Insight into the molecular mechanism of the multitasking kinesin-8 motor

Carsten Peters^{1,3}, Katjuša Brejc^{2,3,4,5},
Lisa Belmont², Andrew J Bodey¹, Yan Lee²,
Ming Yu², Jun Guo², Roman Sakowicz²,
James Hartman² and Carolyn A Moores^{1,*}

¹Institute of Structural and Molecular Biology, Birkbeck College, London, UK and ²Cytokines, San Francisco, CA, USA

Members of the kinesin-8 motor class have the remarkable ability to both walk towards microtubule plus-ends and depolymerise these ends on arrival, thereby regulating microtubule length. To analyse how kinesin-8 multitasks, we studied the structure and function of the kinesin-8 motor domain. We determined the first crystal structure of a kinesin-8 and used cryo-electron microscopy to calculate the structure of the microtubule-bound motor. Microtubule-bound kinesin-8 reveals a new conformation compared with the crystal structure, including a bent conformation of the $\alpha 4$ relay helix and ordering of functionally important loops. The kinesin-8 motor domain does not depolymerise stabilised microtubules with ATP but does form tubulin rings in the presence of a non-hydrolysable ATP analogue. This shows that, by collaborating, kinesin-8 motor domain molecules can release tubulin from microtubules, and that they have a similar mechanical effect on microtubule ends as kinesin-13, which enables depolymerisation. Our data reveal aspects of the molecular mechanism of kinesin-8 motors that contribute to their unique dual motile and depolymerising functions, which are adapted to control microtubule length.

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Introduction

The microtubule cytoskeleton is essential for most cell activities, including motility, cell division and differentiation. Microtubules (MTs) are dynamic polar polymers built from $\alpha\beta$ -tubulin heterodimers. In the cell, the dynamic plus-ends of MTs undergo exploratory polymerisation away from

MT-organising centres, and can interact with and become stabilised by specific cellular structures (Howard and Hyman, 2003). In such dynamic processes, it is essential for the cell to regulate not only MT dynamics, but also the size and extent of the cytoskeleton by controlling MT length. This is particularly important during cell division, when control of MTs within the bipolar spindle is a crucial aspect of successful alignment and separation of sister chromatids (Kline-Smith and Walczak, 2004). Lack of such control can cause chromosomal instability, which is linked to human cancer (Ganem *et al*, 2009). The complex molecular mechanisms by which MT length is regulated during cell division and throughout the cell cycle remain poorly understood.

Recently, kinesin-8s—member of the MT-associated, ATP-dependent kinesin superfamily of molecular motors—have received considerable attention for their MT length-regulating activities (Gupta *et al*, 2006; Varga *et al*, 2006). Kinesin-8s are one of the minority of kinesin classes seen across all eukaryotic supergroups so far examined (Wickstead *et al*, 2010). They are observed on cytoplasmic MTs in interphase (Gupta *et al*, 2006; Mayr *et al*, 2007; Unsworth *et al*, 2008) and at or near kinetochores during cell division (West *et al*, 2002; Mayr *et al*, 2007; Stumpff *et al*, 2008; Savoian and Glover, 2010; Wargacki *et al*, 2010). Disruption of their mitotic function results in unusually long spindle MTs that cause aberrant chromosomal segregation, consistent with the idea that MT length control by kinesin-8s is essential for accurate cell division (DeZwaan *et al*, 1997; Garcia *et al*, 2002; West *et al*, 2002; Goshima and Vale, 2003; Mayr *et al*, 2007; Stumpff *et al*, 2008; Savoian and Glover, 2010; Wargacki *et al*, 2010).

Kinesins were originally identified as dimeric motors that transport cellular cargo by taking directed steps towards the plus-ends of MTs (for review, see Vale and Milligan, 2000). However, different classes within the kinesin superfamily are now known to perform diverse MT-based activities (Miki *et al*, 2005). These include motility in either direction along MTs, different motility speeds, and different degrees of processivity; in addition, some kinesins regulate MT dynamics, including by depolymerising MTs at their ends (Kline-Smith and Walczak, 2004). Different domains characteristic of each kinesin class contribute to these diverse activities, such as cargo-binding domains in transporter kinesin-1s and tetramerisation domains in the MT cross-linking kinesin-5s (Miki *et al*, 2005). However, fundamental aspects of each class's function are defined by their interaction with their MT substrate, and the efficiency with which ATP is hydrolysed by the MT–kinesin complex. This is encoded by the 40-kD kinesin motor domain that contains both the MT- and ATP-binding sites. The motor domain of each kinesin class might thus be expected to perform a single MT-based activity, mirroring the function of the kinesin class. Although the motor domain is the defining feature of all kinesin superfamily members and is highly conserved, sequence-wise and structurally, subtle class-specific differences within the domain have significant functional consequences (Okada and

*Corresponding author. Institute of Structural and Molecular Biology, Birkbeck College, Malet Street, London WC1E 7HX, UK.
Tel.: +44 207 631 6858; Fax: +44 207 631 6803;
E-mail: c.moores@mail.cryst.bbk.ac.uk

³These authors contributed equally to this work

⁴Present address: Howard Hughes Medical Institute, University of California, Berkeley, CA 94720, USA

⁵Present address: Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

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Hirokawa, 2000; Moores *et al*, 2002; Ogawa *et al*, 2004; Bodey *et al*, 2009).

What are the molecular properties of kinesin-8s that enable them to control MT length within cells? The phenotype of long MTs associated with kinesin-8 depletion suggests that kinesin-8s should depolymerise MTs, but consistent evidence for depolymerisation activity as a conserved property of this motor class is lacking (Gupta *et al*, 2006; Varga *et al*, 2006; Mayr *et al*, 2007; Grissom *et al*, 2009; Du *et al*, 2010). In fact, the most clearly defined property of kinesin-8s is their slow, plus-end-directed, lattice-based motility (Gupta *et al*, 2006; Mayr *et al*, 2007; Grissom *et al*, 2009; Du *et al*, 2010), which, for some dimeric molecules, has been shown to be highly processive (Varga *et al*, 2006, 2009). Kinesin-8s do not seem to carry cellular cargo, but their processive stepping takes them to MT plus-ends at which depolymerisation activity could regulate dynamics. Thus, motility seems to be a prerequisite for any depolymerisation that might occur, and the depolymerisation activity that has been observed is proposed to be a passive consequence of motor flux to the MT end (Varga *et al*, 2009). These properties contrast markedly with the well-characterised kinesin-13 motors: kinesin-13s do not step along the MT lattice, but diffuse to MT ends and there catalyse processive depolymerisation of stabilised MTs (Helenius *et al*, 2006).

Thus, a number of outstanding questions remain regarding the molecular mechanism by which kinesin-8s regulate MT dynamics. Do they all indeed depolymerise MTs and, if so, by what mechanism? Is there any similarity with the mechanism of other depolymerising kinesins? How do kinesin-8s undertake the seemingly divergent activities of lattice stepping and MT depolymerisation, and can both stepping and depolymerising activities be encoded by the kinesin-8 motor domain itself? These fundamental questions can be addressed by examining the molecular basis of kinesin-8's multitasking activity. Using the motor core as the minimal function unit of kinesin-8s, we determined the structure of the kinesin-8 motor domain, both alone and in complex with MTs. We probed its MT-based activities to provide insight into the molecular mechanisms of kinesin-8 function.

Results

Crystal structure of kinesin-8 motor domain: conformation of the conserved ATP- and MT-binding elements

To begin to understand the molecular mechanism of the putatively multitasking kinesin-8 motors, we determined the 2.2-Å resolution atomic structure of the human kinesin-8 motor domain (human Kif18A, residues 1–355, which we refer to as kinesin-8-MD) by X-ray crystallography, the first atomic structure of a kinesin-8 (Figure 1; Table I). The kinesin-8-MD has an arrowhead shape that is characteristic of the kinesin superfamily and is built from a central β -sheet with three α -helices on either face (Figure 1B and C; Sack *et al*, 1999). Kinesins share a highly conserved mechanism of nucleotide binding and hydrolysis with actin-based myosin motors and G-proteins (Vale, 1996) built from a P-loop and switch-I and switch-II motifs (Kull and Endow, 2002). In our structure, Mg^{2+} -ADP is bound in the kinesin-8-MD nucleotide-binding site, at which P-loop (loop4) residues interact with the phosphate groups, whereas residues in the β -strand

β 1 (R17, R19 and P20) and α 2 helix (H121) interact with the adenosine base. The switch-I region (loop9), located at the end of helix α 3, forms the binding pocket that can accommodate the γ -phosphate of ATP and adopts a well-defined loop in our structure (Figure 1B). These nucleotide–protein interactions are consistent with those reported for other kinesin–ADP structures (Kull and Endow 2002). At the N-terminus of α 4, loop11, which in addition to being part of the MT-binding interface, is part of the switch-II cluster that coordinates nucleotide binding and MT sensing, is disordered (Vale and Milligan, 2000).

The MT-binding surface is formed from helices α 4, α 5 and α 6 (Figure 1C) but loop8, loop11 and loop12, which also have a conserved role in MT binding (Woehlke *et al*, 1997), are disordered in our structure. Kinesin motor function is inextricably linked to MT binding, and motor ATPase activity is stimulated by binding to MTs (Cross, 2004). This is because interaction with the MT surface induces structural changes within the motor domains that allow precise conformational coupling of MT- and ATP-binding elements (Kikkawa, 2008; Bodey *et al*, 2009; Sindelar and Downing, 2010). In the absence of MTs, variable, nucleotide-independent conformations of these loops and helices are seen in many kinesin motor domain crystal structures (Grant *et al*, 2007), but some capture nucleotide-dependent conformational states (for example, Kikkawa *et al*, 2001; Parke *et al*, 2010). Kinesin crystal structures have, thus, been classified according to the position of their nucleotide sensing, relay helix α 4: structures with helix α 4 in a 'up' position (also known as 'ATP-like') in plus-end-directed kinesins enable neck-linker docking, whereas those with helix α 4 in the 'down' position do not (Rice *et al*, 1999; Vale and Milligan, 2000; Kikkawa *et al*, 2001). In the presence of MTs, the position of the neck-linker (as a result of the orientation of helix α 4) is controlled by the nucleotide bound to the motor, but in the crystal structures the conformation of these features is less correlated. In this context, a comparison of the conformation of the kinesin-8-MD with other kinesin crystal structures indicates that the α 4 relay helix in our structure adopts the 'up' position despite the bound ADP in the nucleotide-binding pocket (Figure 1D and E; Kikkawa *et al*, 2001). The kinesin-8-MD construct terminates at the end of helix α 6 and hence lacks the neck-linker region, although the conformation of α 4 suggests that it would be able to dock against the motor domain.

Identifying a kinesin-8 function-defining structural element?

In seeking to identify structural features in the kinesin-8-MD that could be responsible for its MT dynamics-regulating function in cells, loop2 was an interesting candidate. Previously, studies on mouse, fruit fly and malaria kinesin-13s have revealed the essential role of the kinesin-13 loop2 in MT depolymerisation (Ogawa *et al*, 2004; Shipley *et al*, 2004; Tan *et al*, 2008). Loop2 is located in the small β -lobe connecting β -strands β 1b and β 1c and, in the majority of kinesins, is relatively short. In kinesin-13s, however, loop2 is longer and forms a two-stranded anti-parallel β -sheet structure that ends with a KVD finger (grey structure, Figure 1F) containing conserved KVD residues essential for MT depolymerisation. Point mutation of these three residues specifically abolished the ability of kinesin-13s to perform ATP-dependent MT depolymerisation while leaving their MT

Figure 1 Crystal structure of human kinesin-8 motor domain. (A) Schematic of human kinesin-8 (Kif18A) domain structure; the kinesin-8-MD described in this study is depicted in colour. (B, C) Ribbon representation of (B) front and (C) back view of kinesin-8-MD with rainbow colouring from N- (blue) to C-terminus (red) and structural elements labelled according to standard kinesin nomenclature (Sack *et al*, 1999). The disordered parts of the structure are the N-terminus (residues 1–9), loop2 (L2, 46–67), loop8 (L8, 180–184), loop11 (L11, 266–278) and loop12 (L12, 300–305). Mg^{2+} ADP is shown as a ball and stick model. (D) Structural comparison of kinesin-8 motor domain (rainbow) and kinesin-13 motor domain (grey, Kif2C-ADP, PDB 2HEH), illustrating the conformation of the kinesin-8-MD switch II helix $\alpha 4$ in the ‘up’ conformation (back view). The docked neck-linker of a kinesin-1 (PDB 1MKJ; Sindelar *et al*, 2002) is shown in purple to indicate that the ‘down’ conformation blocks docking while the ‘up’ conformation enables docking. (E) Additional comparison of kinesin-8-MD and kinesin-13 structures with kinesin-3 structures (gunmetal, Kif1a-ADP, PDB 115S; orange, Kif1a-AMPPCP, PDB 116I; Kikkawa *et al*, 2001) reinforces the ‘up’ assignment to the kinesin-8 conformation. (F) Structural comparison between human kinesin-8 (rainbow) and human kinesin-13 (grey; Kif2C PDB 2HEH) side view. The KVD (Lys-Val-Asp) motif in L2 region has been implicated in the MT depolymerisation activity of kinesin-13s (Ogawa *et al*, 2004; Shipley *et al*, 2004). In kinesin-8, this region is 13 residues longer and it is disordered in the crystal structure. UCSF Chimera was used for structure alignment and visualisation (<http://www.cgl.ucsf.edu/chimera/>; Pettersen *et al*, 2004).

Table 1 Crystallographic data and refinement statistics

Space group	P212121
Unit cell (Å)	
a	69.4
b	79.7
c	140.1
Resolution (Å)	2.2
Measurements	355937
Unique reflections	39803
Completeness (%)	98.8 (99.6)
R_{sym} (%)	6.4 (25.1)
$\langle I \rangle / \langle \sigma(I) \rangle$	6.9 (1.5)
$R_{\text{cryst}}/R_{\text{free}}$ (%)	22.1/27.7
r.m.s.d. from ideality	
Bonds (Å)	0.02
Angles (deg)	1.7
Molecules per asymmetric unit	2

lattice-binding and tubulin-stimulated ATPase activity intact (Ogawa *et al*, 2004; Shipley *et al*, 2004; Tan *et al*, 2008). Thus, for kinesin-13s, conserved residues of loop2 and the length of loop2—which presumably brings it close to the MT surface—seem to be important for depolymerisation activity.

Noticeably, kinesin-8 class members also have an extended loop2 (residues 46–67) but it is approximately 13 residues longer than in kinesin-13s and lacks the kinesin-13-conserved KVD residues (also see Supplementary Figure 1). Although computational analysis predicts that the loop2 region of both kinesin-13s and kinesin-8s mainly adopts a coil structure (Supplementary Figure 2), in contrast to kinesin-13s, the kinesin-8 loop2 is flexible and therefore not visible in our structure. Hence, although both kinesin-13s and kinesin-8s have a long loop2 (compared with kinesin-1s), detailed comparison suggests that kinesin-8s have neither the conserved residues nor the structure of the kinesin-13 loop2, which presumably contribute to depolymerising activity. This suggests that the role of loop2 in kinesin-8 function might be distinct from the same region in kinesin-13s and leaves open the question as to whether simply the presence of a long loop2 can be used as a diagnostic feature for depolymerising activity.

MT- and tubulin-stimulated ATPase activity of the kinesin-8-MD

To characterise the activity of kinesin-8-MD, we measured the motor's MT- and tubulin-stimulated ATPase activity. The basal ATPase activity of lattice-based transporter kinesins can be stimulated >1000-fold by MTs, reflecting the efficiency of mechano-chemical coupling in the kinesin-MT complex (Cross, 2004). Tubulin dimers have also been used to probe kinesin ATPases; however, stimulation of the kinesin-1 ATPase by tubulin dimers is approximately 20% of lattice stimulation, and presumably reflects a preference by kinesin-1 for a stable track (Alonso *et al*, 2007). In contrast, the ATPase activity of kinesin-13s is stimulated not by the MT lattice, but by their ends, which is coupled to depolymerisation. In fact, inhibition of kinesin-13-MD ATPase has been reported when unproductive lattice binding competes with binding to MT ends (Moore *et al*, 2003; Helenius *et al*, 2006). As the products of depolymerisation, tubulin dimers are necessarily involved in the kinesin-13 ATPase cycle and thus kinesin-13s also form an ATPase-active complex with tubulin dimers (Hunter *et al*, 2003; Moore *et al*, 2003).

Figure 2 Enzymatic characterisation of kinesin-8-MD. Stimulation of ATPase activity by MTs and tubulin. Kinesin-8-MD concentration was 32.5 nM. Data were fitted to a simple Michaelis–Menten model. The basal rate of kinesin-8-MD ATPase is 0.05/s (data not shown).

What does the kinesin-8 MT-stimulated ATPase activity tell us about its molecular mechanism? With MTs, the kinesin-8-MD shows Michaelis–Menten kinetics with $V_{\text{max,MT}} = 0.56 \pm 0.003 \mu\text{mol/s}$ (17/head/s) and $K_{\text{m,MT}} = 0.11 \pm 0.002 \mu\text{M}$ (Figure 2). There is no kinesin-13-like lattice inhibition, consistent with the evidence for kinesin-8s being lattice-based steppers (Gupta *et al*, 2006; Varga *et al*, 2006; Mayr *et al*, 2007; Grissom *et al*, 2009; Du *et al*, 2010). Kinesin-8-MD also exhibited tubulin-stimulated ATPase activity (which depended on ionic strength; see Supplementary Figure 3) with $V_{\text{max,tub}} = 0.11 \pm 0.001 \mu\text{mol/s}$ (3/head/s) and $K_{\text{m,tub}} = 0.14 \pm 0.006 \mu\text{M}$ (Figure 2), that is, approximately 20% of the MT-stimulated activity, as is also seen for kinesin-1 dimers (Alonso *et al*, 2007). Although our kinesin-8-MD construct has a faster turnover than larger kinesin-8 constructs, the difference in ATPase activation by MTs compared with tubulin, observed by us, is consistent with these other studies (Gupta *et al*, 2006; Mayr *et al*, 2007). This demonstrates that our minimal motor domain reveals aspects of the enzymatic properties of the full-length kinesin-8 protein, and reinforces the idea that the mechanism of MT dynamic regulation by kinesin-8 motors is distinct from that of kinesin-13s.

Structure of the kinesin-8-MD–MT complex

To understand the elements of the kinesin-8 motor that interact with MTs and contribute to its multitasking behaviour, we used cryo-electron microscopy (cryo-EM) and image reconstruction to determine the three-dimensional structure of the kinesin-8-MD–MT complex captured at two points in its ATPase cycle: nucleotide-free and ATP-bound state. We first describe the nucleotide-free structure (at 10 Å resolution; also see Supplementary Figure 4) that reveals details of the kinesin-8–MT interface. The α - and β -tubulin atomic coordinates fit well within the MT portion of the reconstruction and, as would be expected for a reconstruction of this quality, density for the C-terminal helices H11 and H12 of both α - and β -tubulin is clearly visible on the MT surface (Figure 3A; 1JFF, Löwe *et al*, 2001). Helix H12 forms the major points of contact for both α - and β -tubulin with the kinesin-8-MD, which is a conserved feature of all kinesin–MT interactions (for review, see Kikkawa, 2008). To understand the conformation of the kinesin-8-MD in the context of the

Figure 3 Structure of the kinesin8-MD-MT complex in the absence of nucleotide. (A) Overview of the asymmetric unit of the 10 Å helical reconstruction showing the kinesin-8-MD bound to the $\alpha\beta$ -tubulin dimer. Coordinates of α -(blue) and β -(cyan) tubulin are docked and the C-terminal portion of H12 on both α - and β -tubulin is indicated in purple. Purple arrows point to regions of the reconstruction that correspond to the tubulin CTTs. The kinesin-8-MD structure is docked and shown in rainbow colours from N- (dark blue) to C-(red) terminus. (B) The nucleotide-binding pocket shows an open conformation in the MT-bound kinesin-8-MD, with loop9 and 11 forming a well-defined region of density against the MT wall (dotted black oval). $\alpha 3$ of the kinesin-8-MD is not well-accommodated by the EM density, suggesting that a conformational change has been induced by MT binding in this region (green arrow). The P-loop and ADP from the kinesin-8-MD structure are only partially accommodated by the EM density (dotted red oval). (C) Transverse slice through the kinesin-8-MD density, revealing the orientation and discontinuity (marked by an arrow) of $\alpha 4$ (in yellow) in the EM density. $\alpha 4$ seems to stretch from loop9 and 11 at the nucleotide-binding pocket at its N-terminus (on the left) across the back of the motor domain to loop12 at its C-terminus. (D) Longitudinal slice through the kinesin-8-MD density indicating the bend in $\alpha 4$. (E) Loop2 is well-ordered when bound to MTs and forms contact with the surface of α -tubulin. The human kinesin-13 loop2 is shown in grey with the key depolymerisation KVD residues shown as ball and stick representations. The reconstruction is contoured at 1 σ (grey transparency) and 2.5 σ (grey mesh). Inset shows a schematic of the direction of views.

MT-bound complex, we docked our kinesin-8-MD coordinates into the motor density of our reconstruction. The crystal structure fits the density well and allowed us to identify differences between the MT-bound and unbound kinesin-8-MD.

Kinesin-8-MD has multiple points of contact with the MT surface on both α - and β -tubulin. Towards the minus-end side of the intra-dimer interface, $\alpha 6$ lies above α -tubulin H12,

whereas on the plus-end of the motor, a clear bridge of density is formed between loop8 and β -tubulin H12 (Figure 3A). $\alpha 3$ is directly connected to loop8 and protrudes from the cryo-EM density, suggesting that interaction with MT might have induced a local conformational change in this region (green arrow, Figure 3B). In between the contact points of loop8 and $\alpha 6$ lies the $\alpha 4$ relay helix, lying over the intra-dimer interface and in contact mainly with H12 of

α -tubulin (Figure 3A). A key question for the mechanochemistry of kinesin motors concerns the conformation of $\alpha 4$ on binding to MTs, as it is a central, dynamic component of the kinesin motor engine (for review, see Kikkawa, 2008).

The density attributable to $\alpha 4$ is discontinuous at the tubulin intra-dimer interface in our nucleotide-free map (Figure 3C and D), a conformation that is distinct from that described for kinesin-1 (Sindelar and Downing, 2010). In our kinesin-8-MD MT-bound reconstruction, density corresponding to the N-terminus of $\alpha 4$ seems to originate from the nucleotide-binding pocket and presumably incorporates the switch-II (loop11) residues that are disordered in the kinesin-8-MD crystal structure (Figure 1C). This extension of $\alpha 4$ on binding to MTs has also been seen for kinesin-1, kinesin-3 and kinesin-5 (Kikkawa and Hirokawa, 2006; Bodey *et al*, 2009; Sindelar and Downing, 2010) but in contrast to these structures, $\alpha 4$ in the kinesin-8-MD-MT complex breaks after approximately 15 Å for approximately 1.5 turns of α -helix (Figure 3C and D) and resumes again for a further 15 Å at its C-terminus. The fit of our kinesin-8 coordinates suggest that at its C-terminus, $\alpha 4$ in the MT-bound complex is in an 'up' position as is also seen for kinesin-1 (Sindelar and Downing, 2010). However, the trajectory at its N-terminus adjacent to the nucleotide-binding site is at an approximately 60° angle to this against the MT wall (Figure 3D). Thus, binding to the MT surface seems to distort $\alpha 4$. Evidence of the pliability of $\alpha 4$ is also seen in a kinesin-14 motor domain crystal structure (Vinogradova *et al*, 2008), in which the point of flexion of $\alpha 4$ (N1140, equivalent to N280 in kinesin-8-MD) coincides with the end point of the N-terminal portion of $\alpha 4$ density in our reconstruction. However, the N-terminal portion of the kinesin-14 $\alpha 4$ would clash with the MT surface, demonstrating that it is not itself the MT-bound conformation (also see Supplementary Figure 5). Our kinesin-8-MT-bound structure therefore reveals a new conformation that involves stabilisation of a bent, discontinuous $\alpha 4$ helix.

As a consequence of conformational changes in $\alpha 4$, the nucleotide-binding pocket also undergoes additional conformational rearrangements compared with the kinesin-8-MD crystal structure. In particular, interaction with the MT surface causes the flexible, nucleotide-sensing loops (loop9 (switch-I) and loop11 (switch-II)) to form a lobe of density at the bottom of $\alpha 3$ that interacts with both α - and β -tubulin (black oval; Figure 3B and C). The nucleotide-binding pocket has an open conformation (black arrow, Figure 3C) and the nucleotide and the P-loop of the kinesin-8 coordinates protrude from the EM density (Figure 3B). Thus, the overall effect of MT binding on the kinesin-8-MD (which is also seen for kinesin-1 (Sindelar and Downing, 2010), seems to be to pry open the nucleotide-binding site, which presumably contributes to the enhancement of the rate-limiting ADP release from the motor (Cross, 2004).

Noticeably, there is an additional lobe of density at the minus-end of the kinesin-8-MD that contacts α -tubulin between helices H12 and H5 (blue oval, Figure 3A and E). This lobe of density corresponds to the extended kinesin-8-MD loop2, which, in contrast to its disordered conformation in the crystal structure (Figure 1B), adopts a well-defined conformation on the MT surface. The ordered loop2 density seems to be part of a well-defined subdomain at the minus-end of the kinesin-8-MD that also includes $\alpha 0$ and part of $\alpha 6$. Thus, our cryo-EM reconstruction suggests a structural

Figure 4 Structure of the kinesin-8-MD-AMPPNP MT-bound complex. (A) Overview of the asymmetric unit of the 13 Å helical reconstruction showing the kinesin-8-MD bound to the $\alpha\beta$ -tubulin dimer (coloured as previously and in the same orientation as Figure 3A and B)). Coordinates of α - (blue) and β -tubulin (cyan) and the kinesin-8-MD structure are docked as in the nucleotide-free structure. (B) The kinesin-8-MD-MT complex has a similar conformation in the AMPPNP (left) and no nucleotide (right, filtered to 13 Å resolution) reconstructions, with loop9 and 11 forming a well-defined region of density against the MT wall. However, changes in the map density around the nucleotide-binding site of the AMPPNP reconstruction (left) suggest that nucleotide is bound. The reconstructions are contoured at 1 σ (grey transparency) and 2.5 σ (grey mesh).

mechanism by which the new MT contact of the class-specific loop2 can be coupled to changes in the more conserved portions of the kinesin-MT interface.

The resolution of the kinesin-8-MD-MT reconstruction in the presence of AMPPNP was slightly lower than in the absence of nucleotide (approximately 13 Å resolution; also see Supplementary Figure 4). However, docking of the previously fitted coordinates within the +AMPPNP MT-bound structure showed that the overall conformation of the kinesin-8-MD in each reconstruction is very similar. For example, contacts with the MT surface involving $\alpha 4/\alpha 5$, loop8 and loop2 across the MT-motor interface are the same and the sub-domain formed by loops9 and 11 against the surface of the MT beneath $\alpha 3$ remains intact (Figure 4A and B). This also suggests that $\alpha 4$ retains its bent conformation, although, due to the lower resolution of the +AMPPNP reconstruction, this cannot be visualised directly. Nevertheless, a marked

increase in density at the nucleotide-binding pocket suggests that AMPPNP is bound (Figure 4B). This differs from the complete rearrangement of loops 9 and 11 that close the nucleotide-binding pocket in kinesin-1s, kinesin-3s and kinesin-5s on binding AMPPNP (Kikkawa and Hirokawa, 2006; Bodey *et al*, 2009; Sindelar and Downing, 2010). It is currently unclear what the significance of this observation is and may be a consequence of the minimal kinesin-8 motor domain used in our experiments. However, the lack of large conformational change when bound to the MT lattice in the presence of AMPPNP contrasts markedly with the action of kinesin-8-MD at MT ends.

Kinesin-8-MD at MT ends

Our cryo-EM structures capture conformations of kinesin-8-MD when bound to the MT lattice. To address how the motor might behave at MT ends and influence MT dynamics, we incubated kinesin-8-MD with paclitaxel-stabilised MTs and nucleotide analogues, and used a co-sedimentation assay to separate MT polymers and released (depolymerised) tubulin (Figure 5A). In the presence of ATP, little tubulin was seen in the supernatant, in contrast to kinesin-13 motors (Moores *et al*, 2002; Helenius *et al*, 2006). This supports the idea that although multitasking kinesin-8 motors can influence MT dynamics, their mechanism of MT depolymerisation is distinct from that of dedicated depolymerising kinesin-13s. Unexpectedly, however, at approximately stoichiometric protein concentrations and in the presence of the non-hydrolysable ATP analogue AMPPNP (but not other nucleotides), a small amount of tubulin was released (Figure 5A). This suggests that when trapped in an ATP-like state, kinesin-8-MDs—similar to kinesin-13-MDs—can release tubulin from stabilised MTs. Furthermore, similar to kinesin-13s, this activity requires the tubulin C-terminal tails (CTTs) (Figure 5A, right), suggesting that there might, in fact, be a mechanism shared by these two kinesin classes that contributes to tubulin release from MT ends.

To investigate this further, we looked at the mixture of kinesin-8-MD-AMPPNP with paclitaxel-MTs by negative stain EM (Figure 5B). Among a high background of free protein and MT fragments, we observed tubulin rings with dimensions (approximately 400 Å diameter) and morphology similar to the rings produced by kinesin-13 motors in the presence of AMPPNP (Moores *et al*, 2002; Tan *et al*, 2008). We performed computational analysis on several hundred of these rings to reveal more detail of their composition (Figure 5C). Although the rings seem to be heterogeneous due to variation in ring diameter and uneven staining (Supplementary Figure 6A), the 2D averages show that the rings are composed of two bands of protein density: an outer band of curved tubulin dimers (black bracket, Figure 5C) and an inner band of kinesin-8-MD (white bracket, Figure 5C). In addition, the 2D averages show a 1:1 stoichiometry between density corresponding to individual kinesin-8-MDs and tubulin heterodimers, as was previously seen for kinesin-13s. Although the kinesin-8-MD-induced rings are not perfectly circular, imposing symmetry on the class averages reinforces the idea of 1:1 stoichiometry between motor and tubulin dimer and further suggests that, although these rings are heterogeneous, at least a subset of them are composed of 14 kinesin-8-MD-tubulin dimer complexes (Supplementary Figure 6B). Thus, as with kinesin-13s, these rings result from

Figure 5 Microtubule depolymerisation by kinesin-8-MD. (A) SDS-PAGE of a co-sedimentation assay comparing tubulin released to the supernatant (depolymerised, top panel) and tubulin retained as polymer (bottom panel) in the presence of 5 mM ATP and 5 mM AMPPNP. For each condition, 0.8 and 2.5 μ M kinesin-8-MD are shown with 5 μ M paclitaxel-stabilised polymerised tubulin. The right hand panels illustrate that release of tubulin by kinesin-8-MD-AMPPNP is abolished when MTs have been pre-treated with subtilisin to remove their CTTs (validated by western blot, data not shown). (B) Negative stain EM images of tubulin rings formed from paclitaxel-stabilised MTs by kinesin-8-MD-AMPPNP (scale bar, 50 nm). (C) 2D averages of the kinesin-8-induced tubulin rings reveals the two bands of density—the outer tubulin band (black bracket) and the inner kinesin-8-MD ring (white) from which the rings are composed. In the upper average, two white arrows indicate density corresponding to monomers in a tubulin dimer, to which a kinesin-8-MD molecule (white oval) is bound. (D) Negative stain EM images of tubulin rings formed from GMPCPP-stabilised MTs by kinesin-8-MD-AMPPNP (scale bar, 50 nm).

the action of sequentially bound individual kinesin-8-MDs on tubulin dimers at MT ends, trapped by AMPPNP. In addition, kinesin-8-MD-induced tubulin rings were also formed from GMPCPP-MTs (Figure 5D), showing that kinesin-8-MD can influence the conformation of terminal tubulins independently of the nucleotide bound to tubulin.

Discussion

Kinesin-8s are unusual motors because members of this class can both step along, and depolymerise, MTs (Gupta *et al*,

2006; Varga *et al*, 2006, 2009; Mayr *et al*, 2007). Our data now show that this dual functionality is intrinsic to the kinesin-8 motor domain, which has MT-stimulated ATPase activity, an unusual MT-binding conformation and induces nucleotide-dependent tubulin release.

Our kinesin-8-MD structure—the first crystal structure of this kinesin class—shows that, in the absence of MTs, several key MT-interacting loops are disordered, including loop11 at the nucleotide-binding site, loop8 and loop2. When kinesin-8-MD is bound to MTs, however, our cryo-EM reconstructions reveal that all these loops form well-ordered contacts with the MT surface, and probably contribute to motor activity. In this regard, MT-bound kinesin-8-MD is similar to the MT-bound conformations of other plus-end-directed kinesins, including kinesin-1s (Sindelar and Downing, 2010), kinesin-3s (Kikkawa and Hirokawa, 2006) and kinesin-5s (Bodey *et al*, 2009).

An unusual feature of the kinesin-8-MD-MT complex, however, is that $\alpha 4$ —which forms the central MT contact point—adopts a bent conformation. This emphasises that $\alpha 4$ can not only act as a static point of contact with the MT surface (Kikkawa and Hirokawa, 2006; Sindelar and Downing, 2010), but also exhibits conformational flexibility. This has previously been observed in MT-bound minus-end-directed kinesin-14 (Hirose *et al*, 2006), although the conformational flexibility of $\alpha 4$ manifested in this structure as ‘melting’ of its C-terminus, the opposite end of $\alpha 4$ at which we observe a bent conformation in the kinesin-8-MD-MT complex (Figure 3C and D). These structures highlight the potential significance of variable conformations of $\alpha 4$ during the kinesin mechanochemical cycle (also see Vinogradova *et al*, 2008), a theme also seen in the related acto-myosin motors (for review, see Geeves and Holmes, 2005). The new conformation of kinesin-8-MD bound to MTs is particularly striking given the unusual selectivity of the kinesin-8 inhibitor BTB-1, which is a competitive inhibitor of ATP, specifically for MT-bound kinesin-8 (Catarinella *et al*, 2009). One intriguing explanation for these observations is that BTB-1 acts by binding to the MT-bound kinesin-8 conformation we observe in our cryo-EM reconstructions. Future studies will seek to test this idea.

Kinesin-8-MD does not depolymerise stabilised MTs in the presence of ATP (Figure 5A), distinguishing it from depolymerisation by kinesin-13-MD constructs (Moores *et al*, 2002). Surprisingly, though, in the presence of AMPPNP, stoichiometric amounts of kinesin-8-MD do induce formation of tubulin rings from the ends of MTs stabilised by either paclitaxel or GMPCPP (Figure 5B and C), a behaviour that is characteristic of kinesin-13s (Moores *et al*, 2002; Tan *et al*, 2008). As with kinesin-13s, multiple kinesin-8-MD motors artificially trapped by AMPPNP can bind adjacent sites along a protofilament and stabilise a curved tubulin conformation, a common intermediate in MT depolymerisation (Howard and Hyman, 2003). The difference in behaviour of kinesin-8-MD in the presence of ATP or AMPPNP is intriguing. One explanation could be that multiple kinesin-8-MD molecules are needed simultaneously at MT ends for tubulin release: when our monomeric construct is turning over in the presence of ATP, occupancy of the MT end by kinesin-8-MD molecules is likely to be low, and it is only when trapped by AMPPNP that kinesin-8-MD molecules can together induce tubulin release.

The idea of kinesin-8-MD motors acting cumulatively to bring about MT depolymerisation is reminiscent of the cooperative activity described for full-length dimeric kinesin-8 (Varga *et al*, 2009). It is proposed that kinesin-8-bound terminal tubulins are ‘bumped off’ by incoming stepping motors; cooperativity thus derives from the accumulation and action of motile kinesin-8 molecules on each other at MT ends. The long residence time of molecules at MT ends is essential for this behaviour (Varga *et al*, 2009). Our monomeric construct is obviously not capable of processive stepping and we observe ring formation at both ends of stable MTs (data not shown), further demonstrating that plus-end-directed motility is not involved in tubulin release by kinesin-8-MD. This implies that although lattice-based motility is an efficient way to concentrate kinesin-8 molecules at MT ends, stepping-competent kinesin-8s are not absolutely required for MT depolymerisation as long as enough kinesin-8 molecules can access MT ends. This does not exclude the involvement—in the context of dimeric motors at nanomolar concentrations—of stepping kinesin-8s in the efficient removal of terminal (bent) tubulin dimers (Varga *et al*, 2009). The ‘access’ requirement is likely to be sensitive to experimental conditions and may explain some of the discrepancies in the literature regarding MT depolymerisation by kinesin-8s (Varga *et al*, 2006; Mayr *et al* 2007; Du *et al*, 2010). In addition, if individual kinesin-8s stabilise a bent tubulin conformation (as seen in our tubulin rings) at MT ends, this would be predicted to block MT polymerisation (Du *et al*, 2010). Overall, our data suggest that ATP is required for both activities of kinesin-8s: as fuel for stepping along MTs and to stabilise the bent structure of the terminal tubulin at the MT plus-end.

In contrast to kinesin-8’s lattice-based motility, kinesin-13s do not bind tightly to the MT lattice and instead diffuse to either MT end independently of ATP. Here, ATP-coupled depolymerisation occurs and individual kinesin-13 molecules remove multiple tubulin dimers with no evidence of the cooperative team-work required by kinesin-8s (Hunter *et al*, 2003; Helenius *et al*, 2006). However, despite differences in other aspects of their molecular behaviour and cellular roles, ring formation by kinesin-8-MD suggests that the underlying mechanisms of MT depolymerisation—involving induction or stabilisation of a bent tubulin conformation—by kinesin-8s and kinesin-13s are related. Residues in the KVD motif in loop2 are essential for MT depolymerisation (and ring formation) by kinesin-13s (Ogawa *et al*, 2004; Shipley *et al*, 2004; Tan *et al*, 2008) and the length of kinesin-13 loop2 is presumably important for placing these residues close to the MT surface. Loop2 in kinesin-8s is longer than that of kinesin-13s and our cryo-EM reconstructions show that it does indeed contact the surface of the MT lattice. Kinesin-8s are unusually processive (Varga *et al*, 2006, 2009) and the additional contact formed between the MT surface and loop2 could contribute to this. Loop2 contains a number of charged residues and, consistent with this putative role, kinesin-8 processivity is salt-sensitive (Varga *et al*, 2009). Thus, the kinesin-8 loop2 is as likely to be involved in lattice-based motility as MT depolymerisation. Further study will be required to investigate this.

Kinesin-8s are both motile and able to depolymerise, but an apparent consequence of their dual activities is a compromise in their depolymerisation efficacy. This manifests as a

requirement for multiple motors to accumulate at MT ends, which has elegantly been demonstrated to contribute to MT length regulation and is likely to be a crucial aspect of kinesin-8 function (Varga *et al*, 2009). Our study has begun to analyse the molecular basis of this activity and future experiments will be directed towards uncovering more about these molecular multitasking motors.

Materials and methods

Protein preparation

Kinesin-8-MD (residues 1–355) was expressed in *Escherichia coli* strain Rosetta (DE3; Invitrogen). The protein was purified sequentially on an SP Sepharose ion-exchange column, a Superdex 200 gel filtration column and a Mono S 5/5 column. The protein was a stable monomer in solution (data not shown).

Crystallographic methods

Single crystals of kinesin-8-MD were grown at 22°C using the hanging-drop vapour diffusion method by mixing 1 µl of protein at 9 mg/ml and 1 µl of crystallisation condition containing 10–13% w/v PEG 20 000, 0.1 M HEPES (pH 7.8) and 2% v/v dioxane. For the data collection at cryogenic temperature, the crystals were soaked in a gradient of glycerol concentrations (2–15% v/v) in a solution containing 15% w/v PEG 20 000, 0.1 M HEPES (pH 7.8) and 2.5% v/v dioxane. Diffraction data at 2.2 Å resolution were collected on in-house X-ray equipment (Bruker AXS Proteum/R6000 model FR591 rotating Cu anode). Bruker AXS Proteum program suite was used to index, integrate and scale the data. Kinesin-8-MD crystals belong to the space group $P2_12_12_1$, with a unit cell of $a = 69.4$ Å, $b = 79.7$ Å, $c = 140.1$ Å, and two molecules per asymmetric unit. Using MOLREP from the CCP4 suite (Collaborative Computational Project, 1994), we found molecular replacement solution using CENP-E (1T5C) as a search model. The model from the molecular replacement solution was rebuilt with the program O (Jones *et al*, 1991) and refined with Refmac5 (Collaborative Computational Project, 1994). The final model was refined at 2.2 Å resolution to an R_{free} factor of 27.2% and R_{work} of 22.1% (Table I) using Arp/Warp 6.0 (Lamzin and Wilson, 1993). The final model contains 589 residues, 245 water molecules and two ADP/Mg²⁺ molecules. Of the two independent molecules (A and B) in the asymmetric unit, B is slightly better defined than A, so was used for structural analysis.

ATPase assay

The ATPase assays were performed using a coupled enzyme system composed of pyruvate kinase and lactate dehydrogenase as described previously (Moore *et al*, 2003). Unless indicated otherwise, all studies using MTs were performed in PEM25 buffer (25 mM PIPES/KOH; 2 mM MgCl₂; 1 mM EGTA; 1 mM DTT) supplemented with 75 mM KCl and 10 µM paclitaxel. All studies using tubulin were conducted in PEM25 buffer supplemented with 25 mM KCl. Kinetic data were analysed by non-linear fitting using Grafit 3 (Erithacus).

MT polymerisation

MTs were polymerised using bovine brain tubulin (Cytoskeleton). The tubulin, at a final concentration of 5 mg/ml, was incubated with 80 mM PIPES (pH 6.8), 5 mM MgCl₂, 1 mM EGTA, 8% DMSO and 2.5 mM GTP for 2 h at 37°C. Approximately 1 mM paclitaxel (Calbiochem) in DMSO was then added. For GMPCPP MTs, GTP was replaced by GMPCPP (Jena Bioscience); no paclitaxel was added.

MT depolymerisation assays

For MT co-sedimentation assays, paclitaxel-stabilised MTs were incubated with kinesin-8-MD at 25°C for 15 min then centrifuged for 15 min at 90 000 r.p.m. at 25°C. The pellets and supernatants were visualised by SDS-PAGE. Subtilisin cleavage of paclitaxel-stabilised MTs was performed, and evaluated by western blot as previously described (Moore *et al*, 2002).

Cryo-EM

Kinesin-8-MD was pre-incubated with either 2 mM AMPPNP or apyrase (10 U/ml). Paclitaxel-MTs (1 mg/ml polymerised tubulin)

and kinesin-8-MD (approximately 0.5 mg/ml) were applied sequentially to glow-discharged home-made holey carbon or C-flat grids (Protochips), which were then vitrified manually (Dubochet *et al*, 1988). Low-dose images were collected on SO163 Kodak film using a Tecnai F20 FEG microscope (FEI Company) equipped with a Gatan 626.DH Cryotransfer System (Gatan), at $\times 50,000$ magnification. Image defoci were 0.9–3.3 µm and 0.7–1.8 µm for the nucleotide-free and AMPPNP data sets, respectively.

Image analysis for MT reconstruction

Micrographs were digitised on a Zeiss SCAI scanner (Carl Zeiss) at 1.4 Å per pixel at the specimen level. Defoci were measured and phases corrected for the effects of the contrast transfer function (CTF) with BSoft (Heymann and Belnap, 2007). The script system Ruby-Helix was used for helical processing of 15-protofilament MTs (Metlagel *et al*, 2007). Image processing was performed as previously described (Bodey *et al*, 2009). In short, MTs were segmented by repeat length; the number of helical repeats in each segment was optimised according to data quality and was usually 1 or 2. Layer line sets from different segments were aligned and averaged. In total, data representing approximately 36 000 asymmetric units (from 25 MTs) for the Apyrase map and approximately 32 000 asymmetric units (from 24 MTs) for the AMPPNP map were averaged. Reconstruction was performed by Fourier-Bessel synthesis and resolutions were assessed by Fourier shell correlation (FSC) with the RF 3 command in Spider (using the 0.5-FSC criterion, see Supplementary Figure 3A). The map was filtered to correct for over-representation of low frequencies and UCSF Chimera (Pettersen *et al*, 2004) was used for visualisation and rigid-body docking. Fits were first performed manually and were refined computationally.

Negative stain electron microscopy and image analysis

Paclitaxel- or GMPCPP-stabilised MTs were incubated with kinesin-8-MD at 25°C for 15 min then centrifuged for 15 min at 90 000 r.p.m. at 25°C. The re-suspended pellets were applied to continuous carbon electron microscope grids (Pacific Grid-Tech), which had been glow discharged in air, and were negatively stained using 1% uranyl acetate. A total of 294 images of the kinesin-8-MD-tubulin rings formed from paclitaxel-MTs were collected digitally on a Tecnai T12 microscope (FEI Company) electron microscope operating at 120 kV at a nominal magnification of $\times 52,000$ and a final pixel size at the sample of 2.53 Å per pixel using a Gatan charge-coupled device. For image analysis, 386 rings were selected manually using the MRC program Ximdisp (Crowther *et al*, 1996), their defoci were calculated using the MRC program CTFFIND3 and phases were corrected for the contrast transfer function using SPIDER (Frank *et al*, 1996). Individual images were band-pass filtered, normalised and centred using SPIDER and aligned against a total sum of the data set. Centred images were then subjected to multivariate statistical analysis (MSA) and classification in IMAGIC (van Heel *et al*, 1996). Selected class averages were used iteratively for further rounds of alignment until the outcome of MSA had stabilised (approximately two additional rounds of alignment).

Accession numbers

Coordinates for kinesin-8-MD have been deposited in the Protein Data Bank, code 3LRE and both kinesin-8-MD MT-bound reconstructions have been deposited in the EM Data Bank, codes EMD-1701 and EMD-1702 (no nucleotide and AMPPNP, respectively).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: CP collected and processed the cryo-EM data and performed the molecular docking; KB purified the protein, grew the crystals and solved the crystal structure; LB characterised the depolymerisation activity of kinesin-8-MD; AJB processed the cryo-EM data; YL characterised the ATPase activity of kinesin-8-MD; MY assisted in protein purification; JG created the expression constructs; RS and JH designed the biochemical experiments and

guided data interpretation; CAM collected and processed the negative stain EM data and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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