S. pombe kinesin-5 switches direction using a steric blocking mechanism

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Abstract (250/250)

Cut7, the only kinesin-5 in *S. pombe*, is essential for mitosis. Like other yeast kinesin-5 motors, Cut7 can reverse its stepping direction, by mechanisms that are currently unclear. Here we show that for full-length Cut7, the key determinant of stepping direction is the degree of motor crowding on the microtubule lattice, with greater crowding converting the motor from minus end-directed to plus end-directed stepping. To explain how high Cut7 occupancy causes this reversal, we postulate a simple proximity sensing mechanism that operates via steric blocking. We propose that the minus end directed stepping action of Cut7 is selectively inhibited by collisions with neighbours under crowded conditions, whilst its plus end directed action, being less space-hungry, is not. In support, we show that the direction of Cut7-driven microtubule sliding can be reversed by crowding it with non-Cut7 proteins. Thus, crowding by either dynein microtubule binding domain or Klp2, a kinesin-14, converts Cut7 from net minus end-directed to net plus end-directed stepping. Biochemical assays confirm that the Cut7 N-terminus increases Cut7 occupancy by binding directly to microtubules. Direct observation by CryoEM reveals that this occupancy-enhancing N-terminal domain is partially ordered. Overall, our data point to a steric blocking mechanism for directional reversal by which collisions of Cut7 motor domains with their neighbours inhibit their minus end directed stepping action, but not their plus end directed stepping action. Our model can potentially reconcile a number of previous, apparently conflicting, observations and proposals for the reversal mechanism of yeast kinesins-5.

Significance statement (120/120)

Molecular motors organise cells by hauling molecular cargoes along polymer tracks (actin filaments or microtubules). Until recently, the stepping direction of each motor was thought to be fixed. However it now emerges that yeast kinesin-5 motors can reverse their stepping direction. How does this work? We show that the stepping direction of Cut7, a yeast kinesin-5 motor, depends on the level of motor crowding on the microtubule, and that crowding of Cut7 by non-Cut7 proteins can also drive reversal. To explain this, we propose that stepping of

Cut7 in one direction is blocked by collisions with neighbours, whilst stepping in the other direction, being less space-hungry, is not. Crowding-dependent directional reversal is a hitherto-unsuspected aspect of motor-driven self-organisation in cells.

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Kinesin-5 is essential for mitosis in many eukaryotes, from yeast to humans. Its main function appears to be to establish spindle bipolarity by driving the spindle poles apart during mitotic prophase (1). All kinesins-5 so far studied are homotetramers that crosslink and slide microtubules (MTs), and until recently, all were thought to be plus end directed (2-8), meaning that their stepping action drives antiparallel MTs to slide slowly apart, minus ends out. However it was recently shown that a yeast kinesin-5, *S. cerevisae* Cin8, can not only step towards MT plus ends, but can also move rapidly and processively towards MT minus ends (9, 10). The mechanism and biological significance of this bidirectionality are unknown.

Several factors have already been shown to influence kinesin-5 directional switching. Cin8 is a tetramer (11) that steps towards plus ends when linking antiparallel MTs, but towards minus ends when moving as a single molecule on uncrosslinked MTs. Crosslinking of two MTs is required for plus end directed stepping (5), such that engagement of only one MT turns off plus end directed motility but supports minus end directed motility (10, 12). The MT sliding direction of Cin8 has also been shown to reverse according to the surface density of motors in microtubule sliding assays (9) with some dependence also on MT length, suggesting that the total number of motors interacting with each MT may be the key parameter for directional reversal. A related proposal is that the torsional stress and/or strain developed between crosslinked microtubules might turn kinesin-5 motors on and off (13). This proposal stems from the structure of the antiparallel overlap zone of the *Drosophila* Klp61f tetramer (14) which revealed a rotational off-set between the two ends of the tetramer. The stepping direction of Cin8 also depends on ionic strength (12), due in part at least to a Cin8-specific loop L8 sequence (15). Phosphorylation of sites in the Cin8 motor domain can also influence stepping direction (16). Recent evidence also implicates the C-terminal tail of Cin8 (17) in regulating directional reversal. Kip1, the other kinesin-5 in *S. cerevisae*, has also been shown to be bidirectional (18) and so has Cut7, the only kinesin-5 in *S. pombe* (19). Cut7 is essential for spindle bipolarity in *S. pombe* (20), and also plays a role in chromosome congression (20).

Given the critical mitotic role of kinesin-5 motors and their corresponding importance as a target for antimitotic agents (21), it is important to understand how they switch direction. We have studied Cut7. We show here for the first time that full length Cut7 can slide MTs bidirectionally in bundles, and that the MT sliding direction in surface assays depends on the local density of Cut7 motor domains engaged with the MT lattice. Our data indicate that Cut7 has a proximity-sensor that reverses its direction of progress depending on the local density of motors. To explain this, we propose that steric clashes between neighbouring Cut7 motor domains on the MT lattice inhibit their minus end directed mode, but not their plus end directed mode. We validate our steric

blocking model by demonstrating that the stepping direction of Cut7 can be reversed by crowding it with non-Cut7 lattice-binders.

Results and discussion

Cut7FL moves microtubules bidirectionally in bundles. We chose to work with tetrameric Cut7FL because there is evidence that in Eg5, a mammalian kinesin-5, the C-termini of each constituent dimer lie close to the motor domains of its partner (Fig. 1*A*), and can potentially contribute to function. It was recently shown that deleting the C-terminal tail of Cin8 abrogates MT crosslinking (17). For our microtubule sliding experiments we used polarity-marked MTs, assembled by polymerising fluorescent tubulin on to short, stabilised MT seeds that were labelled more brightly or in a different colour (see *Materials and Methods*). Initial experiments in solution established that Cut7FL bundles and parallelises microtubules (Movie S1). In subsequent experiments, we assembled Cut7FL-crosslinked bundles in solution using two classes of polarity-marked MTs, labelled in different colours, one with biotinated tubulin and one without. Bundles consisting of only two MTs were selected, and the sliding of the upper MT relative to the immobile surface-tethered MT recorded. These experiments show that tetrameric (Fig. 1*B*) Cut7FL can drive MTs in bundles to slide in either direction (Fig. 1*C*,*D*; Movies S2-4). This is the first demonstration that a kinesin-5 can drive bidirectional sliding of bundled microtubules.

The direction of Cut7FL-driven MT gliding depends on the concentration of lattice-engaged motors. To try to understand what sets the direction of Cut7-driven MT motility, both in MT bundles and potentially elsewhere, we performed *in vitro* MT gliding assays, using antibodies to link Cut7FL motors to glass coverslips via their C-terminal 6-his tags. Because in our assays Cut7FL tetramers are tethered to the surface, we are effectively testing half-tetramer driven MT gliding (Fig. 1*A*). In these assays, MT velocity and direction depend on both the density of Cut7FL motors on the coverslip and on the ionic strength, as previously reported for Cin8 and other yeast kinesins-5. At low ionic strength in KPEM gliding buffer (Fig. 2*A*) sparse Cut7FL surfaces drive MTs to slide rapidly with their plus ends leading, indicating that the surface-attached Cut7FL motors are stepping towards MT minus ends (Movie S5). On very sparse Cut7FL surfaces, MTs pivot about single points on the surface as they slide (Movie S6), suggesting a minimal processive unit at each locus, presumably a single half-tetramer. Increasing the density of Cut7FL on the cover slip surface under these conditions decreases the MT sliding velocity, and at the highest densities the sliding direction reverses (Fig. 2*A*), so that dense Cut7FL surfaces under these conditions drive only plus end directed sliding (Movie S7). By serially changing the buffer composition, dense Cut7FL surfaces can be switched from net minus end directed to net plus end directed force generation and back again (Fig. 2*C*, Movie S8).

Previous work has shown that directionality of Cin8 depends on motor surface density, MT length (9) and ionic strength (22). Based on the MT length dependence of directional switching, it was proposed that the important parameter for directional switching is the total number of motors engaged by a particular MT (9). For Cut7 we find that if we neglect the intervals during which

the sliding MTs pause, the velocity distributions for Cut7-driven sliding of short (<7.5μM) and long (>7.5μM) MTs are indistinguishable (Fig. 2*C*). Thus our Cut7 data, suggest that the key determinant of directionality is the level of crowding of Cut7 motor domains on the MT lattice. The ionic strength dependence of MT sliding direction is consistent with this idea, because ionic strength will influence the lattice occupancy of kinesin motor domains (23), but not their density on the coverslip. Overall, our results strongly suggest that the stepping direction of Cut7 motor domains is determined by their lattice density, indicating a proximity sensing mechanism.

Monomeric Cut7 motor domains generate only plus end directed sliding force. To further investigate the basis for Cut7 directional reversal, we engineered monomeric (single motor domain) Cut7 constructs, with and without the Cut7-specific ~65 residue N-terminal extension domain (Fig. 1*A*). Both constructs also had a C-terminal 6-his tag. N-terminally truncated Cut7|67-432 monomers linked via his tag antibodies to coverslips drive consistent plus end directed MT sliding at ~10 nm s^{-1}, independent of MT length (Fig. 2*D*, Movie S9). However this N-terminally truncated construct requires low ionic strength (30 mM PIPES) in order to function effectively – at higher ionic strengths, MTs dissociate. Cut7|1-432 monomers with an intact N-terminal extension also drive robust, plus end directed MT sliding (Fig. 2*E*; Movie S10). These data show that monomeric Cut7 motor domains generate only plus end directed steps, implying that minus end directed stepping by Cut7 requires linked pairs of motor domains, as in the intact half-tetramer. Our data also show that the Cut7-specific N-terminal extension domain is not required for plus end directed stepping. Our data are complimentary to recent work from Edamatsu (25), who showed that deletion of the Cut7 N-terminus does not abrogate *in vivo* function, but does affect MT affinity *in vitro*.

The extended N-terminal domain of Cut7 increases MT affinity but also adds drag. To check for an influence of the Cut7 N-terminus on MT affinity, we expressed the Cut7 N-terminal extension as a GFP fusion protein, and performed spin-down assays with MTs (see also (24)). GFP alone did not pellet with MTs, but GFP fused to the Cut7 N-terminus did (Fig. 3*A*), confirming that the Cut7 N-terminus binds directly to MTs. As a further check, we fused the Cut7 N-terminal domain to the N-terminus of kinesin-1. MT sliding velocity for wild type kinesin-1 is ordinarily independent of ionic strength. However with the Cut7 N-terminus attached, MT sliding velocity decreases at low ionic strength (Fig. 3*B*), indicating that the Cut7 N-terminal extension enhances MT binding, but at the expense of increased drag.

Structure of the Cut7 monomer in the MT bound AMPPNP state. Given that our motility assay data indicated a key role for Cut7 occupancy on the MT lattice in determining directionality, we felt it important to visualize the lattice-attached strong-state Cut7 motor domain. We used cryo-electron microscopy to visualize the motor domain in a force generating conformation. We calculated a 9.3 Å resolution reconstruction of Cut7|1-432 bound to MTs in the presence of AMPPNP, a non-hydrolysable ATP analogue, and identified individual secondary structural elements within the motor domain. Our analysis (Fig. 4, Movie S11) demonstrates that the overall conformation of the Cut7-AMPPNP bound to MTs

is very similar to that of the equivalent human kinesin-5 reconstruction (25) consistent with a canonical plus end directed power stroke. Specifically: 1) the major contacts between the motor and the MT are formed by the relay helix α4 (25, 26); 2) with AMPPNP bound, the highly conserved nucleotide sensing loops switch I and switch II adopt a closed configuration around the nucleotide (25, 26); 3) as a consequence, density corresponding to the Cut7 neck linker (red) is in a so-called docked configuration directed toward the MT plus end. However, several distinctive features of the Cut7 sequence compared to human kinesin-5 are also apparent, for example an elongated loop L10 at the plus end of the motor (orange) and a shorter and less distinctively curled loop L5 (pink) (25, 26). In addition, partial density corresponding to the Cut7-specific N-terminal extension is visible in our reconstruction (dark blue/cyan) emerging from the motor domain proximal to the neck linker. However, the N-terminus was sufficiently mobile in our reconstructions that we could only partially model it; indicative locations for its most proximal 10 amino acids are shown in Fig. 4 and Movie S11, only one of which (in dark blue) would allow formation of a cover neck bundle, the structure by which the neck linker and N-terminus of plus end directed motors are thought to collaborate to generate plus end directed force (25-27). Thus, our cryoEM 3D reconstruction of MTs bound by Cut7 establishes that Cut7 is like other kinesin-5 family members in the majority of its structural features, while also containing regions of structural divergence specific to Cut7. In particular, the connection between L10 and the distal part of the neck linker densities in our reconstruction indicates that L10 could play a role in regulating neck linker docking. Overall, our determined structure for Cut7 monomers bound to MTs in AMPPNP closely resembles that of the corresponding Eg5 complex, consistent with an Eg5-like plus end directed conformational cycle in which in AMPPNP the NL anneals into its docking station on the head. Part of the unusual extended N-terminus of Cut7 lies alongside the main part of the head, close to the docked NL, whilst the remainder appears mobile. Thus the N-terminal extension of Cut7 could serve to increase the space occupied by the motor domain and to boost its MT affinity, albeit in the context of the saturated MT lattice of our cryo-EM sample, direct contact of the N-terminus with the MT surface is not evident.

Crowding of Cut7FL with non-Cut7 proteins reverses MT gliding direction. Our finding that directional switching of Cut7 is correlated to lattice crowding suggests that lattice-attached Cut7 motor domains have a proximity-sensing mechanism. To account for this we propose a steric blocking model, in which steric clashes between lattice-engaged motors inhibit minus end directed stepping, but not plus end directed stepping, which we envisage to be less space-hungry. Potentially, the more space-hungry, minus end directed mode of Cut7FL might correspond to processive walking, as suggested by MT pivoting in sparse motility assays (Movie S6). Our steric blocking hypothesis makes a strong prediction, that crowding of Cut7 by a non-Cut7 competitor should also reverse its stepping direction.

To test this prediction, we first used dynein microtubule binding domain (MTBD) (28) as a crowding agent. Adding this non-Cut7 protein to a moderately dense Cut7FL surface reverses the direction of Cut7-driven MT gliding , so that without MTBD, MTs slide with their plus ends leading, (Fig. 5*A*) and with MTBD they slide

with their plus ends trailing (Fig. 5*B*, Movie S12). Raising the ionic strength reverts the sliding direction (Fig. S3). This confirms that a non-Cut7 competitor can obstruct Cut7 and reverse its stepping direction. To test whether a non-Cut7 kinesin can do the same, we used Klp2, a kinesin-14. MTs glide across Klp2 surfaces with their plus ends leading (Fig. 5*D*, Movie S13), independent of Klp2 density. Nonetheless, adding Klp2 to a suitably dense Cut7FL surface does reverse the MT gliding direction (Fig 5*E*, Movie S13), showing that indeed, Klp2 can crowd Cut7 and convert it from minus to plus end directed stepping, despite itself stepping towards minus ends. Together, these data confirm that not only CutFL, but also non-Cut7 competitors, can crowd Cut7FL and switch its stepping direction from minus to plus, consistent with our steric blocking hypothesis.

Our proposed steric blocking model for directional reversal offers a framework within which previous, apparently conflicting, proposals for the basis of kinesin-5 bidirectionality might be reconciled. Thus, the reported buffer-dependence of Cin8 directional switching (15) (which we also find for Cut7) can potentially be due to lower ionic strength favouring increased occupancy of the MT lattice. The dependence of Cin8 directional switching on the sequence of loop 8 (15) and on the phosphorylation of the motor domain (16) might have a similar explanation. The N-terminal extension of Cut7 enhances the MT affinity of the motor domain and so will increase occupancy. Hackney and colleagues previously reported that the extended N-terminus of BimC also enhances MT binding (24). For Cin8 it was previously shown that varying the density of motors on the coverslip surface in motility assays can reverse the direction of MT sliding, leading to the proposal that motor–motor coupling was occurring (9). Our findings suggest a specific mechanism for motor-motor coupling, namely mutual steric interference. It has also been proposed that the crosslinking of two MTs by Cin8 tetramers switches Cin8 from minus end directed to plus end directed motility (22). For Cut7 we can rule this out because we see bidirectional sliding of MTs crosslinked by Cut7. However it is likely that conditions favouring MT crosslinking would also favour lattice crowding, which in our model would in turn favour directional switching. In recent work, Düselder and colleagues show that the Cin8 tail domain is required to regulate its directional switching, because a chimeric Cin8 dimer with a kinesin-1 tail is bidirectional but in an unregulated way. Again our proposed steric blocking model is consistent, in that directionality is regulated by an emergent property, steric interference between neighbours. The earlier data of Edamatsu (19) on the effect of truncation on the directionality of Cut7 are also consistent.

Our finding that directional reversal of Cut7 depends on steric clashes with neighbours adds a surprising new dimension to recent work on the role of "traffic jams" on MTs in the feedback control of molecular motor activity (29), by revealing that the microtubule sliding direction of a mixed population of kinesins can potentially reverse, depending on the pattern and extent of mutual steric interference. Recent optical trapping measurements have shown that for Eg5, which generates plus end directed sliding force and braking force, the forces scale according to the number of engaged motors, showing that each motor has no effect on the forces produced by its neighbours (30). Our work shows that for Cut7, the opposite is true. The action of each motor is strongly dependent on the proximity of its neighbours, and sliding force will scale in a complicated way,

with not only the magnitude but also the direction of the force critically dependent on the proximity of neighbouring motors, and potentially on that of non-motor microtubule binding proteins also.

Methods

Constructs and Proteins. Cut7FL (1085 aa) was PCR amplified from *S. pombe* genomic DNA and cloned into pET17b between NdeI and SacI sites incorporating a C-terminal His6 tag and N-terminal GST tag, cleavable by PreScission protease. Cut7FL cDNA in pET17b was digested with NotI-HF and NdeI (New England Biolabs) and the insert subcloned into PGEX 6P-2, modified by inserting a unique Nde I site immediately following the PreScission site using the Quickchange lightning system (Agilent). Expressed Cut7 proteins were purified initially via their His6 tag according to (32). The resulting fraction was loaded on to an equilibrated 1 ml GSTrap-HP Column (GE) and washed extensively with KPEM100 plus 100 mM NaCl, 1 mM ATP and 10 mM β-mercaptoethanol. 100U PreScission protease (GE) in 1.2 mls of column buffer was run into the column, which was then capped and rotated overnight at 4°C. Next day, groups of eluted fractions were desalted using Zeba columns (Pierce) into either KPEM100 or KPEM100 plus 100 mM NaCl, 10 mM β-mercaptoethanol and 1 mM ATP. KPEM100 contained 100mM PIPES, 1 mM $MgSO_4$, 2 mM EGTA adjusted to pH 6.8 with KOH. For glycerol gradients, the protein was eluted from the GSTrap-HP column at high concentration using a pulse of 50 mM NaCl, 1 mM ATP, 10 mM β-mercaptoethanol and 2 mM $MgCl_2$, 20 mM glutathione, 20 mM Tris HCl pH 8.0 and digested with 100U PreScission protease in a final volume of 1.2 ml. This digest was loaded directly on to glycerol gradients (32). 260 µl fractions were harvested using a 1 ml Gilson Microman positive displacement pipette. For high salt glycerol gradients the above buffer was supplemented with 400 mM NaCl. Protein concentrations were estimated using SYPRO red staining (Life Technologies).

For Cut7 monomeric constructs, residues 1-432 of Cut7 (Cut7|1-432) were PCR amplified from *S. pombe* genomic DNA and cloned into a pET151D-TOPO® vector (Invitrogen), adding a TEV protease-cleavable N-terminal His6-tag. The recombinant His6-tagged protein was expressed in BL21*(DE3) *E. coli* cells in LB media, supplemented with 2% glucose upon induction with 0.5 mM IPTG at 18°C for 5 hours. Cells were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 400 mM NaCl, 1 mM $MgCl_2$, 1 mM ATP, 5 mM 2-mercaptoethanol, 10% v/v glycerol, 0.2 mg/ml lysozyme, 0.1 mg/ml DNAase I, EDTA-free Protease Inhibitor Cocktail (Roche)) and lysed by sonication. Tagged Cut7-MD was purified from the clarified cell supernatant using nickel affinity. The His6-tag was removed using TEV protease and Cut7-MD exchanged into 25 mM PIPES, pH 6.8, 30 mM NaCl, 7 mM $MgCl_2$, 1mM EGTA, 1 mM 2-mercaptoethanol before use in the cryo-EM experiments. For Cut7 monomer gliding assays, an additional monomeric construct, residues 67-432 of Cut7 (Cut7|67-432), was cloned into a pET151D-TOPO® vector introducing a second, non-cleavable C-terminal His6-tag using Site-directed Ligation-Independent Mutagenesis (SLIM) (33). This recombinant, doubly His6-tagged protein was expressed in BL21*(DE3) E. coli cells in LB media, supplemented with 1% glucose upon induction with 0.1mM IPTG at 25°C for 6 hours. Cell pellets were resuspended in lysis buffer (as above) and doubly

tagged Cut7|67-432 purified from the clarified lysis supernatant using nickel affinity chromatography. The N-terminal His6-tag was removed by cleavage with GST-tagged TEV Protease (Sigma) and the cleaved protein separated from the TEV protease using glutathione agarose chromatography. A further doubly His6-tagged Cut7|1-432 construct was cloned in a similar way but to generate proteolysis-protected Cut7|1-432, an additional StrepII tag was inserted immediately before its N-terminus after the TEV cleavage site. This Cut7|1-432 construct was purified using nickel affinity chromatography, followed by TEV cleavage of the N-terminal his-tag and a second purification step using streptavidin beads. The protein was eluted using 5 mM Desthiobiotin in the purification buffer (50 mM Tris, pH 8, 400 mM NaCl, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM ATP) and concentrated for further use. Residues 1-66 of Cut7 (Cut7|1-66) were C-terminally fused with EGFP (followed by a stop codon) by introduction of in-frame BamHI and EcoRI sites in the Cut7|1-432 pET151D-TOPO® vector. This fusion protein was expressed and purified as for Cut7|1-432. EGFP alone was also cloned into pET151D-TOPO® vector, expressed and purified in the same way and used a negative control in the MT binding assay. The N-terminal segment of Cut7 was fused to full length *Drosophila* kinesin-1 using an NEB Gibson kit, and the same vector used to host Cut7FL. The expressed protein was purified and processed as for Cut7FL. Klp2 (31) and dynein p111 MTBD fusion (28) proteins were expressed and purified as previously described .

Polarity Marked biotinylated MTs. GMPCPP seed assembly mix used unlabelled stock tubulin supplemented with either 7% (Hylite 488 TL488M/TL670M/TL590M) tubulin or 10% Biotinylated tubulin (T333) (Cytoskeleton.com) as needed. In protocols using dim seeds, the mix contained 1% label. Seed assembly mix was prepared by mixing stock tubulin into K-PEM-100 buffer, 10 mM β-mercaptoethanol, 1mM GMPCPP to 20 μM tubulin concentration, incubating on ice for 30 min, clarifying at 90,000 g for 5 min and snap freezing the supernatant in 2.5 μl aliquots in liquid nitrogen. To assemble seeds, the thawed seed mix was diluted to 5 μM tubulin in assembly buffer and incubated at 37 °C for 30-45 min in a total volume of 10 μl. Elongation mix was prepared identically to seed mix except that the tubulin was differently fluorescently labelled and stored in 20 μl aliquots. To elongate seeds, elongation mix was diluted to 1 μM tubulin, prewarmed at 37°C and then added to 10 μl of seeds, in a final volume of 200 μl. Elongation was continued at 37 °C for 1.5 to 2 hr. The resulting polarity-marked polymerised MTs were diluted to 0.6 μM (to minimise end-to-end annealing) and held at room temperature. MTs were pelleted in the Airfuge (Beckman Coulter, Inc.) for 5 min, resuspended in gliding buffer (for gliding assay) or K-PEM-100/200/300 (for studying MT bundling) with 20 μM paclitaxel at 0.6 μM or 1.2 μM final tubulin heterodimer concentration and used within 1 hr.

MT Gliding assays. Slides (L4244) and coverslips (L4095-2) (Menzel-Glaser, Agar Scientific) were soaked in 10 M NaOH for 30 mins with shaking, transferred to staining racks, rinsed extensively in Millipore water until pH 7.0 was attained, then transferred to 0.25% Neutricon and soaked for 20 min at 75°C with intermittent sonication, followed by 20 min in Millipore water, again with intermittent sonication. This cycle was repeated twice, followed by extensive washing with Millipore water. Slides and coverslips were stored in Millipore

water at 4°C and used within 3 days, drying using a Spin Clean (Technical Video) before use. Flow cells were made from cover slips adhered to slides using two parallel strips of double-stick tape and sealed using silicone grease (Beckman Coulter). Gliding buffer was 0/100/200/300 mM NaCl, 10 mM β-mercaptoethanol, 10 mg/ml BSA and 20 μM paclitaxel, 1 mM GMP-CPP, 4/1/0.1 mM Mg.ATP, oxygen scavenger (20 mM glucose, 0.2 mg/ml catalase, 0.4 mg/ml glucose oxidase) in KPEM100. Gliding buffer was stored at -80 °C and aliquots freshly thawed for each flow cell. Flow cells were rinsed with 5 volumes of primary mouse anti-hexa histidine antibody (MA1-21315, Thermo Scientific) at 1mg/ml, incubated for 10 minutes and flushed and blocked with gliding buffer. Alternatively, antibody was added at 0.1 mg/ml and only 1 flow cell volume introduced to create a lower-density antibody surface. Kinesin solutions were introduced and incubated for 5 min, then flushed with gliding buffer or K-PEM-100/30/15. MTs in gliding buffer were then introduced at 0.6 μM or 1.2 μM final tubulin heterodimer concentration.

MT sliding in bundles. Flow cells were coated with 5FCV Neutravidin (5 mg/ml) by incubating for 10 min, then rinsed and blocked with gliding buffer for 10 mins. 0.6 μM polarity marked biotinylated MTs (2.5 μl) were mixed with 0.6 μM polarity marked non-biotinylated MTs (7.5 μl) and 1.1 μM Cut7FL and immediately injected into the flow cell. Following 10 min incubation the flow cell was flushed with 5 volumes of gliding buffer in KPEM100 + 100 mM NaCl.

Glycerol Gradients. Glycerol Gradient centrifugation was done as previously described (31).

TIRF Microscopy and analysis. Images were acquired at 25 °C with a humidified environmental stage top incubator (Okolab, Ottaviano, Italy) on an Olympus CELLR/TIRF microscope using a Hamamatsu ImageEM EMCCD camera with a 100x NA 1.49 objective, with or without 1.6x auxiliary magnification giving 160 nm or 100 nm pixels in a 512x512 field. Polarity marked MTs were visualised using 488 nm and 561/640 nm laser lines. MT gliding velocity was measured using RETRAC (32) and/or Metamorph.

MT spin-down assays. Cut7 1-66-eGFP and the eGFP control protein were buffer-exchanged into 25 mM PIPES, pH 6.8, 30 mM NaCl, 7 mM $MgCl_2$, 1 mM EGTA, 1 mM 2-mercaptoethanol and a range of concentrations of each protein titrated against a constant concentration of Paclitaxel (Calbiochem)-stabilised bovine brain tubulin MTs (Cytoskeleton, Inc.). Following incubation at room temperature, the mixture was centrifuged at 392,000g for 10 mins in a Beckman benchtop ultracentrifuge and supernatant and pellet fractions analysed by SDS-PAGE. Protein bands were visualised using SYPRO-Red (Invitrogen) and a Fuji FLA3000 scanner.

Electron microscopy, image analysis and atomic model building. MTs were polymerized from bovine brain tubulin (Cytoskeleton, Inc.), at a final concentration of 5 mg/mL, at 37 °C for 1.5 hr in a buffer containing 100 mM MES pH 6.5, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and 2 mM GTP. MTs were stabilized with 1 mM Paclitaxel in dimethyl sulfoxide for a further 1.5 hr at 37 °C. Cut7|1-432 was incubated with 5 mM AMPPNP for 45 min on ice and mixed with a 10-fold molar excess of MTs (2 μM) for 5 min at room temperature. Subsequently,

3.5 µl were applied to glow-discharged C-flat holey carbon grids (Protochips Inc.) at 24 °C and 100 % humidity, blotted and plunged into liquid ethane (Vitrobot, FEI Company). Low dose micrographs were recorded using a Tecnai F20 FEG microscope (FEI Company) operated at 200 kV, at 0.7-2.6 µm defocus and 68,000x magnification on a 4k x 4k CCD camera (Gatan) with a sampling of 2.2 Å/pixel.

The 3D reconstruction was calculated using a previously described custom single particle procedure (33, 34). 144,300 asymmetric units (Cut7 motor domain bound to an αβ-tubulin dimer) were boxed along 233 13-protofilaments MTs (BOXER, (35)) and the seam orientation was determined by projection matching using custom SPIDER scripts (36) and refined with FREALIGN (37). The final reconstruction was calculated using particles that gave an isotropic angular distribution and by taking into account pseudo-helical symmetry using FREALIGN (37). The resolution was estimated at 9.3 Å using the Fourier Shell Correlation 0.5 criterion. An asymmetric unit of the MT-bound Cut7 reconstruction will be deposited in the EMDB on acceptance for publication.

An initial homology model of the AMPPNP-bound Cut7 motor (residues 70-432) based on human K5-AMPPNP-bound structure (PDB ID 3HQD) was prepared using Modeller. The coordinates of cut7 motor domain bound to an αβ-tubulin dimer (PDB ID 1JFF) were rigidly fitted into the cryo-EM map using UCSF Chimera (38) and refined by flexible fitting using Flex-EM (39). Structural models of loop5 and loop10, respectively shorter and longer than in human K5, were generated using Modeller and selected on the basis of the highest cross-correlation value. The conformation of the neck-linker and the N-terminus were calculated using a conjugate-gradient energy minimization approach implemented in Flex-EM and selected according to the same criterion. Five clusters of N-terminus conformations were produced by ranking 9 models based on the CC value and RMSD clustering Cα cut-off of 1Å. The final cut7 motor domain model was energetically minimized and its stereochemistry was checked. The atomic model will be deposited in the Protein Data Bank on acceptance for publication.

Acknowledgements. CM, AG, SR thank Charles Sindelar (Yale University, USA) for reconstruction algorithms and the BBSRC (BB/H005137/1) and AICR (11-0261) for funding. We thank Andrew Carter for the dynein MTBD fusion construct, and Douglas Drummond and Gillian Fox for the initial Cut7 construct in pET17b. We thank Naomi Sheppard for expressing and purifying the dynein fusion protein. RAC is a Wellcome Senior Investigator (103895/Z/14/Z).

Figure Legends

Fig. 1. Cut7FL (*A*) was subjected to glycerol gradient centrifugation (*B*) in (Left) KPEM100 buffer (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.9) plus 300 mM NaCl and (Right) KPEM100 only to determine its S-value. (*C,D*) Cut7-driven gliding of (magenta) polarity-marked cargo MTs on (green) polarity-marked rail MTs in KPEM100 plus 100 mM NaCl, 4 mM MgATP. (*C*) Magenta cargo MT slides slowly right-to-left along green rail MT, indicating plus end directed motor activity. Note MTs are parallel, implying that one end of the Cut7 tetramer is moving faster than the other. (*D*) Magenta cargo MT slides rapidly left-to-right

with its plus end leading. MTs are antiparallel, so sliding must be driven by minus end directed force. At the end of its travel, the cargo MT pivots for several seconds with its tip attached to the tip of the rail MT. Scale bars 5 μm. See Movies S2-4. The motor locations in the cartoons are speculative.

Fig. 2. *In vitro* motility. (*A*) Directional reversal by increasing the surface density of Cut7FL. Increasing motor density slows MT sliding and ultimately drives directional reversal. Note expanded velocity axis for plus end directed stepping. The concentrations shown are the solution concentrations of Cut7FL used to prepare the surfaces. Control experiments (*B*) show that this concentration scales predictably with the MT landing rate, which in turn is proportional to the surface density of active motor. (*C*) Lawns of Cut7FL motors can be converted back and forth between plus and minus end directed stepping by serially changing the ionic strength. All data are from the same flow cell with a dense surface of Cut7FL. Initial buffer KPEM100 gliding buffer, 1 mM ATP (see Methods), then same plus 100 mM NaCl, then same plus 200 mM NaCl (note directional reversal). Finally, flushing with KPEM30 gliding buffer, 1 mM ATP reverts the motor to plus end directed stepping. Data from short (<7.5 μm) and long (>7.5 μm) MTs are very similar. A MT length of 7.5 μm was chosen to split the data approximately in half. See also Movie S8. (*D*) In 30 mM PIPES pH 6.9, 0.1 mM ATP, Cut7 | 67-432 monomers drive MTs to slide with plus ends trailing, independent of MT length and motor density. See also Movie S9. (E) Cut7 | 1-432 monomers also drive MTs to slide with plus ends trailing (buffer 100 mM PIPES pH 6.9, 0.1 mM MgATP). See also Movie S10.

Fig. 3. (*A*) Spin-down assay showing that Cut7 N-terminal domain C-terminally fused to GFP (filled circles) binds to MTs, whilst GFP alone (open squares) does not. Buffer: 25 mM PIPES, pH 6.8, 30 mM NaCl, 7 mM $MgCl_2$, 1 mM EGTA, 1 mM 2-mercaptoethanol (*B*) MT sliding driven by kinesin-1 dimers fused to the Cut7 N-terminus. MTs move slowly (above) in KPEM100 gliding buffer, and faster (below) in KPEM100 + 250 mM NaCl, gliding buffer, 1 mM ATP.

Fig. 4. Structural analysis of MT-bound Cut7 heads. 9Å Cryo-EM reconstruction and pseudo-atomic model of the MT-bound Cut7-AMPPNP motor domain. (*A, B*) Cryo-EM reconstruction shown in pale yellow surface with density corresponding to the proximal region of the N-terminus shaded in cyan. With AMPPNP bound, the Cut7 neck-linker (red) is docked towards the MT plus end, while density corresponding to the extended N-terminus could contribute to formation of a cover-neck bundle (Nter, in blue) but the majority is flexible; exemplar alternative conformations are shown in cyan. (*B*) View towards the nucleotide binding site. The kinesin-5 specific L5 (pink) protrudes from the surface of the motor and density corresponding to the Cut7-specific elongated L10 (orange) at the plus-end of the motor is clearly defined and close to the docked neck-linker. (*C,D*) Comparison of the Cut7-AMPPNP model (rainbow) and human kinesin-5-AMPPNP (K5, position of key loops indicated in dashed black), superimposed on helix-H4. Cut7-L5 (pink) adopts a distinct conformation compared to K5-L5, while Cut7-L10 is 17 residues longer compared to K5-L10. The MT plus-end is towards the top of this figure. See Movie S11.

Fig. 5. Non-Cut7 competitors can drive directional reversal of Cut7. (*A*) 0.5 μM Cut7FL surface. MTs slide with their plus ends leading. (*B*) Same plus MTBD . MTs slide with their plus ends trailing (*C*) 1.1 μM Cut7FL surface. MTs slide with their plus ends leading. (*D*) 0.35μM Klp2 surface. MTs slide with their plus ends leading. (*E*) Cut7FL + Klp2 surface. MTs slide with their plus ends trailing. Conditions: (*A, B*) KPEM100 gliding buffer, 1 mM ATP (*C-E*) same + 150 mM NaCl. The given motor concentrations are the solution concentrations used to make the surfaces. See Movies S12, S13 , Figs. S2, S3.

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