5 is required for filament crosslinking and sliding. Curr Biol *21*, 154-160.
1360 Zajac, A.L., Goldman, Y.E., Holzbaur, E.L.F., and Ostap, E.M. (2013). Local Cytoskeletal and
1361 Organelle Interactions Impact Molecular-Motor-Driven Early Endosomal Trafficking. Current
1362 Biology *23*, 1173-1180.
1363

Figure 1

A

N-Term cc (dimer)   C-term   Tetrameric core   C-term   N-Term cc (dimer)

34 nm

B

BASS-XL N-term dimerized coiled-coil (620-693)

C

BASS-XL

MBX   Motor neck-linker   CC   N-term CC   Tetra   C-term   Tail

MBXt

D

Motor   Tail   BASS-XL   Tail   Motor

CC (dimer)   C-term   tetramer   C-term   CC (dimer)

N-term CC (dimer)   N-term CC (dimer)

E

Short minifilament

Mini-tetramer ~38-nm

Full-length minifilament

Full-length tetramer   ~ 80-nm

Figure 2

A KLP61F Motor-BASS-XL-NG (kMBX-NG)

B

C KLP61F Motor-BASS-XL-tail-NG (kMBXt-NG)

D

E Eg5 Motor-BASS-XL (hMBX-TMR)

F hMBX-TMR MT   hMBX-TMR

G Eg5 Motor-BASS-XL-tail (hMBXt-TMR)

H hMBXt-TMR MT   hMBXt-TMR

I

J

Figure 3

A

B

	f_{paused}	v, nm/s	D, µm²/s	eps, nm
hMBXt	0.47	76	0.009	90
hMBX	0.03	41	0.012	31

C

hMBXt

$\mu_1 = 0.627$
$\sigma_1 = 0.532$
$\mu_2 = 1.760$
$\sigma_2 = 0.041$

D

hMBX
$\mu = 80.4$ nm/s
$\sigma = 100.3$ nm/s

hMBXt
$\mu = 54.2$ nm/s
$\sigma = 54.8$ nm/s

processive

hMBX
$\mu = 10.3$ nm/s
$\sigma = 90.7$ nm/s

hMBXt
$\mu = 3.9$ nm/s
$\sigma = 52.3$ nm/s

paused

E

$\mu = 0.560$ µm

$\mu_1 = 0.554$ µm
$\mu_2 = 2.942$ µm
$p_1 = 0.897$

Figure 4

Figure 5

Figure 6

A
low torsional flexibility
38 nm
Increased torsional flexibility
80 nm

B: - tail   pausing   processive
+ MT

+ tail   clustering   + end accumulation
+MT

C
MT +
80 nm
Normal sliding velocity
+ MT

D
MT +
38 nm
Rapid sliding velocity
+ MT

E Efficient MT pair alignment

F Poor MT Pair alignment