The mitotic kinesin-14 KlpA contains a context-dependent directionality switch

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Kinesin-14s are commonly known as nonprocessive minus end-directed microtubule motors that function mainly for mitotic spindle assembly. Here we show using total internal reflection fluorescence microscopy that KlpA—a kinesin-14 from Aspergillus nidulans—is a context-dependent bidirectional motor. KlpA exhibits plus end-directed processive motility on single microtubules, but reverts to canonical minus end-directed motility when anchored on the surface in microtubule-gliding experiments or interacting with a pair of microtubules in microtubule-sliding experiments. Plus end-directed processive motility of KlpA on single microtubules depends on its N-terminal nonmotor microtubule-binding tail, as KlpA without the tail is nonprocessive and minus end-directed. We suggest that the tail is a de facto directionality switch for KlpA motility: when the tail binds to the same microtubule as the motor domain, KlpA is a plus end-directed processive motor; in contrast, when the tail detaches from the microtubule to which the motor domain binds, KlpA becomes minus end-directed.
Kinesins are microtubule motor proteins that convert the energy of ATP hydrolysis into mechanical work for various essential cellular processes. The mitotic spindle is a microtubule-based bipolar machine in eukaryotes that separates duplicated chromosomes to ensure that daughter cells each receive proper genetic material during cell division. Several different kinesin motor proteins are orchestrated inside the mitotic spindle for its assembly and maintenance. All of mitotic kinesins, kinesin-14s (KlpA), and kinesin-5s (ref. 25) are commonly considered to be nonprocessive minus end-directed microtubule motors. Loss of kinesin-14s has been shown to cause erroneous chromosome segregation and cell proliferation and survival.

KlpA is a mitotic kinesin-14 from the filamentous fungus Aspergillus nidulans. It is worth noting that A. nidulans is also the model organism for the discovery of BimC, the founding member of mitotic kinesin-5s (ref. 25). Like mitotic kinesin-14s such as Ncd from Schizosaccharomyces pombe and Klp2 from Aspergillus nidulans, KlpA is a plus end-directed processive kinesin-14 motor. With the lone exception of Kar3, which moves processively on single microtubules towards the minus end by forming a heterodimer with the nonmotor proteins Cik1 or Vik1 (refs 30, 33), all other kinesin-14s that have been studied to date are exclusively nonprocessive minus end-directed motors. We thus wanted to determine whether KlpA is a typical kinesin-14 that lacks the ability to move processively on single microtubules as a homodimer. To address this, we performed an in vitro motility assay to visualize the movement of KlpA molecules on surface-immobilized polarity-marked microtubules (Fig. 2a). The assay showed that, contrary to the notion of kinesin-14s as minus end-directed motors, GFP-KlpA molecules unexpectedly formed a steady flux of plus end-directed motion and accumulated at the microtubule plus end (yellow arrow, Fig. 2b and Supplementary Movie 3). Occasionally, there were GFP-KlpA particles moving towards the minus end (white arrow, Fig. 2b), but these minus end-directed particles were significantly brighter than the ones moving towards the plus end, implying that they were aggregates rather than simple homodimers.

Since the aforementioned motility experiments were performed at relatively high input levels of GFP-KlpA (≥4.5 nM; Fig. 2b), we repeated the same motility assay at much lower protein input levels (≤0.2 nM) so that the motile behaviour of individual GFP-KlpA molecules could be distinguished. The assay showed that individual GFP-KlpA molecules moved preferentially towards the microtubule plus end in a processive manner (Fig. 2c and Supplementary Movie 4) with a mean velocity of 300 ± 70 nm s⁻¹ (mean ± s.d., n = 257, Fig. 2d) and a characteristic run-length of 10.5 ± 2.2 μm (mean ± s.e.m., n = 257, Fig. 2e). This run-length likely was an underestimate because most KlpA molecules reached the microtubule plus end. We also performed the mean square displacement (MSD) analysis, which showed that the motility of KlpA is dominated by directional movement (Supplementary Fig. 3). Together, these results demonstrate that, in direct contrast to all other kinesin-14s that have been analysed to date, KlpA is a novel kinesin-14 motor that uniquely exhibits plus end-directed processive motility on single microtubules. It is worth emphasizing that such novel processive motility is not due to coupling, but rather an intrinsic behaviour of a single KlpA homodimer.

KlpA forms a homodimer in our in vitro experiments. We next performed a microtubule-gliding assay to determine the directionality of KlpA (Fig. 1c). Briefly, GFP-KlpA molecules were immobilized on the coverslip via an N-terminal polyhistidine tag, and KlpA directionality was deduced from the motion of polarity-marked microtubules. The assay showed that GFP-KlpA caused polarity-marked microtubules to move with the bright plus ends leading (Fig. 1d and Supplementary Movie 1) and a mean velocity of 309 ± 35 nm s⁻¹ (mean ± s.d., n = 123, Fig. 1e). In a control microtubule-gliding experiment using the plus end-directed human conventional kinesin hKHC, microtubules were driven to move with the bright plus ends trailing (Supplementary Fig. 2 and Supplementary Movie 2). Taken together, these results demonstrate that KlpA, when anchored on the surface via its N terminus, is a minus end-directed motor protein, in agreement with a previous study using KlpA from clarified bacterial lysates.

**Results**

**KlpA glides microtubules with minus end-directed motility.** We set out to determine the directionality of KlpA in vitro using TIRF microscopy. To that end, we purified the recombinant full-length KlpA tagged with an N-terminal green fluorescent protein (GFP-KlpA, Fig. 1a,b). Because KlpA substitutes for Kar3 in S. cerevisiae and Kar3 forms a heterodimer with the nonmotor proteins Cik1 or Vik1 (ref. 30), we performed two different assays—hydrodynamic analysis and single-molecule photobleaching—to determine the oligomerization status of KlpA. The hydrodynamic analysis yielded a molecular weight that is close to the theoretical value of a GFP-KlpA homodimer (Supplementary Fig. 1a,b). The photobleaching assay showed that the GFP fluorescence of GFP-KlpA was photobleached predominantly in a single step or two steps (Supplementary Fig. 1c,d), similar to other dimeric kinesins. Thus, KlpA formed a homodimer in our in vitro experiments.

**Plus end-directed KlpA motility requires its N-terminal tail.** Like other kinesin-14s such as Klp2 in S. pombe and Ncd in D. melanogaster, KlpA was also able to slide apart antiparallel microtubules and to statically crosslink parallel microtubules via its N-terminal nonmotor microtubule-binding tail (Supplementary Fig. 4a–g and Supplementary Movies 5 and 6). As several other...
kinesins are known to rely on nonmotor microtubule-binding domains to either achieve processive motility\(^30\) or enhance processivity\(^37,38\), we sought to determine whether the microtubule-binding tail of KlpA plays a similar role in enabling its unexpected plus end-directed processive motility on single microtubules. To do this, we purified GFP-KlpA-\(\Delta\)tail (Fig. 3a), a truncated construct lacking the N-terminal tail, for \textit{in vitro} motility experiments. Like GFP-KlpA, GFP-KlpA-\(\Delta\)tail formed a homodimer (Supplementary Fig. 5), and exhibited minus end-directed motility in the microtubule-gliding assay (Fig. 3b and Supplementary Movie 7) with a mean velocity of \(287 \pm 10\) nm s\(^{-1}\) (mean \(\pm\) s.d., \(n = 138\), Fig. 3c). This latter observation implies that motor–neck core of KlpA is inherently minus end-directed, as would be expected based on its highly conserved neck\(^32,39–41\). However, the \textit{in vitro} motility assay showed that GFP-KlpA-\(\Delta\)tail did not form a steady flux towards either end of the microtubule, nor did it accumulate at the microtubule ends (Fig. 3d and Supplementary Movie 8). Although some occasional brighter and presumably aggregated particles moved processively towards the microtubule minus ends (white arrow, Fig. 3d and Supplementary Movie 8), individual GFP-KlpA-\(\Delta\)tail molecules behaved like other nonprocessive kinesin-14s (refs 31,36,42) and mostly interacted with the microtubules in a diffusive manner with no apparent directional preference (yellow arrow, Fig. 3d and Supplementary Movie 8). The MSD analysis showed that the motility of GFP-KlpA-\(\Delta\)tail on single microtubules is best described by one-dimensional diffusion (Supplementary Fig. 6). Thus, besides allowing for microtubule-sliding and crosslinking, the tail of KlpA has an additional novel functionality of enabling the kinesin-14 motor to move on single microtubules towards the plus end in a processive manner.

**KlpA exhibits context-dependent directional preferences.** From the opposite directional preference exhibited by KlpA in the ensemble microtubule assays (Fig. 1d and Supplementary Fig. 4c) and the single-molecule motility experiments (Fig. 2b,c), we inferred that KlpA contains a context-dependent mechanism to switch directions on the microtubule. We thus directly compared the motility of GFP-KlpA inside and outside the microtubule overlap on the same track microtubule using a microtubule-sliding assay (Fig. 4a), as has been done previously for \textit{S. cerevisiae} kinesin-5 Cin8 (ref. 43). Briefly, in this assay the track (blue) and cargo (red) microtubules were both polarity-marked but labelled with different dyes; track microtubules were first immobilized on a coverslip inside the motility chamber and bound with purified GFP-KlpA molecules; moreover, cargo microtubules were added into the chamber before three-colour time-lapse imaging was acquired to simultaneously visualize the motility of GFP-KlpA molecules and cargo microtubules on the same track microtubules. Like KlpA, GFP-KlpA was also able to slide antiparallel microtubules relative to each other (Fig. 4b) and to statically crosslink parallel microtubules (Fig. 4c). In both scenarios, when outside the microtubule overlap regions, GFP-KlpA molecules showed a plus end-directed flux and accumulated at the plus end on the track microtubule (yellow arrow, Fig. 4b,c and Supplementary Movies 9 and 10). This matched the behaviour of GFP-KlpA on single microtubules (Fig. 2b). In contrast, inside the antiparallel microtubule overlap regions, GFP-KlpA molecules carried the cargo microtubule towards the minus end of the track microtubule (white arrow, Fig. 4b and Supplementary Movie 9). In the parallel orientation, the cargo microtubule remained stationary on the track microtubule, but GFP-KlpA molecules moved preferentially towards and gradually accumulated at the minus end inside the parallel microtubule overlap (white arrow, Fig. 4c and Supplementary Movie 10). This is similar to the observation that Ncd preferentially accumulates at the minus ends between statically crosslinked parallel
Collectively, these results demonstrate that KlpA can, depending on its context, display opposite directional preferences on the same microtubule: it is plus end-directed outside the microtubule overlap regions and minus end-directed inside the microtubule overlap regions regardless of the relative microtubule polarity.

**Discussion**

Kinesin-14 has been an intriguing kinesin subfamily since the discovery of its founding member Ncd because all kinesin-14s studied to date are exclusively minus end-directed based on the microtubule-gliding experiments. In addition, no kinesin-14 has been shown to be able to generate processive motility on single microtubules without clustering, and it does that by forming a heterodimer with its associated light chains Vik1 or Cik1. To our knowledge, KlpA is the first kinesin-14 that exhibits both plus end-directed processive motility on single microtubules and context-dependent directional switching. Thus, our study markedly expands the diversity of kinesin-14s.

Our results show that while the full-length KlpA clearly moves towards the plus end on single microtubules in a processive manner, a truncated KlpA lacking the N-terminal microtubule-binding tail glides microtubules with minus end-directed motility but becomes nonprocessive on single microtubules. There are several important implications from these observations. First, without the tail, the motor–neck core of KlpA is inherently minus end-directed, which is consistent with the notion that all kinesin-14s share a highly
conserved neck that serves as the minus end directionality determinant. Second, on single microtubules, the tail enables KlpA to exhibit both processive motility and plus end directionality. We propose that on a single microtubule, KlpA assumes a \textit{cis} conformation in which the catalytic microtubule-binding motor domain and the tail bind to the same microtubule. Binding of the tail to the \textit{cis}-microtubule subsequently prevents KlpA from premature dissociation, and this enables the kinesin to move processively on the microtubule, as has been observed previously for Kar3, which depends on the nonmotor microtubule-binding domain in Vik1 or Cik1 for processive motility on microtubules.

At present, it is unclear how exactly the tail enables the full-length KlpA to move preferentially towards the plus end on a single microtubule at the atomic level. Previous studies suggest that kinesin-14 motors use a lever mechanism for minus end-directed motility, where the neck acts as an extended lever arm and undergoes rotation towards the microtubule minus end upon ATP binding. Building on this, we speculate that when KlpA adopts the \textit{cis} conformation on a single microtubule, binding of the tail on the surface of the same microtubule as the motor domain induces a conformational strain through the stalk that causes the neck to rotate towards the microtubule plus end, thereby enabling the kinesin for plus end-directed motility. The precise underlying mechanism of plus end-directed KlpA processivity awaits future single-molecule studies of designed KlpA variants as well as cryo-electron microscopy (cryo-EM) studies of these variants on single microtubules.

Among all the experiments, KlpA exhibits plus end-directed motility only on single microtubules (Fig. 2b,c); moreover, in this scenario, the tail of KlpA and its catalytic motor domain are expected to bind to the same microtubule. In contrast, KlpA exhibits canonical minus end-directed motility when it is either anchored on the coverslip via the N terminus in the ensemble microtubule-gliding experiments (Fig. 1c,d) or between a pair of microtubules in the microtubule-sliding experiments (Fig. 4b,c).
In both scenarios, the N-terminal microtubule-binding tail of KlpA is not attached to the microtubule to which its motor domain binds. These results suggest that the tail of KlpA is a de facto directionality switch: to achieve plus end-directed processive motility, the switch-like tail of KlpA must bind to the same microtubule as its catalytic motor domain; moreover, to achieve minus end-directed motility, the switch needs to be detached from the microtubule to which its motor domain binds.

Our results show that KlpA accumulates at the plus end on single microtubules (Fig. 2b,c), and we suggest that this is enabled by its tail via a mechanism similar to that of the budding yeast kinesin-8 Kip3 (ref. 53). The tail likely binds strongly to the splayed protofilaments at the microtubule plus end to enhance the retention of KlpA there. It is worth noting that such accumulation requires the motor activity of KlpA to reach the microtubule plus end, as purified tail does not accumulate at either end of the microtubule and instead decorates the entire length of the microtubule rather uniformly (Supplementary Fig. 4e).

It has long been established that KlpA counteracts the activity of BimC24, but the underlying mechanism has been largely unknown. How could our results relate to the in vivo functions of KlpA? While KlpA localization inside the mitotic spindle has not yet been studied, one potential site of localization is the spindle pole, as several mitotic kinesin-14s, including HSET, CHO2, Kar3, Ncd, XCTK2 and Pkl1, are all known to localize to the spindle poles54–56. If KlpA does localize to the spindle poles, then how does it localize there? A recent study shows that the Pkl1—a mitotic kinesin-14 from fission yeast—forms a complex with Msd1 and Wdr8 for translocating to and anchoring at the spindle poles55. As homologues of Msd1 and Wdr8-likp-like proteins to KlpA dislodges its N-terminal tail from the cis-microtubule surface, and this activates the kinesin for minus end-directed motility both on single microtubules and at the spindle poles. The spindle midzone is another potential site of localization. In this case, our results suggest that while other mitotic kinesin-14s appear to depend on partner proteins to localize to the microtubule plus end14,47,58,59, KlpA can in principle autonomously localize to the spindle midzone via its inherent plus end-directed processive motility.

Several mitotic kinesin-5s were recently shown to be context-dependent bidirectional motor proteins43,60–62, indicating that context-dependent directional switching is evolutionarily conserved among kinesin-5s. Our current work on KlpA provides the first evidence to suggest that context-dependent directional switching could also exist among some, if not all, mitotic kinesin-14s. Thus, the mechanism and regulation of bidirectional mitotic kinesins will be an important subject for future studies.

**Methods**

**Molecular cloning of recombinant KlpA constructs.** The full-length cDNA of KlpA was codon-optimized and synthesized for enhanced protein expression in bacteria. All recombinant KlpA constructs were integrated in a modified pET-17b vector (Novagen) using either isothermal assembly or the Q5 site-directed mutagenesis kit (NEB) and verified by DNA sequencing. All KlpA constructs were expressed in BL21(DE3) Rosetta cells (Novagen). Cells were grown at 37 °C in tryptone phosphate medium (TPM) supplemented with 50 μg ml⁻¹ Ampicillin and 30 μg ml⁻¹ chloramphenicol until OD600 = 0.8. Expression was induced with 0.1 mM
isopropyl-β-D-thiogalactoside for 12–14 h at 20 °C. Cells were harvested by centrifugation, flash-frozen in liquid nitrogen and stored at −80 °C.

Hydrodynamic analysis. To determine the size of a recombiant KlpA construct, a combination of gel filtration and sucrose gradient centrifugation was used as described previously. For gel filtration, 500 μl of purified protein was applied to a Superdex 200 (GE Life Sciences) column pre-equilibrated with BRB50 (50 mM NaCl, 5 mM MgCl2, 0.5 mM ATP, 10 mM β-mercaptoethanol, 5% glycerol and 20 mM imidazole). Protein was then flash-frozen in liquid nitrogen and stored at −80 °C.

Preparation of polarity-marked microtubules. All taxol-stabilized polarity-marked microtubules (tetrathymethylrhodamine (TMR), Alexa 488, and HiLyte 647) with bright plus ends were prepared as previously described. To make the polarity-marked microtubules, a dim tubulin mix (containing 17 μM unlabeled tubulin and 0.8 μM fluorescently labelled tubulin) was first incubated in BRB80 (80 mM PIPES, pH 6.8, 1 mM EGTA and 1 mM MgCl2) supplemented with 100 mM KCl. The flow chamber was centrifuged in an SW41 rotor at 150,000 g for 18 h at 4 °C. Fractions (420 μl) were collected from the top of the gradient and analysed via SDS–PAGE. For standard proteins, 50 μl of solution containing each standard at 3 mg/ml was applied to the column and analysed similarly. The Stokes radius of a given KlpA construct was determined using the plot of the Stokes radius of standard proteins (Thyroglobulin, 8.5 nm; β-amylase, 5.4 nm; BSA, 3.55 nm) versus peak elution volume.

MSD analysis. To perform the MSD analysis, single-molecule motility data of KlpA on polarity-marked microtubules were acquired with 100-ms exposure time and 130-ms interval for a total duration of 2 min. Sub-pixel xy coordinates of motile KlpA molecules were determined using TrackMate (http://fiji.sc/TrackMate). The MSD values were computed from these xy coordinates using the formula as previously described. The MSD-versus-time plots were analysed by fitting curves to data using nonlinear regression in Matlab (MathWorks).

Other microtubule-based assays. To confirm the microtubule-binding ability of KlpA-tail-GFP, HyLite 647 microtubules were immobilized on the surface of coverglass, and purified KlpA-tail-GFP dilution in BRB50 supplemented with 25 mM KCl, 25 mM taxol and 1.3 mg ml⁻¹ casein was added to the chamber and incubated for 2 min. Unbound KlpA-tail-GFP was washed away before the addition of polarity-marked cargo microtubules. After incubation all non-bundled cargo microtubules were washed away with BRB50 supplemented with 20 μM taxol and 1.3 mg ml⁻¹ casein before the addition of motility buffer (BRB50 supplemented with 25 mM KCl, 1 mM ATP, 25 mM taxol, 1.3 mg ml⁻¹ casein and an oxygen scavenger system). Time-lapse images were taken at 1 frame per second for 5 min.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions
W.Q. conceived, designed and supervised the study; A.R.P. and K.-F.T. performed the experiments; K.-F.T. and P.W. contributed all KlpA constructs. All authors participated in discussing and interpreting the results. P.A.K., X.X. and W.Q. wrote the manuscript with input from all other authors.

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