1   The Orphan Kinesin PAKRP2 Achieves Processive Motility Via Noncanonical Stepping

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25 **Abstract**

26 PAKRP2 is an orphan kinesin in *Arabidopsis thaliana* that is thought to transport vesicles along

27 phragmoplast microtubules for cell plate formation. Here, using single-molecule fluorescence

28 microscopy, we show that PAKRP2 exhibits processive plus-end-directed motility on single

29 microtubules as individual homodimers despite having an exceptionally long (32 residues) neck

30 linker. Furthermore, using high-resolution nanoparticle tracking to visualize motor stepping

31 dynamics, we find that PAKRP2 achieves processivity via a noncanonical stepping mechanism

32 that includes small step sizes and frequent lateral steps to adjacent protofilaments. We propose

33 that the small steps sizes are due to a transient intermediate step that involves a prolonged

34 diffusional search of the tethered head due to its long neck linker. Despite this different stepping

35 behavior, ATP is tightly coupled to each 8-nm step. Collectively, this study reveals PAKRP2 as

36 the first orphan kinesin to demonstrate processive motility and broadens our understanding of the

37 diverse kinesin stepping mechanisms.

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50   **INTRODUCTION**

51   Kinesins constitute a diverse superfamily of ATP-dependent, microtubule-based motor proteins

52   that are known to participate in a variety of intracellular processes, such as microtubule

53   organization and dynamics (1–5) and transport of cellular cargos (6, 7). Previous studies have

54   revealed that some kinesins can move on microtubules by taking many consecutive steps before

55   falling off, allowing them to transport organelles and protein complexes long distances (7–9). A

56   specific example of cellular cargo transport occurs exclusively in plant cell division, which requires

57   the construction of a cell plate at the division site. Plant kinesins hauling cell plate material walk

58   on a microtubule-based structure called the phragmoplast, whose plus-ends are located at the

59   developing cell wall (10, 11). In the model plant organism *Arabidopsis thaliana*, the phragmoplast-

60   associated kinesin PAKRP2 is believed to transport Golgi vesicles to the phragmoplast midzone

61   (12).

62   On the basis of phylogenetic analysis of the motor domains, the kinesin superfamily is

63   divided into 14 subfamilies (kinesin-1 through kinesin-14) and an "orphan" (or ungrouped) family

64   (13). PAKRP2 falls into the orphan family due to divergent structural features, such as a mutation

65   in the conserved nucleotide binding site (12, 14). To date, no processive orphan kinesin has been

66   reported, and many of the characterized orphan kinesins have mutations in the conserved

67   residues essential for motility (15–17). Based on our current knowledge, PAKRP2's predicted

68   function of long-distance vesicle transport is contradictory to its classification as an orphan

69   kinesin, but no investigation of its motility has ever been done. While processive motility isn't

70   necessarily required for organelle transport (18), it is a conserved feature of the prototypical

71   transport kinesins, such as kinesin-1 and kinesin-2 (19, 20). Currently, it is not known if PAKRP2

72   is intrinsically processive or achieves transport of cargo via clustering of several diffusive motors

73   (18, 21) or a non-motor microtubule binding domain that enhances the microtubule affinity of the

74   motor domain via tethering (22, 23).

75   In this study, using a combination of single-molecule fluorescence microscopy, dark-field

76   nanoparticle tracking, and solution ATPase assays, we show that PAKRP2 is an inherently

77   processive kinesin and surprisingly, does not exhibit typical hand-over-hand stepping behavior on

78   a single protofilament. Close examination of the PAKRP2 sequence revealed that it contains a

79   long neck linker that contributes to its processive behavior and does not adversely affect the motor

80   coupling. Overall, this study provides the first glimpse at the motility of an orphan kinesin and

81   broadens current understanding of how kinesin structure and processivity are correlated.

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83 MATERIALS AND METHODS

84 Molecular cloning, protein expression and purification

85   The full-length cDNA of PAKRP2 was codon-optimized for protein expression in *E. coli* and

86   synthesized as two gBlocks (IDT). The construct, containing a C-terminal His-Tag, was integrated

87   in a modified pET17b vector via isothermal assembly and verified by DNA sequencing. All of the

88   truncation constructs were designed for this study except for K560AviC, which was created and

89   characterized previously (24). For protein expression, plasmids were transformed into the BL21

90   Rosetta (DE3) competent cells (Novagen). Cells were grown at 37 °C in TPM (containing 20g

91   tryptone, 15g yeast extract, 8g NaCl, 2g Na_2HPO_4 and 1g KH_2PO_4 per 1 liter) supplemented with

92   50 μg/ml ampicillin and 30 μg/ml chloramphenicol. Expression was induced by cold shock on ice

93   at OD_{600} = 0.8-1 with 0.1 mM IPTG, and incubation was continued for additional 14-17 hours at

94   20 °C. Cell pellets were harvested by centrifugation at 5,500 x g for 10 minutes using a S-5.1 rotor

95   (Beckman Coulter) and stored at -80 °C prior to cell lysis.

96   To purify the His-tagged PAKRP2 and kinesin-1 chimera motors, cell pellets were re-

97   suspended in the lysis buffer (50 mM sodium phosphate buffer, pH 7.2, 250 mM NaCl, 1 mM

98     $MgCl_2$, 0.5 mM ATP, 10 mM ß-mercaptoethanol, 20 mM imidazole, and 1 μg/ml Leupeptin, 1

99   μg/ml Pepstatin, 1 mM PMSF and 5 % glycerol), and lysed via sonication (Branson Sonifier 450).

100   The cell lysate was then centrifuged at 21,000 x g for 35 minutes using a Ti-75 rotor (Beckman

101   Coulter). The supernatant was incubated with Talon beads (Clontech) by end-to-end mixing at 4

102   °C for 1 hour. The protein/beads slurry was then applied to a Poly-Prep column (Bio-Rad) and

103   washed twice with 10 column volumes of wash buffer (50 mM sodium phosphate buffer, pH 7.2,

104   250 mM NaCl, 1 mM MgCl$_2$, 0.1 mM ATP, 10 mM ß-mercaptoethanol, 20 mM imidazole, and 1

105   μg/ml Leupeptin, 1 μg/ml Pepstatin, 1 mM PMSF and 5% glycerol). The protein was eluted with

106   5 column volumes of elution buffer (50 mM sodium phosphate buffer, pH 7.2, 250 mM NaCl, 1

107   mM MgCl$_2$, 0.5 mM ATP, 10 mM ß-mercaptoethanol, 250 mM imidazole and 5 % glycerol). The

108   eluted protein was buffer-exchanged with a PD-10 column into storage buffer (BRB80, 0.5 mM

109   ATP, 100 mM KCl and 5% glycerol), flash frozen in liquid nitrogen, and stored at -80 °C.

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Polarity-marked microtubules

112   To make polarity-marked GMPCPP microtubules, a dim bovine tubulin mix (containing 17 μM

113   unlabeled tubulin, 17 μM biotinylated tubulin, and 0.8 μM HiLyte 647-tubulin) was first incubated

114   in BRB80 with 0.5 mM GMPCPP (Jena Bioscience) at 37° C overnight to make dim microtubules,

115   and then centrifuged at 250,000 x g for 7 minutes at 37° C in a TLA100 rotor (Beckman Coulter).

116   The pellet was re-suspended in a bright bovine tubulin mix (containing 7.5 μM unlabeled tubulin,

117   4 μM HiLyte 647-tubulin, and 15 μM NEM-tubulin) in BRB80 with 2 mM GMPCPP and incubated

118   at 37° C for additional 15 minutes to cap the plus-end of the dim microtubules. The resulting

119   polarity-marked track microtubules were pelleted at 20,000 x g for 7 minutes at 37° C in the

120   TLA100 rotor (Beckman Coulter), and finally re-suspended in BRB80 with 40 μM taxol.

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Total internal reflection fluorescence (TIRF) microscopy

123   All time-lapse imaging assays were performed at room temperature (22-23 °C) using the Axio

124   Observer Z1 objective-type TIRF microscope (Zeiss) equipped with a 100x 1.46 NA oil-immersion

125   objective and a back-thinned electron multiplier CCD camera (Photometrics). All microscope

126   coverslips were functionalized with biotin-PEG as previously described (25) to reduce nonspecific

127   surface absorption of molecules. All time-lapse imaging experiments in this study used flow

128   chambers that were made by attaching a coverslip to a microscope glass slide by double-sided

129   tape.

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131   **Single-molecule motility assay**

132   For single-molecule motility experiments, the chamber was perfused with 0.5 mg/ml Streptavidin

133   to immobilize the taxol-stabilized polarity-marked HiLyte-647/Biotin-labeled microtubules. After

134   removing unbound microtubules by washing the chamber with five-chamber volumes of BRB12

135   supplemented with 20 μM taxol, the chamber was perfused with a BRB80-based (or BRB50 for

136   Kin1_NLswap) motility mixture containing diluted motors, 1 mM ATP, 25 μM taxol, 1.3 mg/ml

137   casein and an oxygen scavenger system based on glucose oxidase/catalase (26). Time-lapse

138   image sequences were recorded at 1 frame per 2 seconds with an exposure time of 200 ms for a

139   duration of up to 10 minutes. Kymographs were generated and analyzed in ImageJ (NIH) for

140   obtaining the velocity and run length information of individual PAKRP2 motors. Reported

141   velocities are the peak of a Gaussian fit to the data, and associated errors are the standard

142   deviations (SD). Characteristic run lengths were calculated by fitting an exponential cumulative

143   distribution function to the data, and creating a bootstrap distribution to find the mean (n = 5000).

144   The mean was then corrected for filament length using the procedure outlined in (27), where the

145   characteristic filament length is the average length of all microtubules used in the analysis.

146   Reported errors are the 95% confidence intervals (CI) of the bootstrap distributions.

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148   **Single-molecule photobleaching assay**

149   For single-molecule photobleaching assays, PAKRP2 molecules were immobilized, in the

150   absence of ATP, on taxol-stabilized polarity-marked HiLyte-647/Biotin-labeled microtubules in

151   BRB80 with 20 μM taxol and 1.3 mg/ml casein. Time-lapse image sequences were continuously

152   recorded with an exposure time of 100 ms until the field of view was completely bleached of

153   fluorescence signal. The number of photobleaching steps of individual PAKRP2 motors was

154   obtained by tracking the fluorescence intensity in ImageJ (NIH).

155

156   **Total internal reflection dark-field microscopy assays**

157   For single-molecule tracking experiments, we used a custom-built total internal reflection dark-

158   field microscope as previously described (28). All experiments were carried out at 22-23 °C.

159   PAKRP2 motors were prepared with a biotinylated C-terminal Avitag or biotinylated GFP binding

160   protein (29), and mixed with 30-nm diameter, Streptavidin-coated gold nanoparticles. The motor

161   was added to the gold particles at the lowest molar ratio that produced landing events (4:1 for

162   PAKRP2 and 3:1 for K560AviC). Taxol-stabilized GDP microtubules were attached to the glass

163   coverslip via a kinesin rigor mutant as previously described (24). High-resolution position vs. time

164   traces were obtained from 100 frame/s (or 1000 frame/s for kinesin-1) movies by fitting the point

165   spread function with a 2D Gaussian using Fiesta software (30). The x and y trajectories were

166   rotated to minimize the standard deviation in the x-direction, resulting in a y-axis that is aligned

167   with the microtubule axis.

168   **Step size determination**

169   The on-axis step sizes were determined by a t-test algorithm (31), for the y-displacement vs time

170   traces. Only the traces with a standard deviation of < 3 nm from the step plateau were chosen for

171   analysis. All reported step sizes are the mean $\pm$ standard deviation of a single Gaussian fit to the

172   forward steps.

173 **ATPase Assay**

174 ATPase assays were carried out by an enzyme-coupled assay protocol adapted from a previous

175 study (32). Assays used 25 nM active dimeric PAKRP2(560), and 5 nM active dimeric

176 Kin1_NLswap, where activity was determined by assessing exchange of mantADP, as previously

177 described (33). Hydrolysis rates at each microtubule concentration (GDP taxol-stabilized) were

178 estimated by a linear fit to steady-state absorbance decreases at 340 nm, as previously described

179 (33). The k_{cat} and K_M were determined by performing a least-squares fit of the ATPase vs. tubulin

180 concentration curve to the Michaelis-Menten equation.

181 **RESULTS**

182 **PAKRP2 is a Processive Kinesin with a Long Neck Linker**

183 PAKRP2 consists of an N-terminal motor domain followed by a neck linker, a coiled-coil central

184 stalk, and an uncharacterized C-terminal tail. Based on previous studies that show that the neck

185 linker is an important component of kinesin processivity (34), and the fact that PAKRP2 is an

186 orphan kinesin (14, 35), we first wanted to determine if there were any structural divergences in

187 the neck linker region. Two coiled-coil prediction programs, COILs (36) and MARCOIL (37), both

188 placed the likely start of the alpha-7 helix between residues 394 and 407 (Fig. 1 *A* and *B*).

189 Examination of the sequence within that range places the first logical heptad repeat of the coiled-

190 coil domain at residue 397, a hydrophobic methionine, followed by negatively charged aspartic

191 and glutamic acids (Fig. 1 *C*). Thus, we conclude that the neck linker of PAKRP2 contains 32

192 residues, considerably longer than the neck linkers of other kinesins, which typically contain 14-

193 18 residues (38).

194 Several studies on kinesin-1 and kinesin-2 demonstrate that increasing the neck linker

195 length can lead to disruptions in the stepping mechanism, such as a decrease in run length or an

196 increase in futile ATP hydrolysis cycles (39–42). To determine whether PAKRP2 is a processive

197   microtubule motor, we engineered PAKRP2(FL), a recombinant full-length PAKRP2 with a C-

198   terminal GFP (Fig. 2 *A* and *B*). We used a single-molecule motility assay to visualize the

199   movement of PAKRP2(FL) on polarity-marked microtubules (Fig. 2 *C* and *D*; Supplementary

200   Movies 1 and 2). The assay showed that individual PAKRP2(FL) molecules moved processively

201   toward microtubule plus-ends with a mean velocity of 65 ± 16 nm s^{-1} (mean ± SD, n = 271; Fig. 2

202   *E*) and a run length of 3.56 ± 0.27 μm (mean ± 95% CI, n = 271, Fig. 2 *F*). The reported run

203   lengths and the associated error are the mean and 95% confidence intervals of the bootstrap

204   distribution. It is well established that nonprocessive kinesin motors can achieve processive

205   motility by clustering to form multi-motor ensembles (18, 21). We thus performed single-molecule

206   photobleaching to determine the oligomerization of PAKRP2(FL). Similar to other dimeric kinesins

207   (18), PAKRP2(FL) was predominantly photobleached in one or two steps (Fig. S1, *A* and *B*).

208   These results show that PAKRP2(FL) contains the ability to exhibit processive plus-end-directed

209   motility on single microtubules as a homodimer.

210   Some kinesins are known to achieve processive motility or to gain enhanced processivity

211   via non-motor microtubule-binding domains (22, 23, 43). To test whether PAKRP2 processivity

212   depends on any domain beyond the head and neck linker domains, we made two additional

213   constructs: PAKRP2(560), a truncation of the full-length at residue 560, and PAKRP2(LZ), a

214   minimal dimer of PAKRP2 containing the motor domain and the neck linker and dimerized through

215   a leucine zipper (Fig. 3 *A-C*). Single-molecule photobleaching experiments confirmed that

216   PAKRP2(560) and PAKRP2(LZ) both exist predominantly as individual homodimers (Fig. S1 *C-*

217   *F*), and exhibited processive plus-end-directed motility on single microtubules (Fig. 3 *D,*

218   Supplementary Movies 3 and 4). The velocities of PAKRP2(560) and PAKRP2(LZ) were within

219   error of PAKRP2(FL) (Fig. 3 *E*, Fig. S2, *A* and *C*). Run lengths of PAKRP2(560) and PAKRP2(LZ)

220   were determined to be 3.35 ± 0.29 μm (n = 266, Fig. 3 *E*, Fig. S2 *B*) and 2.67 ± 0.25 μm (n = 333,

221   Fig. 3 *E*, Fig. S2 *D*), respectively. While the PAKRP2(560) run length was within 5% of the wild

222   type run length, the more significant decrease in run length of the PAKRP2(LZ) construct could

223   indicate the native coiled-coils play a role in motility. Overall, these data show that PAKRP2

224   processivity is largely encoded in the region containing the motor domain and the neck linker.

225

226   **PAKRP2 Frequently Moves Laterally**

227   Based on the knowledge that PAKRP2 is inherently processive despite having a long neck linker,

228   we next investigated its stepping behavior to determine whether and how it may differ from that

229   of kinesin-1. We attached a 30-nm gold particle to the C-terminus of PAKRP2(560) and observed

230   the center-of-mass motion via dark-field nanoparticle tracking (Fig. 4 *A*) (44). Gold nanoparticle

231   attachment did not significantly affect the motor activity, as the single-molecule velocity of

232   nanoparticle-labeled motors on taxol-stabilized GDP microtubules was within 20% of the motors

233   without nanoparticles in the same experimental conditions (Fig. S3). It should also be noted that

234   the velocity of PAKRP2(560) without a conjugated gold nanoparticle on GDP microtubules was

235   36% slower than its velocity without a conjugated gold nanoparticle on GMPCPP microtubules

236   (Fig. S3). This suggests that PAKRP2(560) is sensitive to nucleotide-dependent structural

237   changes of the microtubule lattice (45), which has also been observed in the motility of kinesin-1

238   (46, 47).

239   When imaged at high resolution, PAKRP2(560) appeared to take frequent and sequential

240   lateral steps both to the left and right (Fig. 4 *B*). To rule out that this behavior was due to surface

241   binding of the gold nanoparticle or some other artifact of the assay, we used kinesin-1 with a gold

242   nanoparticle on the C-terminus as a control (K560AviC, Fig. 4 *C*), since kinesin-1 has been

243   demonstrated to walk on single protofilament unless it is navigating a roadblock (48, 49). The

244   different lateral stepping characteristics of PAKRP2(560) with a gold nanoparticle on the C-

245   terminus can be seen clearly when compared to K560AviC (Fig. 4 *D*). To quantify the lateral

246   stepping behavior, a distribution of the lateral displacement per 40 nm of on-axis displacement

247   was generated for both motors (Fig. 4 *E*). The standard deviations for the Gaussian fits to the

248   histograms of average off-axis displacements were 12.3 nm (n = 99) and 5.1 nm (n = 113) for

249   PAKRP2 and kinesin-1, respectively, confirming that PAKRP2 steps laterally more than kinesin-

250   1. Interestingly, the respective means were -1.5 nm and 0.7 nm, indicating that neither motor has

251   a significant off-axis directional bias.

252   Canonical processive kinesins are known to take sequential 8-nm center-of-mass steps

253   for the duration of their run lengths , due to the periodicity of tubulin binding sites on a single

254   protofilament (50–52). Using point-spread-function fitting to the gold nanoparticle position and a

255   t-test step-finding algorithm (Fig. 4 *F*), we measured an on-axis, center-of-mass step size of 5.0

256   ± 2.0 nm for PAKRP2(560) (mean ± SD, n = 958, Fig. 4 *G*). In comparison, the kinesin-1 control

257   displayed an on-axis, center-of-mass step size of 8.0 ± 3.0 nm (mean ± SD, Fig. S4 *A* and *B*),

258   consistent with previous results (49, 51). Thus, PAKRP2 stepping is distinct from that of kinesin-

259   1, in both the lateral movement and the average step size.

260

261   **PAKRP2 Takes Intermediate Steps**

262   To better understand the individual head dynamics during PAKRP2 stepping and to confirm the

263   small step size seen in the center-of-mass data, we attached the gold nanoparticle to one motor

264   domain (head) of PAKRP2(560) via an N-terminal Avi-tag (Fig. 5 *A*). Similar to the center-of-mass

265   construct, the head-tagged motor stepped processively along the microtubule with clearly

266   observable steps (Fig. 5 *B*). Using the t-test step-finding algorithm, we measured an average step

267   size of 7.6 ± 3.4 nm (mean ± SD, n = 230, Fig. 5 *C*). This value is larger than the center-of-mass

268   step size, as expected, but is half of the 16.4 nm expected for canonical hand-over-hand stepping

269   (39, 53). There are two plausible explanations for a step size smaller than the distance between

270   successive binding sites on a single protofilament: the labeled head is binding to an adjacent

271   protofilament or the steps represent transient intermediates in which the labeled head is between

272 binding sites. For instance, Stepp *et al.* demonstrated that head-labeled kinesin-2 motors take 13-

273 nm steps on axonemes, compared to 16.4-nm steps on single microtubules (54). This discrepancy

274 was explained by 50% of the steps landing on an adjacent site 8.2 nm away. In contrast, high-

275 resolution nanoparticle tracking revealed that the motor domain of kinesin-1 takes intermediate

276 steps, or substeps, at saturating ATP conditions that result in an average step size of 8.2 nm (24).

277   In order to resolve whether the PAKRP2 motor domains are taking 8.2-nm steps to

278 adjacent protofilaments or pausing midway through 16.4-nm steps, we measured the ATPase

279 activity of PAKRP2. Canonical steppers are known to take one step per ATP molecule hydrolyzed,

280 as ATP binding initiates the power stroke (55, 56). It is important to remember here that the

281 displacement of the motor's center-of-mass is approximately half the displacement of one motor

282 head if the second head remains bound to the microtubule. Based on the measured velocity of

283 40 ± 12 nm s^{-1} on GDP taxol-stabilized microtubules (Fig S3), 8.2-nm steps of the center-of-mass

284 correspond to a stepping rate of 4.9 s^{-1} and 4.1-nm steps correspond to a stepping rate of 9.8 s^{-}

285 ^{1}. In the ATPase assay the k_{cat} for a PAKRP2(560) dimer was 3.5 ± 0.6 ATP/s (Fig. 5 *D*), which is

286 close to the rate for 8.2 nm center-of-mass steps (Fig. 5 *E*) and not consistent with the motor

287 hydrolyzing multiple ATP per step. Therefore, we conclude that PAKRP2 stepping includes an

288 intermediate step, with the final position of the head being ~16.4 nm from the starting position.

289 This result also necessitates that PAKRP2 uses one ATP per step, despite having a long neck

290 linker, which is contrary to previous studies on kinesin-1 in which long neck linkers lead to

291 uncoupling of the ATPase from stepping (39, 42).

292

293 **PAKRP2 Neck Linker Disrupts Kinesin-1 Stepping**

294   The finding that PAKRP2 contains a long neck linker domain yet retains tight coupling

295 between its ATPase and stepping activities raises the possibility that sequence-specific structural

296   features in its long neck linker contribute to the coupling. To test whether the PAKRP2 neck linker

297   confers tight coupling to other motors, we designed a kinesin-1 chimera, Kin1_NLswap, in which

298   the native 14 residue neck linker was replaced by the 32 residue PAKRP2 neck linker (Fig. 6 *A*,

299   S5 *A*). A photobleaching assay confirmed that this construct is a homodimer in solution (Fig. S5,

300   *B* and *C*). From kymograph analysis (Supplementary Movie 5), the single molecule velocity of

301   Kin1_NLswap was 103 ± 20 nm s^{-1} (n = 172, Fig. 6 *B*), and the run length was 1.64 ± 0.19 μm (n

302   = 172, Fig. 6 *C*). Under identical conditions, the velocity and run length of wild-type kinesin-1 are

303   670 ± 70 nm s^{-1} and 1.21 ± 0.16 μm, respectively (44). Thus, swapping the PAKRP2 neck linker

304   into kinesin-1 significantly disrupts the stepping rate but does not substantially alter motor

305   processivity.

306   In principle, this slower velocity could result from either slowing of the ATPase cycle or

307   uncoupling of the hydrolysis cycle from the stepping cycle. To test between these possible

308   mechanisms, we measured the microtubule-stimulated ATPase of Kin1_NLswap. The k_{cat} of the

309   Kin1_NLswap was 121 ± 7 s^{-1} (Fig. S6), which is significantly higher than the center-of-mass

310   stepping rate of ~ 12 s^{-1}, assuming it takes 8.2-nm steps. However, even if the steps were 4.1-nm,

311   the stepping rate of ~ 25 s^{-1} would still be considerably lower than the ATP hydrolysis rate. Thus,

312   replacing the kinesin-1 neck linker with the longer PAKRP2 neck linker led to uncoupling of the

313   kinesin-1 from its stepping activity. It follows that, although PAKRP2 maintains tight

314   mechanochemical coupling despite having a long neck linker, tight coupling results from features

315   of the motor catalytic domain rather than specific structural features of the neck linker domain.

316

317   **PAKRP2 Neck Linker Enhances Its Processivity**

318   Given that the PAKRP2 neck linker greatly disrupts the stepping cycle of kinesin-1, it could also

319   negatively impact the stepping cycle of PAKRP2. To determine if the neck linker extension affects

320   the processivity of PAKRP2, we made a mutant, PAKRP2_NL14, in which neck linker residues

321 beyond the first fourteen amino acids in the sequence were deleted (Fig. 6 *A* and S5 *D*). The

322 construct was also confirmed to be a homodimer (Fig. S5, *E* and *F*). In contrast to the change in

323 velocity resulting from swapping the neck linker into Kinesin-1, shortening the PAKRP2 neck linker

324 did not affect the stepping rate. The velocity of PAKRP2_NL14 on GMPCPP microtubules was

325 determined to be 59 ± 19 nm s^{-1} (n = 168, Fig. 6 *D*), within 10% of wild-type PAKRP2 (Fig. 2 *E*).

326 Additionally, single-molecule motility assays showed that the neck-shortened motor maintains

327 processivity, but the run length is decreased to 1.96 ± 0.27 μm (n = 168, Fig. 6 *E*), which is 45%

328 less than wild-type (Supplementary Movie 6). The deleted region of the neck linker has a neutral

329 charge, making it unlikely that the reduction in run length is due to weaker electrostatic interactions

330 between the motor domain and the microtubule (57). Therefore, unlike in other kinesins where

331 longer neck linkers reduce inter-head coordination leading to decreased processivity, the long

332 neck linker of PAKRP2 contributes to its substantial processivity.

333

DISCUSSION

335 In this study, we have demonstrated that the orphan kinesin PAKRP2 is inherently processive,

336 which sets it apart from all other characterized orphan kinesins but is consistent with its putative

337 role in vesicle transport (12). Like other orphan kinesins (17, 58–60), PAKRP2 has a divergent

338 nucleotide-binding motif and hydrolyzes ATP more than 10-fold slower than dimeric kinesin-1 (61,

339 62). It also contains a 32-residue neck linker that is much longer than most other members of the

340 kinesin superfamily (38). Despite these variations in structural features that are integral to motility

341 (40, 63), PAKRP2 is more processive than kinesin-1, and it achieves this processivity via a

342 noncanonical stepping mechanism that includes a long one-head-bound intermediate state and

343 frequent lateral steps.

344   In the kinesin-1 motility cycle, ATP turnover is tightly coupled to its stepping activity (55,

345 56, 64). This coupling is thought to be facilitated by conformational changes in the neck linker,

346   which transmits tension between the two heads (41, 42). Evidence for this tension-based

347   mechanism can be seen in the uncoupling of the heads in kinesin-1 with neck linker insertions,

348   where in some cases the ATP turnover rate becomes much higher than the stepping rate (39,

349   42), and in some cases the stepping rate slows relative to wild-type (39, 40). Our study confirms

350   this behavior in kinesin-1, as the ATP hydrolysis rate of Kin1_NLswap is much higher than the

351   predicted stepping rate (Fig. S6). In contrast, PAKRP2 maintains tight mechanochemical coupling

352   despite its long neck linker (Fig. 5 *E*), suggesting that the mechanism for motor coupling is

353   different than in other kinesins. Consistent with this, shortening the PAKRP2 neck linker had no

354   effect on the stepping rate but significantly decreased the run length (Fig. 6 *D* and *E*). These

355   results suggest that the PAKRP2 motor domain and long neck linker have coevolved to achieve

356   tight mechanochemical coupling through an alternative mechanism than the one that has been

357   determined for kinesin-1.

358   High-resolution particle tracking of PAKRP2 revealed tail displacements of 5.0 nm (Fig. 4

359   *G*), which is considerably smaller than the 8.2-nm tubulin periodicity, and motor domain

360   displacements of 7.6 nm (Fig. 5 *C*), which is considerably smaller than the 16.4 nm periodicity

361   expected from a classical hand-over-hand mechanism (39, 42, 53). The small step size in the

362   PAKRP2 head-labeled traces is characteristic of an inchworm-like stepping model whereby the

363   two motor domains walk on adjacent protofilaments, such as the stepping observed in dynein

364   motility (26, 65). Although dynein is capable of taking coordinated hand-over-hand steps, it has

365   also been observed to be a stochastic stepper, wherein each head hydrolyzes ATP and moves

366   independently of the other (26, 65). Inchworm-like stepping in this case differs from the originally

367   proposed inchworm model in that it involves two catalytically active motor domains (66, 67), and

368   therefore two ATP molecules are burned per 8.2 nm step. This type of stepping in dynein is

369   facilitated by the flexibility between the two heads (68), making it a plausible mode of motion for

370   a kinesin with a long neck linker. However, the ATPase data that show PAKRP2 burns one ATP

371   molecule per 8.2 nm step oppose this explanation for the unusual step sizes seen in PAKRP2

372   (Fig. 5 *E*). This is not to say that PAKRP2 does not step on adjacent protofilaments, which was

373   clearly shown in the center-of-mass data, but that the step does not end at a binding site 8.2 nm

374   away.

375   An alternative explanation for the small PAKRP2 step sizes is that particle tracking is

376   detecting an intermediate step (or substep) of the tethered motor head before it reaches the next

377   binding site. A 2015 study on kinesin-1 labeled on one motor domain at saturating ATP observed

378   8.2-nm substeps that were attributed to a transient one-head-bound state following ATP binding

379   and preceding ATP hydrolysis (24). In the present study, detection of these intermediate states

380   by particle tracking could be facilitated by the slow stepping rate of PAKRP2, which is ~20-fold

381   slower than kinesin-1. Step sizes smaller than 16.4 nm have been observed before in kinesin-1

382   mutants with long neck linkers, but they were not attributed to substeps (39). In support of a

383   transient one-head-bound state, a recent study demonstrated that the duration of the one head-

384   bound state can be increased by increasing the neck linker length in kinesin-1 and kinesin-2

385   motors, an effect that may result from an increase in the area of diffusional search taken by the

386   tethered head before binding (44). Taken together, the PAKRP2 data align best with a substep

387   model, though the exact mechanism of this substep may be different than what is seen in kinesin-

388   1. In canonical processive steppers, such as kinesin-1 and kinesin-2, an increase in the one head-

389   bound state duration leads to a higher probability that the bound head detaches before the trailing

390   head binds, and consequently, causes a reduction in processivity of the motor (44). In contrast,

391   shortening the neck linker of PAKRP2, which presumably decreases the one-head-bound state,

392   resulted in reduced processivity (Fig. 6 *E*).

393   Another example of a kinesin with an unusually long neck linker is Zen4, a member of the

394   kinesin-6 family that plays a role in microtubule organization during cytokinesis (69). The neck

395   linker of Zen4 is 75 residues long and includes a binding site for GTPase activating proteins (70),

396   but does not prevent the motor from processively stepping along microtubules (71). The crystal

397   structure of the Zen4 motor domain in a nucleotide-free state revealed that the initial segment of

398   the neck linker docked in a backward conformation; this conformation is thought to relieve inter-

399   head tension and allow for more stability in the two head-bound state (71). Zen4 functions

400   primarily in crosslinking microtubules, so the long neck linker, coupled with backward docking in

401   the two head-bound state, could potentially allow both motor heads to remain bound for long

402   periods of time. However, despite both motors having long neck linker domains, PAKRP2 does

403   not contain the "arginine gate" that facilitates the backward docking of the neck linker, and it is

404   not clear how stabilization of the two head-bound state would benefit a transport motor. Thus,

405   parallels that can be drawn between Zen4 and PAKRP2 are limited.

406   Although long neck linkers have primarily been viewed as a disadvantage for processivity

407   (20, 38–40), there is evidence to suggest that they are an advantage for obstacle avoidance.

408   Kinesin-2, which contains a 17-residue neck linker, has been shown to step laterally to adjacent

409   protofilaments (48) and to be less affected than kinesin-1 by the addition of roadblocks, such as

410   tau protein or rigor kinesin-1 motors on the microtubule track (72, 73). These studies propose that

411   a long neck linker makes kinesin-2 sufficiently flexible to step to many of the adjacent binding

412   sites, and less likely to dissociate from the microtubule at a roadblock. In the cell, microtubules

413   are decorated with microtubule-associated proteins (MAPs) that could potentially block the paths

414   of processive motors (74–77), and so this obstacle avoidance could provide a selective advantage

415   in maximizing transport. Given that PAKRP2 is thought to transport material on the phragmoplast

416   microtubules, it seems likely that it encounters MAPs and side stepping would be a useful feature.

417   As a final point, consecutive side-steps have also been observed for kinesin-8, which achieves

418   superprocessivity despite having a long neck linker domain (23, 73, 78, 79); thus side-stepping

419   does not necessarily correlate with decreased processivity.

420   Based on the data presented here, we propose that PAKRP2 steps via a hand-over-hand

421   mechanism that includes a transient intermediate state in which one head is bound to the

422   microtubule, and that the motor takes frequent lateral steps to adjacent protofilaments. We

423   propose that this stepping behavior results from the long neck linker domain, however the tight

424   mechanochemical coupling also suggests that the neck linker and catalytic core of the motor have

425   coevolved to achieve processivity using a stepping mechanism that is different from the classical

426   hand-over-hand mechanism of kinesin-1 (50, 53, 67). Further single-molecule work is needed to

427   precisely determine how PAKRP2 achieves processive stepping and tight mechanochemical

428   coupling despite having an extended neck linker domain. It is also not known how the long neck

429   linker might impact the ability of PAKRP2 to generate sustained forces. From a biological

430   perspective, there is still no direct evidence that PAKRP2 can associate with vesicle membranes

431   and it is not clear whether intracellular transport by PAKRP2 generally results from a small

432   population of motors or a large ensemble of motors attached to the cargo. Overall, these results

433   add to the developing model of kinesin stepping, wherein each motor steps in a way that is

434   optimized for its structure and role in the cell.

435

436 **Acknowledgments**

440

441 **Author Contributions**

442   W.Q conceived and supervised the study. A.G. and P.W. performed all experiments. K.J.M. built

443   the microscope used for high-resolution tracking and assisted with experiments and analysis.

444   A.G., W.O.H. and W.Q. wrote the manuscript with input from all authors.

445

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644

645 **Figure Legends**

646 **Figure 1: PAKRP2 contains a long neck linker.** (A) The coiled-coil profiles of full-length

647 PAKRP2 predicted by COILs (red) and MARCOIL (blue). Black arrow denotes the end of the

648 motor domain. (B) Zoomed-in coiled-coil profiles of residues just before the coiled-coil region.

649 Black arrow denotes the end of the motor domain. (C) Sequence of residues between 394 and

650 414 to show first heptad repeat starting at residue 397 and ending at residue 403.

651   **Figure 2: Full-length PAKRP2 is a processive plus-end-directed kinesin.** (A) Schematic

652   diagrams of full-length PAKRP2 and PAKRP2(FL). (B) SDS-PAGE gel of PAKRP2(FL).

653   Arrowhead indicates the expected band of PAKRP2(FL), MW = 126 kDa. (C) Schematic diagram

654   of the single-molecule motility assay. (D) Representative kymographs of individual PAKRP2(FL)

655   molecules moving processively towards the plus-ends of single microtubules. Scale bars: 2

656   minutes (vertical); 5 µm (horizontal). (E) Velocity histogram of PAKRP2(FL). Red line corresponds

657   to a Gaussian fit with a mean value of 65 ± 16 nm s^{-1} (n = 271). (F) Cumulative distribution of

658   PAKRP2(FL) run length with a characteristic run length of 3.56 ± 0.27 μm (n = 271). Black circles

659   correspond to experimental data and the red line corresponds to an exponential cdf fit.

660   **Figure 3: PAKRP2 processivity does not require C-terminal tail or stalk domain**. (A)

661   Schematic diagram of full-length PAKRP2, PAKRP2(560) and PAKRP2(LZ). (B) SDS-PAGE gel

662   for PAKRP2(560). Arrowhead indicates the expected band of PAKRP2(FL). MW = 91 kDa. (C)

663   SDS-PAGE gel for PAKRP2(LZ). Arrowhead indicates the expected band of PAKRP2(FL). MW =

664   76 kDa. (D) Representative kymographs of individual PAKRP2(560) and PAKRP2(LZ) molecules

665   on single microtubules. Scale bars: 2 minutes (vertical); 5 µm (horizontal). (E) Bar graphs showing

666   the relative velocities and run lengths of PAKRP2(FL), PAKRP2(560) and PAKRP2(LZ) with

667   corresponding errors.

668   **Figure 4**: **PAKRP2 takes frequent lateral steps.** (A) Schematic diagram of the gold nanoparticle

669   placement on the C-terminus of PAKRP2(560) (B) Sample x-y traces for PAKRP2(560), where

670   the x-axis corresponds to the microtubule axis and the y-axis corresponds to lateral movement.

671   (C) Schematic diagram of the gold nanoparticle placement on the C-terminus of K560AviC. (D)

672   Sample x-y traces for PAKRP2(560), where the x-axis corresponds to the microtubule axis and

673   the y-axis corresponds to lateral movement. (E) Off-axis distance distributions for PAKRP2(560)

674   and K560AviC. Red and blue lines correspond to a Gaussian fit to the data. (F) A representative

675   on-axis distance vs. time plot. Raw data is shown as blue lines and steps detected by the t-test

676   step finding algorithm are shown in black. Data was acquired at 1 mM ATP every 10 ms. (G) Step

677   size histogram of PAKRP2(560) fit to a single Gaussian (n = 958).

678   **Figure 5: PAKRP2 takes intermediate steps.** (A) Schematic diagram of the gold nanoparticle

679   placement on the N-terminal motor domain of Avi-PAKRP2(560). (B) A representative on-axis

680   distance vs. time plot. Raw data are shown as purple lines and steps detected by the t-test step

681   finding algorithm are shown in black. Data were acquired at 1 mM ATP every 10 ms. (C) Step

682   size histogram of Avi-PAKRP2(560) fit to a single Gaussian (n = 230). (D) The ATPase rate per

683   dimer for PAKRP2(560) as a function of tubulin concentration. Data correspond to the mean $\pm$ SE

684   (n = 3 or 5 determinations per point). The solid line represents a fit to the Michaelis-Menten

685   equation, giving a maximum k_{cat} of 3.5 ± 0.6 s^{-1}. (E) Comparison of the ATP hydrolysis rate per

686   dimer and the stepping rate per dimer assuming different step sizes. Stepping rates were

687   calculated from the division of the center-of-mass velocity by the center-of-mass step size.

688   **Figure 6**: **PAKRP2 neck linker affects processivity.** (A) Schematic diagram of Kin1_NLswap

689   and PAKRP2_NL14. (B) Velocity histogram of Kin1_NLswap. Red line corresponds to a Gaussian

690   fit with a mean of 103 ± 20 nm s^{-1} (n = 172). (C) Run length histogram of Kin1_NLswap. Red line

691   corresponds to an exponential fit with a characteristic run length of 1.64 ± 0.19 μm (n = 172). (D)

692   Velocity histogram of PAKRP2_NL14. Blue line corresponds to a Gaussian fit with a mean of 59

693   ± 19 nm s^{-1} (n = 168). (E) Run length histogram of PAKRP2_NL14. Blue line corresponds to an

694   exponential fit with a characteristic run length of 1.96 ± 0.27 μm (n = 168).

695   **Figure S1: PAKRP2(FL), PAKRP2(560) and PAKRP2(LZ) form homodimers in solution.** (A)

696   Representative photobleaching traces of individual PAKRP2(FL) molecules. (B) Photobleaching

697   histogram of PAKRP2(FL) (n = 441). (C) Representative photobleaching traces of individual

698   PAKRP2(560) molecules. (D) Photobleaching histogram of PAKRP2(560) (n = 214). (E)

699   Representative photobleaching traces of individual PAKRP2(LZ) molecules. (F) Photobleaching

700   histogram of PAKRP2(LZ) (n = 266).

701 **Figure S2: PAKRP2(560) and PAKRP2(LZ) velocity and run lengths.**

702 (A) Velocity histogram of PAKRP2(560). Red line corresponds to a Gaussian fit with a mean of

703 61 ± 15 nm s^{-1} (n = 266). (B) Run length histogram of PAKRP2(560). Red line corresponds to an

704 exponential fit with a characteristic run length of 3.35 ± 0.29 μm (n = 266). (C) Velocity histogram

705 of PAKRP2(LZ). Blue line corresponds to a Gaussian fit with a mean of 66 ± 20 nm s^{-1} (n = 333).

706 (D) Run length histogram of PAKRP2(LZ). Blue line corresponds to an exponential fit with a

707 characteristic run length of 2.67 ± 0.25 μm (n = 333).

708 **Figure S3: Gold nanoparticle does not affect motor activity.** Bar graphs showing the relative

709 velocities of PAKRP2(560) on GMPCPP or taxol-stabilized GDP microtubules without gold, and

710 on taxol-stabilized GDP microtubules with gold. The velocities are 65 ± 16 nm s^{-1} (n = 271), $40 \pm$

711 12 nm s^{-1} (n = 47) and 32 ± 11 nm s^{-1} (n = 57), respectively.

712 **Figure S4: Step size determination for K560AviC.** (A) A representative on-axis distance vs.

713 time plot. Raw data is shown as red lines and steps detected by a step finding algorithm are

714 shown in black. Data were acquired at 1 mM ATP every 1 ms. (B) Step size histogram for

715 K560AviC with a single Gaussian fit (n = 664).

716 **Figure S5: PAKRP2_NL14 and Kin1_NLswap both form individual homodimers in solution.**

717 (A) SDS-PAGE gel of Kin1_NLswap. Arrowhead indicates the expected band of Kin1_NLswap.

718 MW = 75 kDa. (B) Representative photobleaching traces of individual Kin1_NLswap molecules.

719 (C) Photobleaching histogram for Kin1_NLswap (n = 149). (D) SDS-PAGE gel of PAKRP2_NL14.

720 Arrowhead indicates the expected band of PAKRP2_NL14. MW = 89 kDa. (E) Representative

721 photobleaching traces of individual PAKRP2_NL14 molecules. (F) Photobleaching histogram for

722 PAKRP2_NL14 (n = 289).

723 **Figure S6: Kin_NLswap ATP hydrolysis and stepping rate are uncoupled.** The microtubule-

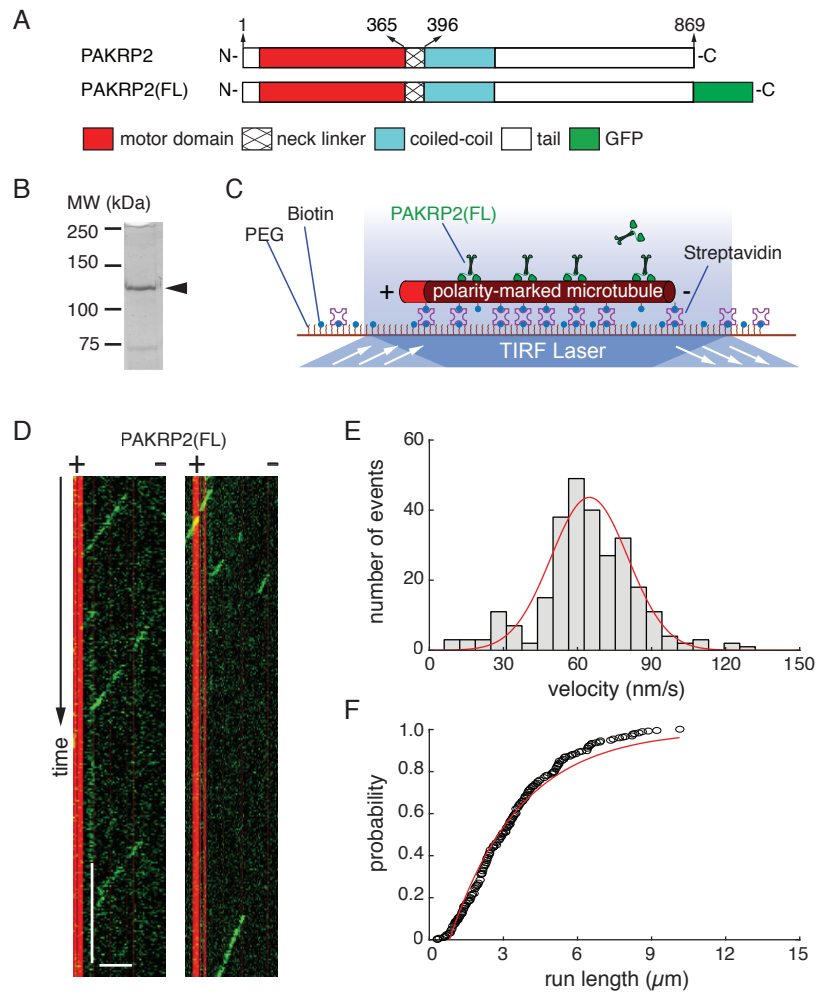724 stimulated ATPase rate per dimer for Kin1_NLswap as a function of tubulin concentration. Data

725   correspond to the mean $\pm$ SE (n = 3 determinations per point). The solid line represents a fit to

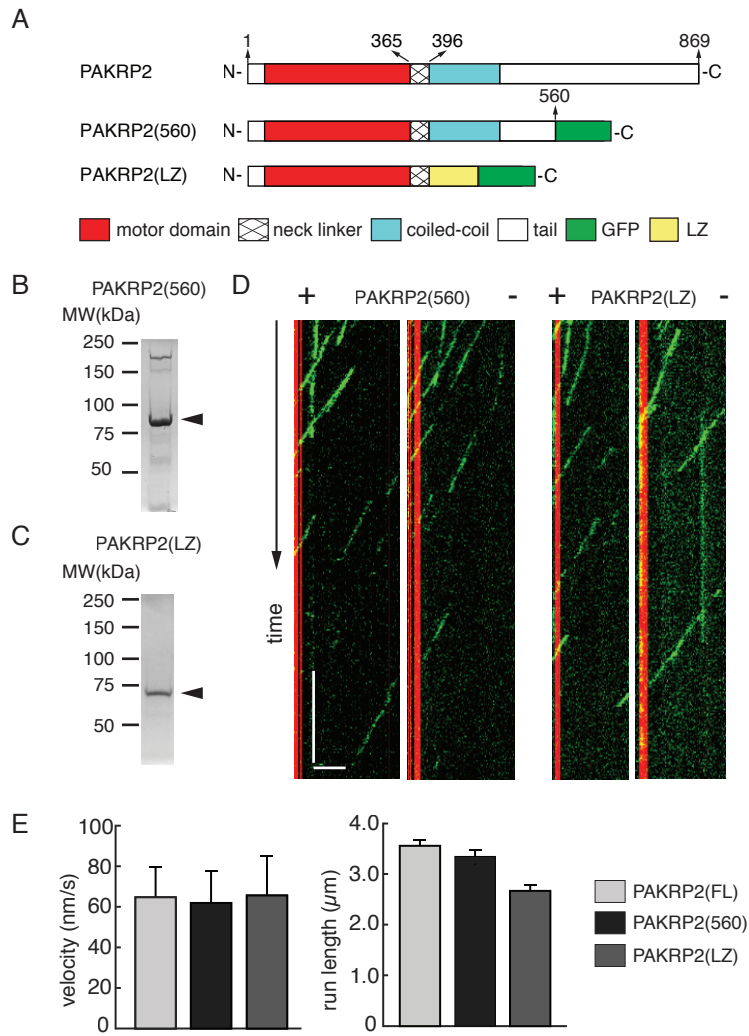726   the Michaelis-Menten equation, giving a maximum k_{cat} of 121 ± 7 s^{-1}.

Figure 1
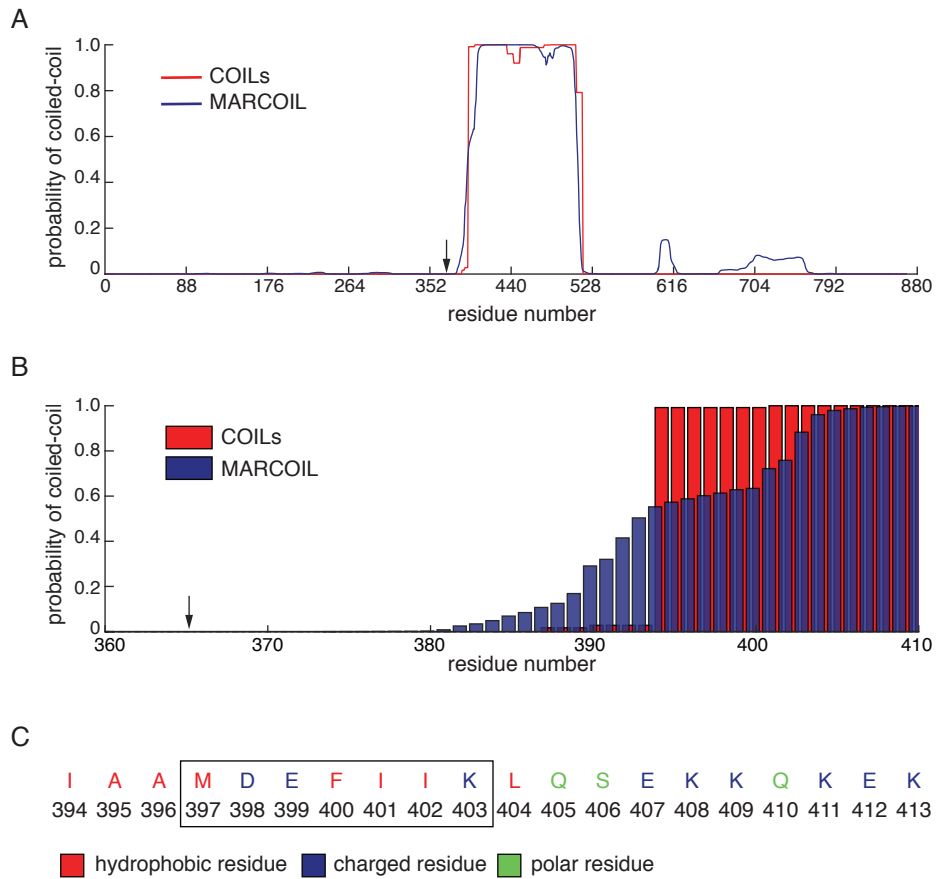
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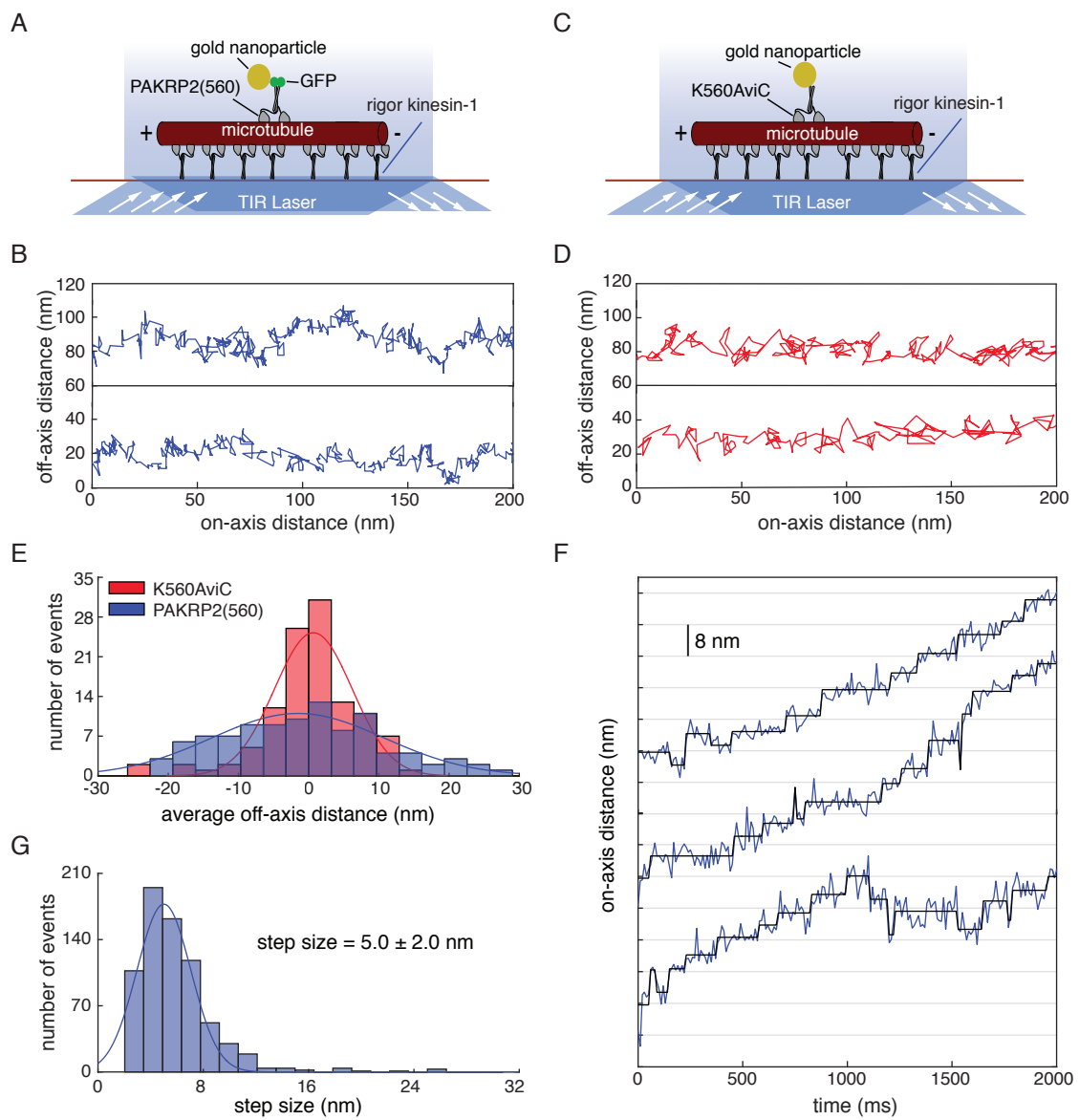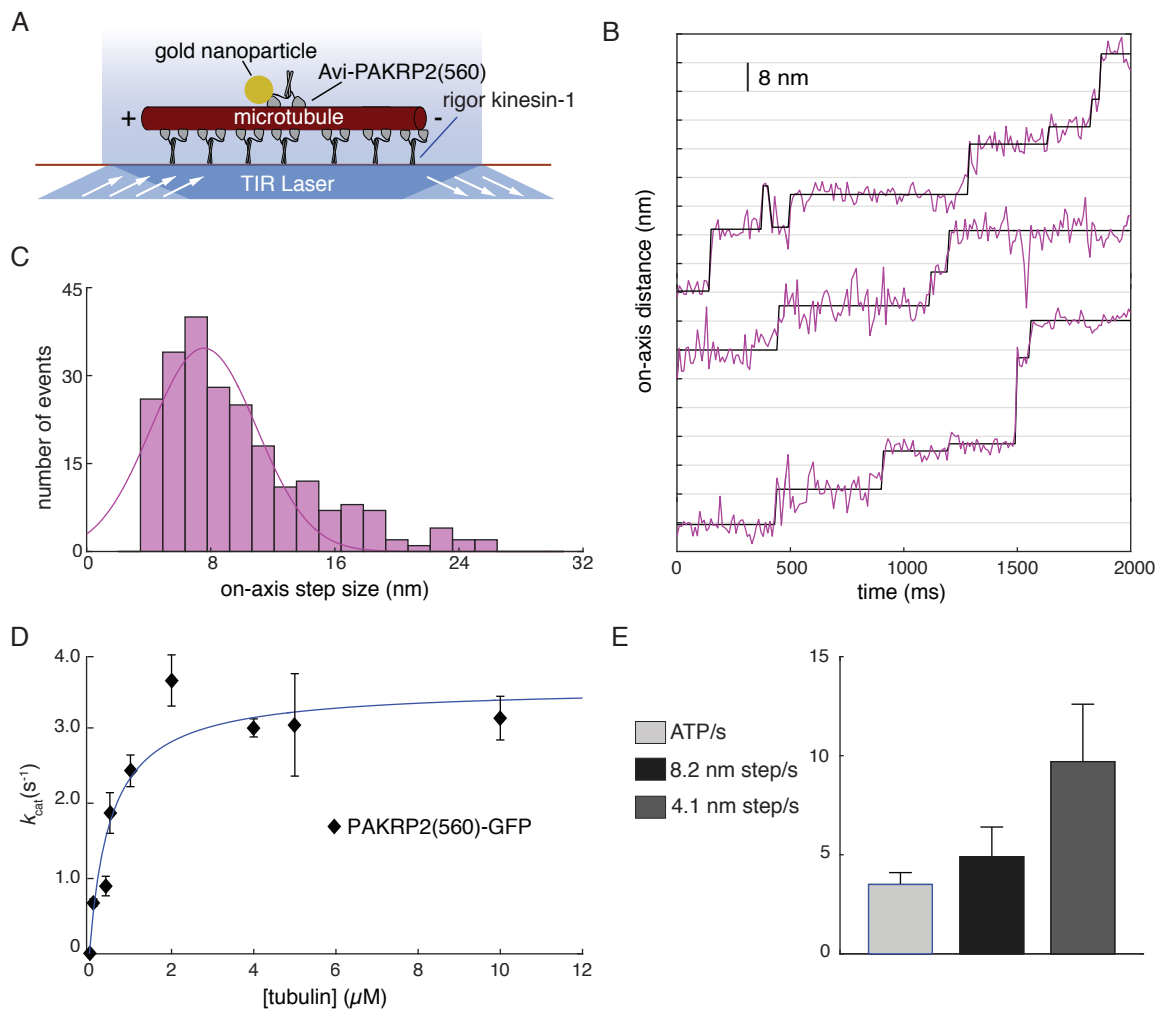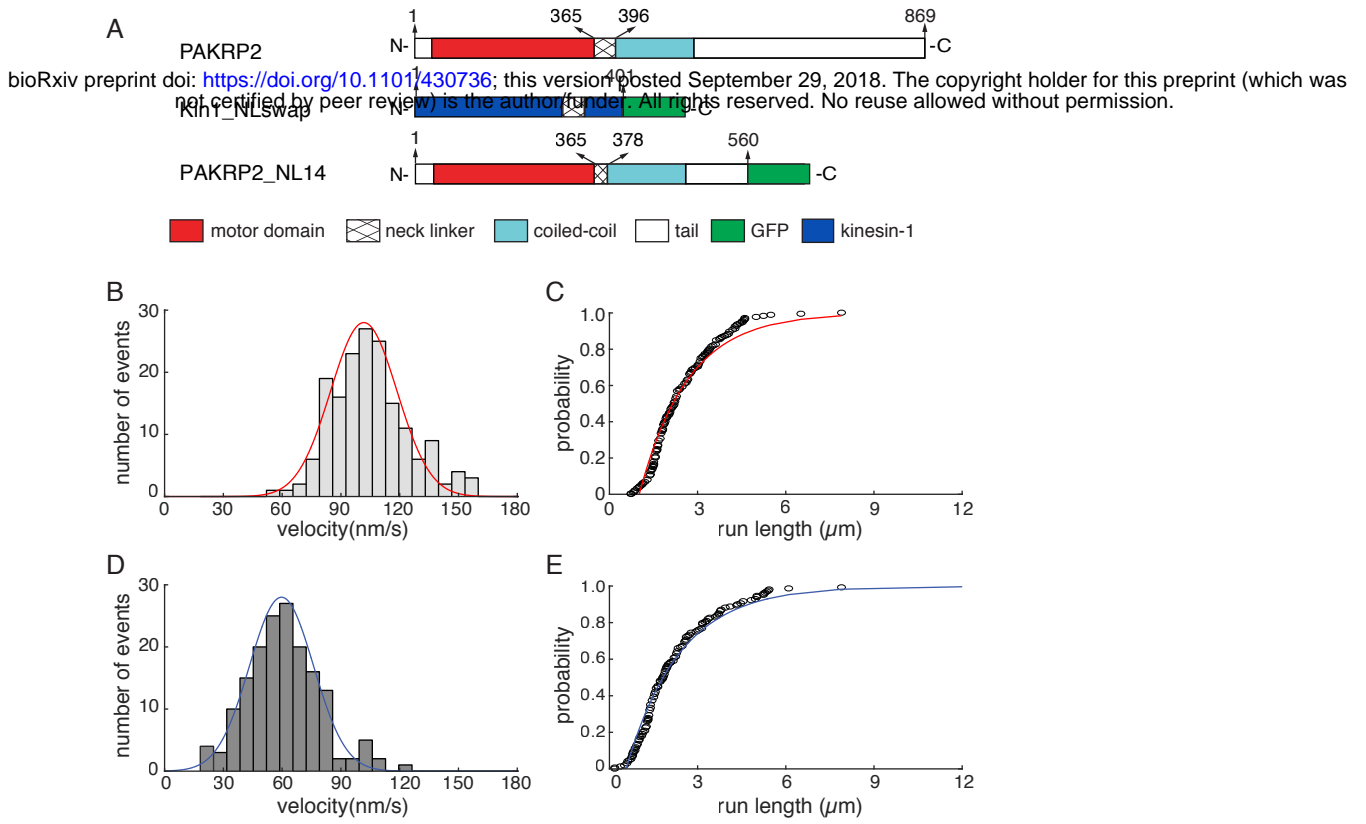
Figure 3

Figure 4

Figure 5
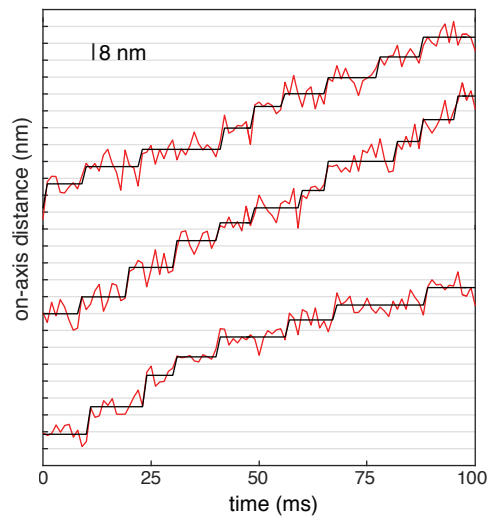
Figure 6

A

PAKRP2

Klh1_NLswap

PAKRP2_NL14

motor domain · **neck linker** · **coiled-coil** · **tail** · **GFP** · **kinesin-1**
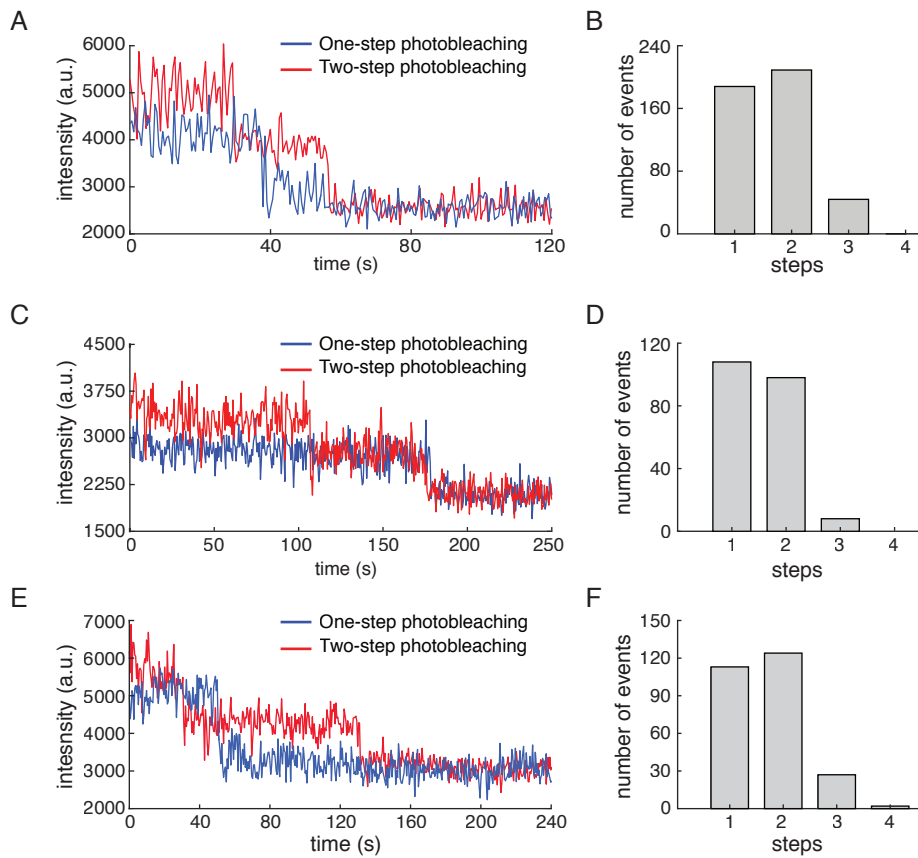
Figure S1

Figure S2

Figure S3

Figure S4

A

| 8 nm

on-axis distance (nm)

time (ms)

B

number of events

step size = 8.0 ± 3.0 nm

step size (nm)

Figure S5

Figure S6