Mechanochemical tuning of a kinesin motor essential for malaria parasite transmission

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Plasmodium species cause malaria and kill hundreds of thousands annually. The microtubule-based motor kinesin-8B is required for development of the flagellated Plasmodium male gamete, and its absence completely blocks parasite transmission. To understand the molecular basis of kinesin-8B’s essential role, we characterised the in vitro properties of kinesin-8B motor domains from P. berghei and P. falciparum. Both motors drive ATP-dependent microtubule gliding, but also catalyse ATP-dependent microtubule depolymerisation. We determined these motors’ microtubule-bound structures using cryo-electron microscopy, which showed very similar modes of microtubule interaction in which Plasmodium-distinct sequences at the microtubule-kinesin interface influence motor function. Intriguingly however, P. berghei kinesin-8B exhibits a non-canonical structural response to ATP analogue binding such that neck linker docking is not induced. Nevertheless, the neck linker region is required for motility and depolymerisation activities of these motors. These data suggest that the mechanochemistry of Plasmodium kinesin-8Bs is functionally tuned to support flagella formation.

Malaria—of which there were 241 million cases globally and 627,000 deaths in 2020 (https://www.who.int/publications/i/item/9789240040496)—is caused by apicomplexan Plasmodium parasites. Plasmodium spp. are intracellular parasites with a complex life cycle that alternates between mammalian hosts and mosquito vectors. The microtubule (MT) cytoskeleton plays a number of important roles throughout this life cycle, including formation of the mitotic/meiotic spindles during the several replicative stages1, during invasion of and egress from host cells and tissues2, and in forming the motile flagella in male gametes1. Given this diversity of functions, precise regulation of MT dynamics and organisation by cellular factors is absolutely essential for parasite survival. In particular, the flagella-driven motility of male gametes, which develop from male gametocytes in the mosquito gut immediately on ingestion of a blood meal, is required to fertilise female gametes for onward progression of the life cycle. If male gamete motility is compromised, parasite transmission is blocked3. Therefore, understanding the molecular processes involved in male gamete development and flagella formation is of fundamental interest, and may offer new avenues for development of disease control4.

Kinesin-8B—a member of the kinesin superfamily of ATP-driven, MT-based molecular motors1— is required for flagella formation in P. berghei male gametes, and its knockout completely disrupts parasite transmission6,7. Specifically, kinesin-8B localises to the basal body of male gametes and also localises along the assembling flagellum. While singlet and doublet axonemal microtubules are observed in the cytoplasm of P. berghei parasites deleted of kinesin-8B, the classical 9 + 2 axonemes do not assemble and exflagellation of male gametes does not occur8,9. Kinesin-8s are one of the fifteen kinesin families identified across apicomplexa that, together with kinesin-13s, are important regulators of MT dynamics9. Kinesin-8s are phylogenetically subclassified into kinesin-8As (e.g. mammalian KIF18A, KIF18B, S. cerevisiae Kip3), kinesin-8Bs (mammalian KIF19A) and kinesin-8Xs9.
(Plasmodium kinesin-SX)\(^\text{10}\). Nine kinesins have been phylogenetically identified and were recently functionally characterised in \(P.\) \textit{bergei},\(^\text{11,12}\) while at least 1 other may also be present in \(P.\) \textit{berghei} spp.\(^\text{6,8}\). Intriguingly, with genes encoding one kinesin-13 and two kinesin-8 isoforms (kinesin-8B and kinesin-8X), at least one third of \(P.\) \textit{berghei} kinesins are potential regulators of MT dynamics\(^\text{9}\). This likely reflects the requirement for frequent and often rapid remodelling of the MT cytoskeleton during the parasite life cycle.

Although eukaryote-wide kinesin families have been distinguished based on phylogenetics\(^\text{9,11}\), we don’t yet know if individual families such as kinesin-8s have conserved molecular activities and function. Previously characterised kinesin-8s (primarily from mammals and yeast) move towards the plus ends of MTs and regulate dynamics of these ends on arrival\(^\text{10}\). While lattice-based kinesin-8 movement requires the ATPase activity of the motor domains, ATP binding but not necessarily hydrolysis appears to be required for MT end regulation\(^\text{13}\). Kinesin-8s also exhibit MT depolymerisation activity, but their depolymerisation mechanism and the extent of conservation of this activity is not currently clear\(^\text{14–17}\). The best understood function of kinesin-8s is regulation of spindle MT dynamics during chromosome alignment\(^\text{18}\). \(P.\) \textit{berghei} kinesin-8X is spindle-associated in the mosquito stages of the parasite life cycle and is needed for oocyst development and sporozoite formation. This motor exhibits plus-end directed motility and MT depolymerisation activity, supporting a classical role for this motor in regulating spindle dynamics\(^\text{11}\).

**Results**

**\(Pb\)kinesin-8B-MD and \(Pf\)kinesin-8B-MD have MT-stimulated ATPase and motility activities**

To investigate the molecular properties of \(P.\) \textit{berghei} kinesin-8Bs, we expressed and purified \(Pb\)kinesin-8B-MD and \(Pf\)kinesin-8B-MD (Supplementary Fig. 1b), and measured their steady state MT-stimulated ATPase activities (Fig. 1b). The activities of each of these constructs equation, from which the \(K_{cat}\) and \(K_m\) were calculated in Prism 9. Both \(Pb\)kinesin-8B-MD (top—green) and \(Pf\)kinesin-8B-MD (bottom—blue) exhibit MT-plus end directed gliding activity. For \(Pb\)kinesin-8B-MD, the velocity = 41.3 ± 8.8 nm/s (mean ± SD; \(n = 36\)), and for \(Pf\)kinesin-8B-MD = 44.3 ± 8.4 nm/s (mean ± SD; \(n = 104\)). Paclitaxel-stabilised MTs were used and data are plotted on the left, while the representative TIRF-M kymographs on the right shows gliding of a single polarity-marked GMPCPP-MT consistent with plus-end directed motility; MT schematic above.

![Fig. 1](https://doi.org/10.1038/s41467-022-34710-x)
were similar: $K_{cat} = 1.9 \pm 0.2$ ATP/s and $K_m = 2.5 \pm 0.6 \mu M$ for $Pb$ kinesin-8B-MD, while $K_{cat} = 1.4 \pm 0.2$ ATP/s and $K_m = 1.2 \pm 0.5 \mu M$ for $Pf$ kinesin-8B-MD.

To begin to understand how the ATPase activity of these motors is harnessed, we investigated their behaviour in a multi-motor gliding assay using TIRF microscopy (TIRF-M), in which motors are attached to the assay coverslip and labelled, stabilised MTs are flowed into the assay cell. Both kinesins generated paclitaxel-stabilised MT movement, with average velocities of $Pb$ kinesin-8B-MD $= 41.3 \pm 8.8$ nm/s and $Pf$ kinesin-8B-MD $= 44.3 \pm 8.4$ nm/s (Fig. 1c, left). Using polarised GMPCPP MTs, we observed that this gliding activity was plus-end directed for both motors (Fig. 1c, right) and with the same average velocity as paclitaxel stabilised MTs (Supplementary Fig. 2). These data establish that the overall biochemical properties of these parasite kinesin-8B-MD constructs are conserved, and that they are capable of driving ATP-dependent plus-end directed motility along MTs.

$Pb$ kinesin-8B-MD and $Pf$ kinesin-8B-MD are MT depolymerases

We also used TIRF-M to investigate the influence of $Pb$ kinesin-8B-MD and $Pf$ kinesin-8B-MD on MT ends. In this assay, we incubated unlabelled motor protein with tethered, labelled, paclitaxel-stabilised MTs and monitored MT length. Both kinesin-8B constructs cause MT shortening in the presence of ATP or the non-hydrolysable ATP analogue AMPPNP (Fig. 2a). In all cases, shortening is observed at both MT ends showing that the MT depolymerisation activity of $Pb$ kinesin-8B-MD and $Pf$ kinesin-8B-MD is not restricted by the plus-end directed motility of these constructs (Fig. 1c). Our data are consistent with depolymerisation occurring as a result of monomeric motors encountering both MT ends by diffusion from solution. It also supports previous observations that dimeric motor-mediated stepping along the MT lattice is not required for $kinesin-8$-mediated depolymerisation at MT ends.

In the presence of ATP, the average depolymerisation rate by $Pb$ kinesin-8B-MD is $1.3 \pm 0.7$ nm/s and by $Pf$ kinesin-8B-MD is $1.1 \pm 0.7$ nm/s (Fig. 2b). In the presence of the non-hydrolysable ATP analogue, AMPPNP, the depolymerisation is slower—by $Pb$ kinesin-8B-MD it is $0.4 \pm 0.2$ nm/s and $0.5 \pm 0.3$ nm/s by $Pf$ kinesin-8B-MD (Fig. 2b). Faster depolymerisation in the presence of ATP compared to AMPPNP demonstrates that ATPase cycle turnover of $Pb$ kinesin-8B-MD and $Pf$ kinesin-8B-MD is coupled to catalytic MT depolymerisation. During ATP turnover, these motors can interact with MT ends, induce tubulin release, themselves release from this tubulin and thus be recycled for further depolymerisation. The observation that some depolymerisation occurs in the presence of AMPPNP shows that the ATP-binding step of the motor’s ATPase cycle can be sufficient for tubulin release. However, the slower overall depolymerisation without ATP hydrolysis at the same motor concentration suggests the motors could be trapped on depolymerisation products. Consistent with this, we observed formation of tubulin rings and peeling protofilaments when $Pb$ kinesin-8B-MD was incubated with stabilised MTs and AMPPNP but not ATP (Fig. 2c). Such curved structures do not form from stabilised MTs in the absence of $Pb$ kinesin-8B-MD. Similar but more plentiful rings and spirals were also observed on incubation of $Pb$ kinesin-8B-MD with AMPPNP and unpolymerized tubulin (Fig. 2d). Although these oligomers are flexible and heterogeneous, 2D image analysis showed that $Pb$ kinesin-8B-MD molecules bind to curved tubulin dimers around the inner circumference of these rings (Fig. 2e). These observations demonstrate that the ATP-binding step of malaria kinesin-8B motors can induce or stabilise a bent tubulin conformation which drives tubulin release from MT ends, an activity that is not typical of other kinesin-8s.

To further investigate the relationship between MT depolymerisation activity and nucleotide hydrolysis, we prepare a $Pb$ kinesin-8B-MD ATPase inactive mutant, in which the Glu residue in the conserved switch 2 motif (DXXGXE) is mutated to Ala ($Pb$ kinesin-8B-MD E203A). As expected, $Pb$ kinesin-8B-MD E203A exhibited no ATPase activity (Fig. 2f). This mutant was nevertheless able to depolymerise stabilised MTs in the presence of ATP, with a mean rate of $0.7 \pm 0.3$ nm/s (Fig. 2g), which is 65% of the WT + ATP rate (one-way ANOVA; $p < 0.0001$), compared to 31% of the WT + ATP rate observed in the presence of AMPPNP. This further supports the idea that while ATP turnover by Plasmodium kinesin-8Bs is not essential for MT depolymerisation, it supports catalytic MT depolymerisation by these motors.

Individual tubulin dimers have been suggested to share some structural properties with tubulins located at MT ends, so we also measured the tubulin-stimulated ATPase activity of $Pb$ kinesin-8B-MD (Supplementary Fig. 3). Although tubulin was found to stimulate the motor ATPase to some extent, motor turnover is much slower than with MTs ($K_{cat} = 0.1 \pm 0.0$ ATP/s) and its interaction is also weaker ($K_m = 7.6 \pm 2.4 \mu M$). The ratio of MT-stimulated ATPase $K_{cat}$ compared to tubulin is thus much higher (19x) for $Pb$ kinesin-8B-MD than mammalian kinesin-8s ($K_{cat}/K_m$ ratio = 5.0; $K_{cat}/K_m$ ratio = 1.82$^{20,21}$). Although tubulin may not optimally mimic the configuration of the MT end substrate for Plasmodium kinesin-8s ATPase activity, these data suggest that the lattice-based ATPase activity of the parasite motor domains dominates compared to the depolymerisation activity at MT ends.

Nucleotide-dependent structures of lattice-bound Plasmodium kinesin-8Bs

To investigate the mechanistic basis for these activities, we used cryo-EM to determine the structures of $Plasmodium$ kinesin-8Bs bound to GMPCPP MTs (Fig. 3). $Mt$-bound structures of $Pb$ kinesin-8B-MD are two different nucleotide states—no nucleotide (NN) and AMPPNP—were imaged and their structures determined to overall resolutions of $4.3$ and $3.3$ Å, respectively (Table 1; Supplementary Fig. 5). Resolutions in the kinesin motor domain of each reconstruction ranged between $4$ and $8$ Å (Supplementary Fig. 5). MT-bound complexes of $Pb$ kinesin-8B-MD in the absence of nucleotide (NN) were also imaged and used to calculate a reconstruction with an overall resolution of $4.1$ Å with resolution in the kinesin motor domain of $4.4$–$5$ Å (Supplementary Fig. 6a). To facilitate interpretation of these structures, we built molecular models of the motor-MT complexes (Table 2).

All the reconstructions show that $Pb$ kinesin-8B-MD and $Pf$ kinesin-8B-MD contact a single tubulin dimer in the MT lattice, with motor binding centred on the intradimer tubulin dimer interface (Fig. 3; Supplementary Fig. 6b). In the NN structure of $Pb$ kinesin-8B-MD, density corresponding to nucleotide is indeed absent from the nucleotide binding site (NBS) (Fig. 3a, b). The conserved nucleotide binding loops – P-loop, loop 9 (containing the switch 1 motif) and loop 11 (containing the switch 11 motif) – adopt a canonical conformation previously described for the NN state of a number of other plus-end directed kinesins, including human KIF18A$^{19}$. In this conformation, density corresponding to the P-loop is visible in the empty NBS, while loop 11 is retracted from the NBS. The C-terminal end of loop 11 adopts a turn and interacts with α-tubulin before it leads into helix-α4, a major contact point with the MT surface. Density corresponding to loop 9 is visible between the P-loop and loop 11 but is poorly defined. Adjustment of the reconstruction density threshold reveals some evidence of connectivity between loop 9 and both the P-loop and the helical turn of loop 11, supporting the idea that loop 9 is partially flexible in the absence of bound nucleotide. Consistent with this conformation of the NBS, on the opposite side of the kinesin motor domain, helix-α6 abuts the C-terminal end of helix-α4 and density corresponding to the beginning of the C-terminal neck linker peptide of $Pb$ kinesin-8B-MD is visible protruding towards the MT minus end and adjacent to the P-lobe (Fig. 3c, d). The N-terminal peptide of the motor can also be visualised protruding in the opposite direction towards the MT-plus.
end. The NN *P* kinesin-8B-MD reconstruction is very similar to that of *P* kinesin-8B-MD (Supplementary Fig. 6b, c), including the empty NBS and undocked neck linker. Overall, the configuration of MT-bound NN *P*lasmodium kinesin-8Bs in these conserved parts of their motor domains are similar to a number of NN states of other plus-end directed motors.\(^{19,24-26}\)

Surprisingly, the structure of MT-bound *P* kinesin-8B-MD in the presence of AMPPNP is overall similar to that of the NN state (Fig. 3e, Supplementary Movie 1.2). While density corresponding to bound nucleotide is clearly present (Supplementary Fig. 7), the overall open configuration of the NBS is very similar to that in the motor's NN conformation (Fig. 3e, f). Consistent with this, helix-α6a gains but the C-terminal end of helix-α4 and no neck linker docking is observed (Fig. 3g). However, differences in EM density are visible between the 2 nucleotide states such that density corresponding to a number of loops around the motor domain—including loop 2, loop 9, loop 11 and...
the neck linker—are more flexible in the presence of bound AMPPNP and, as a consequence, were not included in the AMPPNP model (Fig. 3f, h dashed lines). Because this variation in density is not attributable to resolution differences, we conclude that it reflects small structural adjustments of the motor domain to the bound nucleotide, but these are not converted into larger conformational changes. As a result, overlay of the NN and AMPPNP models of \( \text{Pb} \) kinesin-8B-MD (aligned on helix-\( \alpha \)4) shows only minor structural variations around the NBS, \( \beta \)-lobe and in helix-\( \alpha \)6 (Fig. 3i). Such changes are very small compared to the changes that have been observed in the NN-AMPPNP transition in other plus ended kinesins\(^{25-28}\).

MT-binding interface of \textit{Plasmodium} kinesin-SBs and distinct contributions of interface regions to \textit{Pb}kinesin-8B-MD function

The interaction between \textit{Plasmodium} kinesin-SBs and \( \alpha \)- and \( \beta \)-tubulin is centred on helix-\( \alpha \)4 (Fig. 3, Fig. 4a, Supplementary Fig. 6). Contacts are also formed with \( \beta \)-tubulin by the C-terminal part of loop 12, helix-\( \alpha \)5 and \( \beta \)-lobe/loop 8, and between \( \alpha \)-tubulin and helix-\( \alpha \)6 (Fig. 4a). The MT interaction in all these regions is not detectably different between the \( \text{Pb} \)kinesin-8B-MD-NN and AMPPNP reconstructions (Supplementary Fig. 8a). These elements are well-conserved points of MT contact in kinesins from different families\(^{24,26-29}\), although loop 12 often exhibits family-specific insertions, including in \textit{Plasmodium}...
Fig. 3 | Cryo-EM reconstructions of MT-bound Ptkinesin-8B-MD. a Asymmetric unit of GMPCPP-MT-bound NN Ptkinesin-8B-MD as solid surface towards NBS (threshold = 0.0322). Ptkinesin-8B-MD-NB is dark green, α/β-tubulin dark/light grey, respectively; region around NBS depicted in b is boxed. b Zoom-in of NN Ptkinesin-8B-MD NBS with docked model, showing contact formed between the helical turn (arrow) in loop 11 (yellow) and α-tubulin, P-loop (orange) in the empty NBS, and density corresponding to flexible-appearing loop 9 (blue). Ptkinesin-8B-MD-NN model is dark green, α/β-tubulin dark/light grey, respectively. c MT-bound NN Ptkinesin-8B-MD as solid surface towards the neck linker region (threshold = 0.0322). Ptkinesin-8B-MD-NB is dark green, α/β-tubulin are dark/light grey respectively; region around the neck linker depicted in d is boxed. d Zoom-in of NN Ptkinesin-8B-MD-NN neck linker region with docked model, showing density corresponding to loop 12 (fuchsia) at C-terminus of helix-α4 that contacts β-tubulin, adjacent to which is density corresponding to the N-terminal end of the neck linker (red), which is directed towards the MT minus end. e Asymmetric unit of GMPCPP-MT-bound AMPPNP Ptkinesin-8B-MD as solid surface towards NBS (threshold = 0.0249). Ptkinesin-8B-MD-AMPPNP is light green, α/β-tubulin dark/light grey respectively; region around NBS depicted in f is boxed. f Zoom-in of AMPPNP Ptkinesin-8B-MD NBS with docked model, showing weaker loop 11 density (dashed yellow line), the P-loop (orange) adjacent to density corresponding to AMPPNP in NBS and density corresponding to flexible-appearing loop 9 (dashed blue line). AMPPNP Ptkinesin-8B-MD model is light green, α/β-tubulin dark/light grey, respectively. g MT-bound AMPPNP Ptkinesin-8B-MD depicted as solid surface towards the neck linker region (threshold = 0.0249). Ptkinesin-8B-MD-AMPPNP is light green, α/β-tubulin are dark/light grey respectively; region around the neck linker depicted in h is boxed. h Zoom-in of AMPPNP Ptkinesin-8B-MD neck linker region with docked model, showing density corresponding to loop 12 (fuchsia) at the C-terminus of helix-α4 that contacts β-tubulin, and weaker neck linker density (red dotted line), directed towards the MT minus end. I Ca RMsD (Å) of Ptkinesin-8B-MD-NB compared to AMPPNP models aligned on helix-α4 of Ptkinesin-8B-MD, depicted on the NN model; the small range of RMSD observed illustrates that only minor structural changes are detected when AMPPNP binds.

Table 1 | Cryo-EM data collection, 3D image processing statistics

| Data collection and reconstruction | Ptkinesin-8B-MD-NN | Ptkinesin-8B-MD-AMPPNP | Ptkinesin-8B-MD-NN |
|-----------------------------------|--------------------|------------------------|--------------------|
| Grid type | C-Flat 2/2-4 C | C-Flat 2/2-4 C | C-Flat 2/2-4 C |
| Microscope | Polara | Krios | Krios |
| Detector and mode | K2 counting mode | K2 counting mode | K2 counting mode |
| Collection software | Serial EM | EPU | EPU |
| Magnification | 160 K | 130 K | 130 K |
| Voltage (Kv) | 300 | 300 | 300 |
| Electron exposure (e/Å²) | 50.81 | 47.11 | 46.50 |
| Exposure time (s) | 15 | 8 | 8 |
| Dose rate (e/pixel/s) | 6.54 | 6.80 | 6.71 |
| Total frame | 50 | 32 | 32 |
| Fraction dose (e/Å²) | 1.02 | 1.47 | 1.45 |
| Defocus range (µm) | ~0.5 to ~2.5 | ~0.5 to ~2.5 | ~0.5 to ~2.5 |
| Pixel size (Å) | 1.35 | 1.05 | 1.05 |
| Particle number for final reconstruction | 196,084 | 87,906 | 205,687 |
| Map resolution (Å, FSC 0.143) | 4.3 | 3.3 | 4.1 |
| Local resolution range (Å) | 4.2–7.6 | 3.3–6.5 | 3.6–5 |
| B factor | ~120 | ~58 | ~150 |

Table 2 | Model building statistics

|                      | Ptkinesin-8B-MD-NN | Ptkinesin-8B-MD-AMPPNP | Ptkinesin-8B-MD-NN |
|----------------------|--------------------|------------------------|--------------------|
| **Global cross-correlation** | | | |
| Homology model | 0.89 | 0.91 | \ |
| Final model | 0.91 | 0.94 | 0.89 |
| **QMEAN** | | | |
| Homology model | ~2.94 | ~2.94 | \ |
| Final model | ~0.08 | ~0.05 | ~0.25 |
| **MolProbity** | | | |
| Homology model | 3.45 | 3.45 | \ |
| Final model | 0.93 | 0.96 | 1.8 |
| RMS deviations bound length (Å) | 0.0169 | 0.202 | 0.0163 |
| RMS deviations bound angles (°) | 1.65 | 3.38 | 1.63 |
| Clashscore | 1.09 | 1.21 | 12.37 |
| Poor rotamer (%) | 0.32 | 0.36 | 0.32 |
| Ramachandran outliers (%) | 0.29 | 0.33 | 0.29 |
| Ramachandran favoured (%) | 97.39 | 97.34 | 96.81 |

kinesin-8Bs (Fig. 4b),35,36. In both *Plasmodium* kinesin-8Bs NN reconstructions, there is an additional connection between α-tubulin and loop 2, which protrudes from the β-lobe of the motor domain and appears to adopt a partially helical configuration (Fig. 3a, d, Fig. 4a). In the Ptkinesin-8B-MD-AMPPNP reconstruction, the density corresponding to loop 2 is less distinct due to the above described motor domain flexibility, although at more inclusive density thresholds, connectivity with the MT surface is also visible (Supplementary Fig. 8b). *Plasmodium* kinesin-8B loop 2 is the same length as, and relatively well conserved compared to, loop 2 in mammalian kinesin-8B KIF19A (Supplementary Fig. 9a, b), although shorter compared to loop 2 in the mammalian kinesin-8A KIF18A (Supplementary Fig. 9a). Most kinesin-8 proteins so far characterised form an additional MT contact via loop 221,21, a characteristic we now show *Plasmodium* kinesin-8Bs also share.

To test the functional contributions of distinct MT contact regions of Ptkinesin-8B-MD, we engineered mutants in loop 2 and loop 12. These loops are shorter in kinesin-1 compared to canonical kinesin-8 (Fig. 4b, c), and we spliced the shorter loops of human kinesin-1 (KIF5B) into the Ptkinesin-8B-MD sequence. Both loop substitution mutants exhibited ~40–50% of the WT ATPase activity, with Kcat-Ptkinesin-8B-MD-L2ΔThr = 0.9 ± 0.1 ATP/s and Kcat-Ptkinesin-8B-MD-L12ΔThr = 0.8 ± 0.3 ATP/s compared to Kcat-WT = 1.9 ± 0.2 ATP/s. Both mutants also exhibited a higher Kₘ,MT compared to...
Fig. 4 | The MT-binding interface of Pbkinesin-8B-MD and contributions to motor function. a Middle, ribbon depiction of the Pbkinesin-8B-MD and tubulin dimer NN state model, with Pbkinesin-8B-MD in green, α-tubulin in dark grey and β-tubulin in light grey; left, zoomed view of the Pbkinesin-8B-MD MT-binding surface coloured according to contacts with α-tubulin (dark grey) and β-tubulin (light grey); right, MT footprint of Pbkinesin-8B-MD on α- and β-tubulin indicated in dark green (tubulin residues <5 Å distance from the bound motor). Labels indicate the specific contacting secondary structure elements in tubulin dimer and Pbkinesin-8B-MD(in bracket). b Structural alignment of the Pbkinesin-8B-MD model (green) and KIF5B motor domain model (PDB 6OJQ, white), focusing on the loop 12, with the Pbkinesin-8B-MD-NN cryo-EM density shown in mesh representation. A sequence alignment of this region, and the sequence of the swap mutant, is depicted below. Pbkinesin-8B-MD loop 12 is coloured pink with positively charged residues coloured blue. Negatively charged residues in the adjacent H12 of β-tubulin are coloured red; c Structural alignment of the Pbkinesin-8B-MD model (green) and KIF5B motor domain model (PDB 6OJQ, white), focusing on loop 2, with the Pbkinesin-8B-MD loop 2 is coloured purple with positively charged residues coloured blue, negatively charged residues coloured red and hydrophobic residues coloured orange. Negatively charged residues in the adjacent H12 of α-tubulin are also coloured red, indicating the potential electrostatic interactions between loop 2 and the MT surface. d GMPCPP-MT-stimulated ATPase activity of Pbkinesin-8B-MD, Pbkinesin-8B-MD-L2KIF5B and Pbkinesin-8B-MD-L12KIF5B. Data (n = 3 for each point, mean ± SD) was fitted using Michaelis-Menten equation, from which the kcat and Km were calculated in Prism9; e Paclitaxel-stabilised-MT depolymerisation rate (nm/s) for Pbkinesin-8B-MD, Pbkinesin-8B-MD-L2KIF5B and Pbkinesin-8B-MD-L12KIF5B in the presence of ATP. Error bars represent the mean ± SD and individual measurements are also plotted. Ordinary one-way ANOVA was performed in Prism. Significance values are displayed as asterisks, ****p-values < 0.0001; ns not significant, p = 0.5029. Npbkinesin-8B-MD = 97 ends, Npbkinesin-8B-MD-L2KIF5B = 86 ends, Npbkinesin-8B-MD-L12KIF5B = 100 ends, Nno-kinesin-8B = 85 ends.
motors

The importance of the kinesin-8B neck linker sequence for all of these Pf and Pb lysates of Pf1.2 ± 0.5 activity (Fig. 5c). Furthermore, the MT depolymerisation activity of Pf kinesin-8B-MD was also much reduced compared to WT and was not significantly different from the no-kinesin control (Fig. 4e). Surprisingly, Pf kinesin-8B-MD-L2KIF5B retained MT depolymerisation activity, albeit slower than WT. This was reinforced by the fact that, on incubation of this mutant with tubulin and AMPPNP, tubulin rings with dimensions indistinguishable from those of WT Pf kinesin-8B-MD were observed using negative stain EM (Supplementary Fig. 9c, d). In contrast, while a few tubulin rings were observed in the presence of the Pf kinesin-8B-MD-L12KIF5B mutant, they did not exhibit the double-layer appearance arising from stable association of the motor construct with individual tubulin dimers around the ring (Fig. 2e); this is presumably because of this mutant’s low apparent affinity for tubulin/MTs (Fig. 4d). Taken together, these data show that loop 2 of Pf kinesin-8B-MD is not required for the specific interaction and stabilisation of curved tubulin that is correlated with MT depolymerisation activity but that stable association with tubulin—which is disrupted in the Pf kinesin-8B-MD-L12KIF5B mutant—is required for depolymerisation activity.

The role of the kinesin neck linker in kinesin-SB-MD function

We also investigated the contribution of the kinesin-SB neck linker to motor function and compared the activities of Pf kinesin-8B-MD and Pf kinesin-8B-MD with and without (Pf kinesin-8B-MDΔNL and Pf kinesin-SB-MDΔNL) their C-terminal neck linkers (Fig. 5a). In the ATPase assay, while the Km of Pf kinesin-8B-MDΔNL (1.8 ± 1.0 μM) is only slightly lower than that of Pf kinesin-8B-MD (2.5 ± 0.6 μM), its Kcat was substantially reduced, at 0.3 ± 0.1 A/μs compared to 1.9 ± 0.2 A/μs (Fig. 5b). Likewise, the Km, MT and Kcat of Pf kinesin-8B-MDΔNL are also lower than that of Pf kinesin-8B-MD (Km,MT: 0.5 ± 0.0 μM vs 1.2 ± 0.5 μM; Kcat: 0.5 ± 0.1 A/μs vs 1.4 ± 0.2 A/μs) (Fig. 5b). Neither Pf kinesin-8B-MDΔNL nor Pf kinesin-SB-MDΔNL generated MT gliding activity (Fig. 5c). Furthermore, the MT depolymerisation activity of both of these constructs was significantly lower than Pf kinesin-8B-MD and Pf kinesin-SB-MD (Fig. 5d). Together, these data demonstrate the importance of the kinesin-SB neck linker sequence for all of these motors’ functions.

Kinesin-SB interaction partners in parasites

Finally, to begin to understand the cellular context in which Pf kinesin-8B performs its multi-tasking functions, we immunoprecipitated endogenously expressed Pf kinesin-8B-GFP from parasite lysate and identified potential native interacting partners using mass spectrometry (Supplementary Fig. 10). Specifically, we used lysates of P. berghei gametocytes 6 min after activation because of the high expression of, and functional relevance for, the motor at this parasite life cycle stage. Proteomic analysis of these samples identified a number of microtubule-associated proteins that are linked with male gamete maturation, axoneme formation and function, and are expressed specifically in male gametocytes, including dynein heavy chain, kinesin-13, calcium-dependent protein kinase 4 and PF16 (Supplementary Fig. 10a, b). These observations are consistent with the inferred role of Pf kinesin-8B based on disruption of male gamete formation in knockout parasites. Detailed dissection of the functional significance of these interactions and their perturbation by specific mutation of the motor domain suggested by our structural work will be important future directions of study.

Discussion

Kinesin-Ss are among the most widely distributed kinesin subfamilies across eukaryotes, perhaps because of their functional adaptability to both move processively along MTs and to influence MT dynamics. To understand the molecular basis of P. berghei kinesin-SB function in parasite transmission, we characterised its motor domain and compared it with kinesin-SB from P. falciparum. Our biochemical and structural data provide evidence of conserved and precisely tuned mechanochemistry in these motors, which is distinct compared to other kinesin-Ss characterised to date, including those in the parasite’s mammalian hosts.

Pf kinesin-8B-MD and Pf kinesin-8B-MD behave very similarly to each other and share the ability with other kinesin-Ss—including kinesin-SX from P. berghei and P. falciparum—to drive ATP-dependent plus-end directed MT gliding (Fig. 1). While the MT substrates used in the ATPase assay for Pf kinesin-8B-MD and Pf kinesin-8B-MD were stabilised in different ways (Fig. 1b), the gliding velocities for each motor on the non-polarity-marked paclitaxel-MTs and the polarity-marked GMPCPP MTs are not statistically significantly different (Supplementary Fig. 2), and suggest that Plasmodium kinesin-SB ATPase activity is not detectably sensitive to different modes of in vitro MT stabilisation. Pf kinesin-8B-MD and Pf kinesin-8B-MD both require the neck linker sequence for this activity (Fig. 5c), consistent with models of plus-end directed kinesin motility. Pf kinesin-8B-MD and Pf kinesin-8B-MD also depolymerised stabilised MTs and, as monomeric constructs, access both MT ends via diffusion and depolymerise them. Depolymerisation by both Pf kinesin-8B-MD and Pf kinesin-8B-MD, as well as mammalian kinesin-SB (also called KIF19A) is faster in the presence of ATP—i.e. it is catalytic (Fig. 2). Catalytic depolymerisation was also observed by Plasmodium kinesin-SXs. In contrast, S. cerevisiae Kip3, a kinesin-SA, MT depolymerisation is linked to suppression of motor ATPase activity and in the case of HsKIF19A M (another kinesin-SA), depolymerisation is more robust in the presence of the non-hydrolysable ATP analogue AMPPNP than ATP. This suggests that kinesin-8s and other kinesin-Ss is that kinesin-8As exhibit non-catalytic depolymerase activity. In different functional contexts, this may manifest more as modulation of MT dynamics rather than MT depolymerisation per se. Characterisation of kinesin-8s from a range of organisms is required to solidify this distinction.

We used cryo-EM to determine the MT-bound NN and AMPPNP structures of Pf kinesin-8B-MD and of NN Pf kinesin-8B-MD (Fig. 3). Only a few other structures of motor domains from kinesin-8s have been determined to date, and we compared our Plasmodium kinesin-SB reconstructions to Hs KIF19A_MD, for which the most comparable experiments were performed. In the NN reconstructions, the overall MT-binding footprint of both Pf kinesin-8B-MD and Pf kinesin-8B-MD are essentially indistinguishable from KIF19A_M at the resolutions of the available reconstructions. One of the distinctive features of kinesin-Ss compared to other plus-end directed kinesins is an extended loop 2. In both Pf kinesin-8B-MD and Pf kinesin-8B-MD, density corresponding to loop 2 is clear, well-structured and contacts the C-terminal end of α-tubulin. Loop 2 of Hs KIF19A_MD, which is 28 amino acids longer, contacts the MT surface at a similar site—however, even when contacting the MT surface, it is flexible and lacks a clearly defined structure, and is thereby distinct from the shorter and structurally well-defined loop 2 of kinesin-Ss visualised to date. In both KIF19A, S. cerevisiae and C. albicans Kip3, loop 2 residues are not absolutely required for MT depolymerisation activity, but do contribute to motor processivity, MT-plus-end residence time or MT depolymerisation efficiency, respectively. In contrast, loop 2 residues are crucial for KIF19A depolymerase activity. We show, however, that elimination of the Pf kinesin-8B-MD loop 2 sequence
reduces MT affinity and depolymerase activity but does not eliminate them. We also note that there is no evidence for the role of loop1 in Plasmodium kinesin-8Bs in mediating cooperativity as has been recently described for C. albicans Kip3—continuous density for this short loop in Plasmodium kinesin-8Bs proximal to the motor domain core is seen in all our reconstructions, in clear contrast to the extended sequence seen in C. albicans Kip3. In summary, while the extended nature of kinesin-8 loop 2 sequences likely contributes to their phylogenetic co-classification and can form an additional contact point with the MT surface, this region differently modulates motor function in different kinesin-8s.

A further striking difference between MT-bound Pbkinesin-8B-MD and HskIF18A_MD is its structurally minimal response to AMPPNP binding (Fig. 3i). In contrast, AMPPNP binding to HskIF18A_MD induces rearrangements within the motor domain that support neck linker docking towards the MT-plus end (maximum RMSD = 17.4°).
The minimal structural response of Plkinesin-8B-MD is surprising given the neck linker dependence of MT gliding activity by this motor (Fig. 5c) and its relatively conserved neck linker sequence (Supplementary Fig. 10). It is possible that the observed small shift in the P-loop domain on AMPNNP binding (Fig. 3d) is sufficient to bias the neck linker towards the MT-plus end and thereby support ATP-driven MT gliding (Fig. 1c). However, the characteristic hydrolysis-competent ‘closed’ NαS conformation7 is not observed in our AMPNNP reconstruction; it is thus also possible that AMPNNP as an analogue does not induce motility-relevant conformational changes in MT lattice-bound Plkinesin-8B-MD. AMPNNP binding is, however, sufficient to stabilise tubulin in a curved conformation and induce depolymerisation at MT ends (Fig. 2). Two recent studies of fungal Kip3s reported similar overall observations38–39; AMPNNP binding did not induce canonical conformational changes in lattice-bound motors, but observations of curved tubulin oligomers or molecular dynamics simulations indicated that such an ATP-dependent canonical conformational change would occur at MT ends. Furthermore, a motility-relevant conformation was observed in C. albicans MT-bound Kip3 motor domain in the presence of an ADP.Pr-like analogue (ADP.AfX)40, reinforcing the sensitivity of kinesin mechanochemistry to both underlying MT substrate and bound nucleotide. However, a crucial difference in the behaviours observed is that AMPNNP binding to S. cerevisiae Kip3 does not induce MT depolymerisation (not reported for C. albicans), further emphasising that the precise mechanochemistry of these motors is distinct38–40.

Intriguingly, a minimal structural response to AMPNNP binding by lattice-bound kinesin-13s has also been observed10, but AMPNNP binding does induce depolymerisation at MT ends, kinesin-13s are well-conserved MT catastrophe factors with regulatory roles in both interphase and dividing cells41. In contrast to kinesin-8s, however, kinesin-13s do not take steps along the MT lattice but diffuse to either MT end to stimulate MT depolymerisation, an activity that depends on the motor ATPase42. In addition—and as is the case for Plkinesin-8B-MD, PfKinesin-8B-MD and KIF19A–kinesin-13s are catalytic depolymerases43,44. Despite the mechanistic differences with respect to lattice-based stepping, we hypothesised that the minimal response by lattice-bound kinesin-13s and Plkinesin-8B-MD to AMPNNP binding could reflect a distinct mechanochemical sensitivity of catalytic depolymerases to the underlying tubulin substrate. Intriguingly, when motor domain structures of NN MT-bound Plkinesin-8B-MD, HskKIF15A-MD and the MT-bound Drosophila melanogaster kinesin-13 KLP10A-MD (DmKLP10A-MD) are overlaid by alignment on their tubulin-binding subdomains (Fig. 6a), the position of the rest of Plkinesin-8B-MD (by relative angle between α-helices and helix-c4) is more similar to DmKLP10A-MD than to HskKIF15A-MD (Fig. 6b). Thus, while at the primary sequence level, kinesin-8s are more similar to each other (as expected from their family classification—Supplementary Fig. 11), the structural comparison of motor domains, suggests that the configuration of Plasmodium kinesin-8Bs—and we speculate kinesin-8Bs more generally—shares some features with kinesin-13s and specifies their mechanochemistry (Fig. 6c).

Taken together, these data suggest that the motor activity of Plasmodium kinesin-8Bs has evolved to be both motile and capable of catalytic MT depolymerisation (Fig. 6d). ATPase-dependent conformational changes in lattice-bound motors—probably not all of which were captured in our current study—bias motor movement towards the MT-plus end (Fig. 6d, step 1–3). At the MT end, larger conformational changes are enabled and drive catalytic ATP-dependent depolymerisation (Fig. 6d, step 4). In parasites, the context in which the kinesin-8B motor domain operates is likely to influence this finely tuned activity—in the context of the full-length motor, when interacting with Plasmodium tubulin11 and in the cellular environment, potentially modulated by binding partners and cellular regulators12,13.

**Methods**

**Ethics statement**

The animal work required to prepare gametocyte material passed an ethical review process by the Animal Welfare and Ethical Review Body of the University of Nottingham and was approved by the United Kingdom Home Office. Work was carried out under UK Home Office Project Licenses 657 (30/3248 and PDD2D5182) in accordance with the UK Animals (Scientific Procedures) Act 1986. Six- to eight-week-old female CD1 outbred mice from Charles River laboratories were used for all experiments. Mice were kept with a 12 h light/12 h dark cycle (07:00–19:00), ambient temperature was between 20 and 24 °C and ambient humidity was between 40 and 60%.

**Molecular cloning**

DNA encoding the motor domain of Plkinesin-8B (PBANKA_020270), residues 760–1130, referred to as Plkinesin-8B-MD, was codon optimised, synthesised (Gene Oracle, Inc.) and cloned into the pNLC28-Bsa4 vector (Structural Genomics Consortium, Oxford, UK) using ligation independent cloning. Plkinesin-8B-MDΔNL (residues
760-1115) was prepared by introduction of a stop codon using site-directed mutagenesis after the codon for residue 1115. For the Pb kinesin-8B-MD-SNAP construct and Pb kinesin-8B-MD ΔNL-SNAP construct, Gibson assembly (NEB E2621) was used to insert a SNAPf-tag at the C-termini of these constructs. A flexible linker of (GGGS)2 was added immediately before the C-terminal SNAPf-tag of Pb kinesin-8B-MD ΔNL-SNAP construct. The Pb kinesin-8B-MDE1023A was generated using site-directed mutagenesis. The Pb kinesin-8B-MD_L2KIF5B construct was generated by replacing the loop 2 sequence (LDPSDNTDNVLRQNRTKE) in Pb kinesin-8B-MD with the corresponding sequence (AS) from human KIF5B using Gibson assembly (NEB E2621). Similarly, the Pb kinesin-8B-MD_L12KIF5B construct was generated by replacing the loop 12 sequence (SRSKGTSKSNFIPF) of Pb kinesin-8B-MD with the corresponding sequence (EGSTYVPY) from human KIF5B using Gibson assembly. For Gibson assembly, the insert DNA fragments for Pb kinesin-8B-MD ΔNL-SNAP, Pb kinesin-8B-MD_L2KIF5B and Pb kinesin-8B-MD_L12KIF5B constructs were synthesised (Eurofins genomics), and the insert DNA fragment for Pb kinesin-8B-MD-SNAP and all vector fragments were produced using PCR. Primer sequences are provided in the Supplementary Data.

DNA encoding the motor core of Pf kinesin-8B (PF3D7_0111000), residues 917–1265, referred to as Pf kinesin-8B-MDΔNL, was codon optimised, synthesised (Gene Oracle, Inc.) and cloned into the pNIC28-Bsa4 vector using ligation independent cloning. The putative neck linker sequence, residues 1266–1276, was incorporated into this construct using Gibson assembly and referred to as Pf kinesin-8B-MD. Insertion of a SNAPf-tag at the C-terminus of the constructs was also performed using Gibson cloning (NEB E2621). Due to poor solubility of Pf kinesin-8B-MD_SNAP on expression in E. coli, an N-terminal NusA solubility tag59 was also incorporated into this construct, C-terminal of the 6xHis tag but N-terminal of the TEV protease cleavage site so that it could be removed during purification. PCR was used to generate the insert DNA fragment and vector DNA fragment for all constructs. Primer sequences are provided in the Supplementary Data.
Protein expression

For expression of all Pf kinesin-8B proteins, expression plasmids were transformed into BL21 Star™ DE3 E. coli competent cells (Invitrogen C601003). Cells were grown to OD 0.6–0.8 at 37 °C then induced with 20 μM IPTG. After induction, the temperature was lowered to 20 °C and cells were incubated overnight before pelleting by centrifugation at 6235 × g for 20 min.

For expression of Pf kinesin-MD and Pf kinesin-MDΔNL proteins, expression plasmids were transformed into BL21 Star™ DE3 E. coli competent cells (Invitrogen C601003). Cells were grown to OD 0.6–0.8 at 37 °C then induced with 1 mM IPTG. After induction, the temperature was lowered to 26 °C and cells were incubated overnight before pelleting by centrifugation at 6235 × g for 20 min. For expression of Pf kinesin-MDΔNL, proteins were collected, concentrated, snap frozen and stored at 18 °C or 26 °C, respectively, overnight post-induction.

Protein purification

For purification of all Pf kinesin-8B-related proteins, pelleted cells were resuspended in lysis buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 5 mM MgCl2 with EDTA-free protease inhibitor (Roche 5056489001)), sonicated using 25% amplitude and pulse of 5 s on and 5 s off for 2 min with 30 min each interval and then centrifuged at 48,384 × g, 4 °C for 30 min. The His₆-tagged proteins were purified using Immobilised Metal Affinity Chromatography (IMAC) with 2 mL Ni-NTA His-Bind® Resin (Merck 70666), followed by incubation with TEV protease for 12 h at 4 °C to remove the His₆ tag. The protein was exchanged into low-salt buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl2) and subjected to a further reverse-IMAC step, followed by application to HiTrap Q HP IEX column (GE Healthcare) to remove any residual bacterial proteins, which bind to the Q column. The Q column flow-through was collected and concentrated using Amicon Ultra-0.5 ml Centrifugal Filters (Millipore UFC501024) then separated into single-use aliquots, snap frozen in liquid nitrogen and stored at −80 °C. In the case of Pf kinesin-MD-L23614 and Pf kinesin-8B-MDΔNL, proteins were collected, concentrated, snap frozen and stored right after the reverse-IMAC step.

For purification of Pf kinesin-8B proteins, pelleted cells were resuspended in lysis buffer (50 mM Tris pH 7.0, 400 mM NaCl, 2 mM MgCl₂, 1 mM ATP, 2 mM beta-mercaptoethanol) together with TEV protease treatment to cut both His₆ and NusA tags. The Pf kinesin-MD-MDΔNL, proteins were collected and concentrated to around 30 μM using Amicon Ultra-0.5 ml Centrifugal Filters (Millipore UFC501024) then separated into single-use aliquots, snap frozen in liquid nitrogen and stored at −80 °C.

MT polymerisation

For assays, porcine brain tubulin was purchased as a lyophilised powder (Cytoskeleton, Inc. T240) either unlabelled, X-rhodamine-labelled or biotinylated. The protein was solubilized in BRB80 buffer (80 mM Pipes-KOH pH 6.8, 1 mM EGTA, 1 mM MgCl₂) to ~10 mg/ml (tubulin dimer concentration).

Paclitaxel-stabilised MTs. Reconstituted tubulin was polymerised at 5 mg/ml final concentration in the presence of 5 mM GTP at 37 °C for 1 h. After this, a final concentration of 1 mM paclitaxel (Calbiochem S80555) dissolved in DMSO was added and the MTs incubated at 37 °C for a further 1 h.

GMPCPP-stabilised MTs. GMPCPP MTs were prepared using a double-cycling protocol as follows to maximise GMPCPP occupancy. Reconstituted tubulin was polymerised at 5 mg/ml final concentration in the presence of 1 mM GMPCPP at 37 °C for 1 h. Polymerised MTs were pelleted at 313,000 × g for 10 min at 25 °C using TLA100 rotor (Beckman Coulter), and the pellet was washed with BRB80 buffer. The MT pellet was then resuspended with BRB80 buffer, followed by incubation on ice for 20 min to depolymerise the MTs. The mix were incubated on ice for another 5 min with an additional 1 mM GMPCPP. The reaction mix was then incubated at 37 °C for 30 min.

For ATPase assays, Paclitaxel- or GMPCPP-stabilised MTs were polymerised as above, and free tubulin was removed by pelleting the MTs by centrifugation at 313,000 × g for 10 min at 25 °C using TLA100 rotor (Beckman Coulter) through a sucrose cushion, the supernatant removed and the MT pellet was resuspended in BRB80 buffer. Protein concentration was determined using a Bradford assay.

For depolymerisation assays, paclitaxel-stabilised MTs containing 10% X-rhodamine-labelled (Cytoskeleton TL620M) and 10% biotinlabelled tubulin (Cytoskeleton T333P) were polymerised as above and were left at room temperature for 48 h before use in the TIRF assay.

Paclitaxel-stabilised MTs and GMPCPP-polarised MTs were used in the gliding assay. Paclitaxel-MTs containing 10% X-rhodamine-labelled tubulin were polymerised as above and left for 48 h at room temperature before use in a TIRF assay. To prepare polarised MTs to detect gliding directionality, long “dim” MTs were first polymerised by mixing X-rhodamine-labelled tubulin and unlabelled tubulin at a 1:9 ratio to a final concentration of 2 mg/ml. This mix was incubated at 37 °C for 2 h in the presence of 0.5 mM GMPCPP. MTs were then pelleted by centrifugation at 17,000 × g in a bench-top centrifuge for
15 min. To add bright plus end caps to the MTs, X-rhodamine-labelled tubulin and unlabelled tubulin were mixed in a 1:1 ratio. The unlabelled tubulin in this reaction had been previously incubated with 1 mM N-ethyl maleimide (NEM) on ice for 10 min, followed by incubation with 8 mM beta-mercaptoethanol on ice for 10 min to block growth from the MT minus end. This “bright” mix was pre-warmed then added to the polymerised long, dim MTs and incubated at 37 °C for 15 min. MTs were pelleted by centrifugation and resuspended in BRB80 with 40 μM paclitaxel.

**MT- and tubulin-stimulated ATPase assay**

MT/tubulin-stimulated kinesin ATPase activity was measured using an NADH-coupled assay. The assay was performed using 250 nM PbKinesin-8B-MD or PbKinesin-8B-MD titrated with paclitaxel-stabilised MTs (Pf), GMPCPP MTs (Pb) or tubulin dimers(PB) in 100 μl ATPase reaction buffer containing an ATP regeneration system: For PbKinesin-8B-MD: BRB80 buffer, 5 mM ATP(Sigma), 5 mM phosphoenolpyruvate (PEP), 2 mM NADH, 12 U pyruvate kinase and 16.8 U lactate dehydrogenase; for PbKinesin-8B-MD: BRB80 buffer, 5 mM ATP(Sigma), 50 mM NaCl, 5 mM phosphoenolpyruvate (PEP), 2 mM NADH, 12 U pyruvate kinase and 16.8 U lactate dehydrogenase. NADH depletion was monitored by the decrease in absorbance at 340 nm in a SpectraMax M5 plate reader at 26 °C operated by SoftMax Pro 5 software. The Michaelis-Menten equation was used for curve fitting of the ATPase data using Prism 9. To compare ATPase rates between PbKinesin-8B-MD and its mutants, all PbKinesin-8B proteins were buffer exchanged to BRB80 buffer before use in the ATPase assay and the assay was performed in BRB80 buffer containing the above ATP regeneration system without addition of any NaCl.

**MT gliding assay**

SNAP-tagged kinesin-8B-MD proteins (20 μM) were biotinylated in 50 μl reaction volumes by incubating with 40 μM SNAP-biotin (NEB S9110) at 4 °C overnight. Proteins were purified from excess SNAP-biotin by size-exclusion chromatography on a Superdex 75 Increase 3.2/300 column using an AKTA micro system (GE Healthcare) in gel filtration buffer (20 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM MgCl2, 1 mM DTT). Peak fractions were pooled, snap frozen in liquid nitrogen and stored at −80 °C.

Flow chambers for Total Internal Reflection Fluorescence (TIRF) microscopy were made between glass slides, biotin-PEG coverslips (Microsurfaces Inc.), and double-sided tape. Chambers were sequentially incubated with: (1) blocking solution (0.75 % Pluronic F-127 (Sigma P2443), 5 mg/ml casein (Sigma C7078) for 5 min, followed by two washes with assay buffer (BRB80 buffer, 1 mM DTT and 20 μM paclitaxel); (2) 0.5 mg/ml neutralavidin (Invitrogen™ A2666) for 2 min, followed by two washes with assay buffer (BRB80 buffer, 1 mM DTT and 20 μM paclitaxel); (3) biotinylated kinesin-8B-MD, incubated for 2 min, followed by two washes with assay buffer supplemented with 1 mg/ml casein; (4) the reaction mixture containing 5 mM ATP together with 10% X-rhodamine-MTs (or polarity-marked GMPCPP MTs to determine directionality) in assay buffer supplemented with an oxygen scavenging system (20 mM glucose, 300 μg/ml glucose oxidase (Sigma G2133), 60 μg/ml catalase (Sigma C40)).

An Eclipse Ti-E inverted microscope was used with a CFI Apo TIRF 1.49 N.A. oil objective, Perfect Focus System, H-TIRF module, LU-N4 laser unit (Nikon) and a quad band filter set (Chroma). Movies were collected at 26 °C under illumination at 561 nm for 10 min with a frame taken every 2 s with 100 ms exposure on a iXon DU888 Ultra EMCCD camera (Andor), using the NIS-Elements AR Software (Nikon). The gliding rates of single MTs were measured from kymographs using Fiji software.

**MT depolymerisation assay**

Flow chambers were treated and incubated with blocking solution and washed twice with assay buffer (BRB80 buffer, 1 mM DTT and 20 μM paclitaxel), followed by incubation with 0.5 mg/ml neutralavidin and two washes with assay buffer as above in MT gliding assay. 1:100 dilution of X-rhodamine and biotin-labelled paclitaxel-stabilised MTs were flowed into the chamber and incubated for 2 min, followed by two washes with assay buffer supplemented with 1 mg/ml casein; 5 μM unlabelled kinesin-8B-MD and mutants in assay buffer supplemented with 5 mM nucleotide (as indicated) and an oxygen scavenging system (20 mM glucose, 300 μg/ml glucose oxidase, 60 μg/ml catalase) were introduced into chamber right before observation. For assays comparing PbKinesin-8B mutants, all PbKinesin-8B related proteins were buffer exchanged into BRB80 buffer prior to use in the assay. The microscope and camera used were the same as for the MT gliding assays. Movies were collected at 26 °C under illumination at 561 nm for 30 min with a frame taken every 10 s with 100 ms exposure. MT depolymerisation rates were determined from kymographs using Fiji software.

**Negative stain sample preparation, data collection and analysis of tubulin ring structures**

60 μM PbKinesin-8B-MD, PbKinesin-8B-MD-L2KIF5B or PbKinesin-8B-MD-L2KIF5B was incubated with 20 μM tubulin in BRB80 buffer in the presence of 5 mM AMPPNP at room temperature for 1 h. The reaction mix was diluted into BRB80 buffer 10-fold, followed by application of 4 μl of the mix onto glow-discharged continuous carbon electron microscopy grid (400 mesh, EMS) and was incubated on grid for 1 min. The sample drop was blotted using filter paper (Whatman) before 4 μl of 2% uranyl acetate was applied. After incubation on grid for a further 1 min, the stain was blotted with filter paper and the grid was allowed to dry. All negative stain micrographs were collected using a Tecnai Ti2T transmission electron microscope (Thermo Fisher Scientific) with a 4 × 4 K CCD camera (Gatan) at 120 kV, using magnification of 52,000, with an image pixel size of 2.09 Å and defocus around −5 μm. Data were collected using Digital Micrograph™ software (Gatan). The diameters and rim thickness of tubulin rings were measured in Fiji.

**Cryo-EM sample preparation**

25 μM PbKinesin-8B-MD or 50 μM PbKinesin-8B-MD was incubated in BRB80 buffer containing apyrase (10 units/ml, Pb and Pf) or 5 mM AMPPNP (Pb) at room temperature for 15 min. 4 μl of 10 mM GMPCPP-MT (polimerised as described above) were applied to a glow-discharged C-flat 2/2-4 C grid (EMS CF224C) at room temperature. After incubation on the grid for 1 min, 3.5 μl of MTs were removed by pipetting, followed by double application of 3.5 μl of the PbKinesin-8B-MD mix. Grids were then plunge frozen using Vitrobot Mark IV (Thermo Fisher Scientific) with the following setting: blot force of 5, blot time of 5 s, humidity of 100% and temperature of 22 °C.

For the tubulin ring samples, 60 μM PbKinesin-8B-MD was incubated with 20 μM tubulin and 5 mM AMPPNP at room temperature for 1 h. Four microliters were applied to a glow-discharged C-flat 2/2-4 C grid (EMS CF224C). Grids were then plunge frozen using Vitrobot Mark IV (Thermo Fisher Scientific) with the following setting: blot force of 5, blot time of 5 s, wait time 10 s, humidity of 100% and temperature of 22 °C.

**Cryo-EM data acquisition**

For the PbKinesin-8B-MD-NN dataset, 329 movies were collected on a Tecnai G2 Polara microscope (Thermo Fisher Scientific) with K2 Summit detector operating in counting mode with a GIF Quantum LS Imaging Filter (Gatan). The microscope was operated at an accelerating voltage of 300 kV with nominal magnification of 160 K and pixel size of 1.35 Å. 50 frames for each micrograph were collected using serialEM software, 15 s exposure time, 51 e−/Å² total electron
exposure dose and 7 e-/pixel/s dose rate. The defocus range is from −0.5 to −2.5 μm.

For Pbkinesin-8B-MD-AMPPNP dataset, 1026 movies were collected on a Titan Krios microscope (Thermo Fisher Scientific) with K2 Summit detector operating in counting mode with a GIF Quantum LS Imaging Filter (Gatan). The microscope was operated at an accelerating voltage of 300 kV with nominal magnification of 105,000 and pixel size of 1.37 Å. 40 frames for each micrograph were collected using EPU (Thermo Fisher Scientific), 12 s exposure time, 40 e−/Å² total electron exposure dose and 8e-/pixel/s dose rate. The defocus range is from −0.5 to −2.5 μm. The Pbkinesin-8B-MD-NN dataset consisted of 4075 movies which were collected similarly, using 8 s exposure time, 47 e−/Å² total electron exposure dose and 7e-/pixel/s dose rate. The defocus range is from −0.5 to −2.5 μm.

For the Pbkinesin-8B-MD-tubulin cryo-EM dataset, 8148 movies were collected on a Titan Krios microscope (Thermo Fisher Scientific) with K2 Summit detector operating in counting mode with a GIF Quantum LS Imaging Filter (Gatan). The microscope was operated at an accelerating voltage of 300 kV with nominal magnification of 105 K and pixel size of 1.37 Å. 40 frames for each micrograph were collected using EPU (Thermo Fisher Scientific), 12 s exposure time, 40 e−/Å² total electron exposure dose and 8e-/pixel/s dose rate. The defocus range is from −0.5 to −2.5 μm.

Data processing
Movie frames were motion-corrected using MotionCor2 as follows: Pbkinesin-8B-MD-NN: frame 2–24, Pbkinesin-8B-MD-AMPPNP: frame 2–16, Pbkinesin-8B-MD-NN: frame 1–32. The parameters of the contrast transfer function (CTF) for each micrograph were determined using CTFFIND4.46. Particles with box size of 432 pixels (Pbkinesin-8B-MD-NN), 576 pixels (Pbkinesin-8B-MD-AMPPNP, Pbkinesin-8B-MD-NN) and non-overlapping region of one tubulin dimer size were picked using EMAN2 v2.13 e2helixboxer.py. All picked particles were imported into RELION v3.0 and performed using the MiRP pipeline.67 Lattice results in a resolution decay in the processing was performed in Cryosparc v2.11.0.70. Frames were motion-corrected using patch motion, followed by CTF estimation using CTFFIND4. Particles were initially selected manually for 2D classification. The best classes were selected as templates for template picking. 89,836 particles were picked and extracted for multiple rounds of 2D classification. For the final round of 2D classification, 48,171 particles were classified into 100 classes.

Model building
100 comparative models of Pbkinesin-8B-MD were calculated using MODELLER v9.23,71 using multiple known structures as templates (PDB IDs: 5GS2,42 4LNO35 3HQP15 and 4OZQ36). The top 10 models were selected using SOAP scoring,72 then the top model selected using QMEAN.73 The comparative models were rigidly fitted into no nucleotide and AMPPNP reconstructions using the Fit-in-Map tool in Chimera74. To improve the fit to the density, a local all-atom fit to density step was performed using Rosetta Relax, incorporating a fit to density term. To improve models of loop 2 (16 Amino Acids(AAs)), loop 5 (6 AAs), loop 11 (13 AAs), the visible neck linker (5 AAs), and the N-terminus (5 AAs), loop conformations were predicted using Rosetta. First, 500 models using cyclic coordinate descent with fragment insertion were calculated,75 then the model with highest cross-correlation to the cryo-EM density was selected. From this top model a further 500 models were calculated using kinematic closure with a fit-to-density term and the top model selected based on cross-correlation76. A Pbkinesin-8B-MD-NN model was generated by mutating amino acids using the “mutate” tool in Coot v0.9.8.146 from Pbkinesin-8B-MD-NN model, which exhibits 85% sequence identity and 94% similarity (Supplementary Fig. 1). Cross-correlation with the cryo-EM density was calculated showing a good fit, while the calculated QMEAN value and Molprobity score77 demonstrated the good geometry of the model (Table 2).

Sequence alignment
Sequence alignments were performed with Clustal Omega, with residue colouring according to the Clustal X scheme.80

Purification of gametocytes
The purification of P. berghei gametocytes from kinesin-8G-GFP (two biological replicates) and GFP-only expressing controls was achieved by injecting parasites into phenylhydrazine treated mice and enriched by sulfadiazine treatment after 2 days of infection.81 The blood was collected on day 4 after infection and gametocyte-infected cells were purified on a 48% v/v Nycodenz (in PBS) gradient (Nycodenz stock solution: 27.6% w/v Nycodenz in 5 mM Tris-HCl, pH 7.20, 3 mM KCl, 0.3 mM EDTA). The gametocytes were harvested from the interface and activated.

Immunoprecipitation and mass spectrometry
Purified gametocytes activated for 6 min were used to prepare cell lysates. Immunoprecipitation was performed using GFP-Trap® A Kit (Chromotek) following the manufacturer’s instructions. Proteins bound to the GFP-Trap® A beads were digested using trypsin and the peptides were analysed by LC-MS/MS. Briefly, to prepare samples for
LC-MS/MS, wash buffer was removed and ammonium bicarbonate (ABC) was added to beads at room temperature. We added 10 mM TCEP (Tris(2-carboxyethyl) phosphine hydrochloride) and 40 mM 2-chloroacetamide (CACA) and incubation was performed for 5 min at 70 °C. Samples were digested using 1 µg Trypsin per 100 µg protein at room temperature overnight. Reversed phase chromatography was used to separate tryptic peptides prior to mass spectrometric analysis. Two columns were utilised, an Acclaim PepMap µ-precolumn cartridge 300 µm i.d. × 5 mm 5 µm 100 Å and an Acclaim PepMap RSLCnano system (Thermo Fisher Scientific). Mobile phase buffer A was composed of 0.1% formic acid in water and mobile phase B 0.1% formic acid in acetonitrile. Samples containing 0.1% trifluoroacetic acid for 5 min at 10 µL/min after which peptides were eluted onto the analytical column at 250 nL/min by increasing the mobile phase B concentration from 8% B to 25% over 36 min, then to 35% B over 10 min and to 90% B over 3 min, followed by a 10 min re-equilibration at 8% B. Eluting peptides were converted to gas-phase ions by means of electrospray ionisation and analysed on a Thermo Orbitrap Fusion (Q-OT-qIT, Thermo Scientific). Survey scans of peptide precursors from 375 to 1575 m/z were performed at 120 K resolution (at 120 μm) with a 50% normalised AGC target and the mass injection time was 150 ms. Tandem MS was performed by isolation at 1.2 Th using the quadrupole, HCD fragmentation was performed with normalised collision energy of 33, and rapid scan MS analysis in the ion trap. The MS² was set to 50% normalised AGC target and the max injection time was 200 ms. Precursors with charge state 2–6 were selected and sampled for MS². The dynamic exclusion duration was set to 45 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 2 s cycles.

The raw data were searched using MaxQuant (version 2.0.3.0) or MSFragger (version 18.0) against the P. berghei protein sequences from the PlasmoDB database (release 58, www.plasmodb.org) and a common contaminant database. For the database search, peptides were generated from a tryptic digestion with up to two missed cleavages, carbamidomethylation of cysteines as a common modification, and methionine oxidation and N-terminal acetylation as possible modifications. Results were analysed using Scaffold (version S1.2, Proteome Software). Proteins and peptides having minimum threshold of 95% were used for proteomic analysis. Only proteins present in both experimental samples were taken as probable interacting partners.

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Author contributions

T.L. and F.S. performed and analysed cryo-EM experiments and undertook motor activity experiments in consultation with A.J.R.; A.D.C. performed structural model calculations in collaboration with T.L.; M.Z. and D.B. performed parasite experiments and analysed proteomics data; C.A.M., C.J.S., A.J.R. and T.T. coordinated the project; T.L. and C.A.M. prepared the first manuscript draft and all authors contributed to manuscript editing and revisions.

Competing interests

F.S. declares that she is now an employee of AstraZeneca. The remaining authors declare no competing interests.

Additional information

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