Prime movers: the mechanochemistry of mitotic kinesins

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Preface

Mitotic spindles are self-organising protein machines that harness teams of multiple force generators to drive reliable chromosome segregation. Kinesins are key members of these force-generating teams. Different kinesins walk directionally along dynamic microtubules; anchor, crosslink, align and sort microtubules into polarized bundles; and influence microtubule dynamics by interacting with microtubule tips. The mechanisms of these kinesins are specialized to allow each type to make a specific contribution to spindle self-organisation and chromosome segregation. Here, we review recent progress in our understanding of the mechanochemical mechanisms of these mitotic kinesins and how they contribute to the key events of spindle assembly.

Introduction

Mitotic spindles are highly complex protein machines, assembled once per cell cycle in order to segregate chromosomes accurately to the two daughter cells (FIG. 1). The remarkable ability of the spindle machinery to self-organise **[G]** [1-3] and adaptively re-organise[4] derives from the force-generating interactions of dynamic microtubules with the microtubule molecular motors kinesins and dynein.

Together with dynein, the mitotic kinesins constitute a kind of society or eco-system of interacting force-generators, having relatively simple individual behaviours but complex collective behaviour. There is evidence that the spindle contains multiple motor-driven subsystems with overlapping, balanced functions, each contributing slightly different engineering approaches to the same problem. For example, it is clear from experiments in yeast that there is functional antagonism (force-balance) between pairs of oppositely-directed mitotic kinesins, such that the deletion of one motor activity abrogates spindle function, and the further deletion of an oppositely-directed motor rescues function[5, 6]. This property of the spindle as a collection of balanced systems has allowed the field to construct several useful models[7-9] for spindle mechanics (see[7] for a recent review), using the known and inferred single molecule mechanical properties of various key players. The task facing the field now is to extend this approach, by learning more about the detailed mechanisms of individual force generators and by carefully analysing how these mechanisms interact with each other and with regulated microtubule dynamics to produce the emergent self-organisation[3] and complex, adaptive mechanics of the full spindle machinery. Moreover, the mechanisms of several mitotic motors are emerging as potential targets for anti-cancer therapeutics (BOX 1).

This task of delineating the detailed mechanisms of cytoskeletal molecular motors is important because motorized self-organisation is a central feature of eukaryotic life. There is evidence that kinesins, together with actins, myosins, dyneins and tubulins, were all present at the root of the eukaryotic tree of life, 2.2 billion years ago[10]. Spindle subsystems and the balance between their activities have therefore had a long time to evolve and diverge, and the eukaryota have developed a wide variety of approaches to chromosome segregation, some of which work quite differently to the mainstream model systems (*Xenopus laevis*, *Drosophila melanogaster, Caenorhabditis elegans, Schizosaccharomyces pombe, Saccharomyces cerevisiae* and mammalian cells) that are studied by the field[11]. The common threads that link apparently divergent systems are the mechanochemical mechanisms of the individual motors. Recent work using *in vitro* reconstitution of kinesin–microtubule interactions has revealed unexpected new complexity even at this level, throwing new light on spindle biology and emphasizing the urgent need to ground the field firmly in knowledge of the mechanics of the individual motors and subsystems of the spindle.

With this in mind, we focus here on recent progress in defining the individual mechanisms of the mitotic kinesins as they interact with dynamic microtubules and how they contribute to key mechanistic events during bipolar spindle assembly and chromosome segregation in animal cells. (FIG. 1). Kinesin motors in general haul cellular traffic towards the plus ends of microtubules **[G]**, but some kinesin superfamily members move in the opposite direction and some modulate microtubule dynamics. The other key player in spindle dynamics is cytoplasmic dynein, which hauls cargo towards minus ends of microtubules **[G]**, in the opposite direction to most kinesins. Dynein has important functions in mitosis (FIG. 1) but space precludes a review of its mechanochemistry here. The reader is referred to a recent review of dynein mechanism[12].

Structure and domain organization of mitotic kinesins

Kinesins are modular. Each kinesin consists of head domain(s) (also known as motor domains; each of ~300 residues) joined to a tail that typically consists of sections of alpha helical coiled coils, interspersed with unstructured, natively unfolded[13] sequences. Two different naming conventions for the kinesins are in simultaneous and widespread use. A simplified classification defines 14 major subfamilies of kinesins[14], albeit recent work indicates that this scheme may need expansion[10]. The KIF classification[15] is finer grained but refers only to mammalian kinesins. Figure 2 shows the topology of those kinesins that are known to have a role in mitosis. Kinesin heads are built on a structurally-invariant beta sheet backbone, flanked by alpha helices. The active site is on one side of this backbone, and the microtubule-binding interface on the other (FIG. 2c). This topology is recognizably similar to RAS family small G-proteins and to myosin heads; indeed, both kinesin and myosin are thought to have evolved from an ancestral small G-protein[16].

Kinesins, myosins and small G proteins are all P-loop ATPases, in which docking of Mg•ATP against the P-loop element causes the Switch 1 and 2 elements that flank the active site to close together into a catalytically active configuration (FIG. 2b). The P-loop contains the highly conserved GXXXXGK(T/S) Walker A sequence motif. The Switch 1 contains a characteristic SSRSH motif, and Switch 2 contains a LAGSE motif. During ATP hydrolysis, a critical salt bridge links these two elements, forming the phosphate tube. This salt bridge positions two water molecules that are proposed to split the beta–gamma phosphate bond of ATP by extracting a proton[17]. The Mg^{2+} ion of Mg•ATP is essential for catalytic activity of the kinesin ATPase. The Mg^{2+} ion bridges the beta and gamma phosphates of ATP and, once docked into the active site, is coordinated by residues from the P-loop and (both directly and via water molecules) from the two switch elements[17] (FIG. 2b).

Kinesin tails can be very different in length (the longest is the tail of the kinesin-7 family member KIF10 (also known as CENP-E) at 230 nm[18]) (FIG. 2a). The tails contain recognition sequences for co-proteins, regulatory kinases and cargo[19]. For some kinesins, such as KIF10, hinges allow the motor tail to bend back on itself, contact and inhibit the heads[20] Most kinesins have a recognizable neck linker (kinesin-6[21] family members are an exception), a short sequence at the C-terminus of the head domain that in all kinesins other than those of the kinesin-14 subfamily (KIFC1 in mammals) links the motor domain to its coiled-coil tail.

Between different subfamilies of kinesins, the heads have the strongest sequence similarities, whereas the tails are more divergent. Even within the heads, however, there are subfamily-specific features, with some subfamilies containing large inserts of uncertain function in specific surface loops. N-terminal extensions of the head are present in most subfamilies.

In summary, the kinesins share a common active site geometry and P-loop ATPase enzymatic mechanism with G-proteins and with myosins. This mechanism is harnessed by different kinesins to generate force and motion in the direction of the plus or minus ends of microtubules, to drive binding and unbinding of microtubules, or to trigger microtubule catastrophe **[G]**.

Generic features of the kinesin mechanism

Kinesins use a generic ATPase mechanism to drive subfamily-specific activities. In this section we briefly review this generic mechanism, which is based on work largely carried out on the non-mitotic Kinesin-1 family (human KIF5B).

A cycle of ATP turnover drives a mechanical cycle

Many aspects of the mechanism(s) of mechanochemical coupling in kinesins remain to be determined, but it is clear that in simplified form the mechanical cycle consists of ‘diffusional

search', 'bind', 'kick' (conformational change(s)) and 'release' phases (FIG. 3) and that this mechanical cycle is driven by the biochemical cycle of ATP turnover. A key feature of the mechanochemical coupling is that the binding of the kinesin motor domain to microtubules markedly increases the ATPase activity of the kinesin. ATP turnover by kinesin heads is essentially blocked until they bind to microtubules. Once they do bind to a microtubule, mechanical and chemical events alternate, with each event being dependent to some extent on the completion of the preceding event. For all kinesins (and for dyneins and myosins also), a second generic feature of mechanochemical coupling is that the 'ground state' (the most stable state) of the motor is the apo (empty) state, also known as the rigor state, in which the motor is strongly bound to the microtubule and there is no nucleotide in its active site. The subsequent binding of ATP (in the form of Mg•ATP) at the active site and its conversion to ADP destabilizes this ground state, relaxing the grip of the motor on its microtubule track and allowing it to unbind and move to a new site.

The active site Mg^{2+} ion is required for ATPase activity and is key to another generic feature of the kinesin mechanism, the establishment of a trapped-ADP state in which Mg•ADP is stably bound into the kinesin active site. In the absence of microtubules, the ATPase cycle pauses at this trapped-ADP state, such that purified kinesin motors (and most kinesin crystal structures) tend to retain Mg•ADP in their active sites. In kinesin-1, auto-inhibition of the head by the folded tail super-stabilises this trapped-ADP state [22]. Kinesin heads in the trapped-ADP state bind only weakly (unstably) to microtubules (FIG. 3a, step 2). But once in the weakly bound state, they gain access to a strongly bound state in which microtubule binding activates Mg^{2+} release from the kinesin active site[23, 24], triggering ADP release and converting the motor heads into their apo ground state (FIG. 3a, steps 3 and 4). Microtubule binding typically accelerates the basal cycle of ATP turnover by 10^3 fold or more. For some mitotic kinesins, Mg•ADP release can also be activated by free tubulin heterodimers[25]. Once Mg•ADP is released, the active site of the kinesin is in its apo state, and a new cycle of turnover can begin. For most, but not all[26, 27] kinesins (below), ATP binding to the apo state does not immediately relax the grip of the motor on the microtubule (FIG. 3a, step 2). Instead, the ATP must first be hydrolysed, and the product phosphate released, leaving Mg•ADP in the active site (FIG. 3a, step 4). Both hydrolysis and phosphate release have conformational effects on the kinesin head, but the evidence so far indicates that it is phosphate release that drives the major change, resulting in collapse of the phosphate tube[17, 27-29] (FIG. 2b) and conversion of the kinesin head from strong (stable) to weak (unstable) microtubule binding[23, 30].

The mechanical cycle generates force and movement

Two main types of mechanism are envisaged to explain how track-following molecular motors such as kinesins can generate force and movement: conformational change (lever arm) mechanisms and biased binding (Brownian ratchet) mechanisms. In a conformational change

mechanism, impulses of force and motion are produced by one or more shape changes in the kinesin motor head that occur after it attaches stably to its track. For kinesins, there is firm evidence for the lever-like docking action of a neck linker domain[31] (FIG. 3), and for rocking and twisting of the entire kinesin head[32, 33]. The neck linker changes conformation by docking reversibly into a docking station on the main part of the head, with the docked state being further stabilized by an N-terminal cover strand that overlies the docked neck linker (FIG. 2d) to an extent that varies between kinesins. Docking of the neck linker is favoured by nucleotide binding and to a greater extent by ATP binding than by ADP binding[31, 34;35]. In kinesin dimers, the bias towards forward stepping is thought to be due at least in part to neck linker docking. The docked neck linker lies almost exactly along the microtubule axis, so that docking can exert a lever action (FIG. 3b)[26, 31], both steering the tethered head towards its next site and generating a force impulse directed to the microtubule plus end. Mutations that disrupt neck linker docking in kinesin-1 monomers strongly inhibit motility in microtubule sliding assays[36]. Cover strand mutations in kinesin dimers reduce stall force **[G]** but not unloaded velocity **[G]**[26], indicating that the ability of the motor to do work is specifically affected by cover strand mutations.

By contrast in biased binding models, the kinesin head produces force and movement by scanning along its microtubule track, detecting its position relative to the microtubule, and binding/unbinding with a directional bias[37]. A biased binding model was initially proposed to explain muscle crossbridge action[38]. There is some evidence that the binding[39] of individual kinesin heads by microtubules is directionally biased[40, 41], but most current kinesin models emphasise the conformational changes that occur following tight binding of the motor head to the microtubule[30] as the main means by which directional impulses of force are produced[24, 26, 31, 33]. In our view, it is likely that the actual mechanism involves both processes.

Processive and non-processive kinesins

Processive kinesins 'walk' along microtubules as single molecules (FIG. 3a, FIG.4). In these kinesins, the intrinsic diffuse–bind–kick–release cycles (FIG. 3b) of the two kinesin heads are coordinated so that one head supports the load whilst the other is free to move to its next microtubule binding site, establish itself, and then reverse roles with its partner. Figure 3a shows a generic processive (walking) kinesin, based on kinesin-1, but with major features in common with all the kinesins that are capable of single molecule walking under load (examples among the mitotic kinesins are members of the kinesin-5 and kinesin-7 families; below). Walking is controlled by two gating mechanisms. At the ATP-gate **[G]**, the kinesin pauses with one head (shaded in FIG. 3a) attached to the microtubule in an apo state, and the other unattached head (white) in a trapped-ADP state (step 1). This so-called ATP waiting state of the kinesin dimer redevelops after each step and serves to resynchronise the ATPase cycles of the two coupled heads. Forward stepping is dependent on, and triggered by, ATP binding to the apo head (Fig. 3a, steps 1 and 2). This ATP-gating process licenses or drives

the other head to undergo tethered diffusion to its next binding site, 8 nm away along the microtubule in the progress direction, potentially guided by neck linker docking (below). Backsteps and slips are rare except at high backwards loads, and represent a failure of the directional biasing mechanism. Backsteps require ATP (although nucleotide-independent slips can occur[42]). Stall occurs when the backwards load is such that progress stops, because the probability of forward steps equals the probability of backward steps[43, 44]. At the strain-gate **[G]**, backwards tension acting on the leading kinesin head of a walking dimer inhibits ATP binding[45], giving the other head time to cycle round to its ADP-bound state and detach from the microtubule (FIG. 3a, steps 4 and 5). Forwards tension on the rear head can accelerate its detachment, but only by about twofold[46]. Interhead tension enables communication between the two heads and is a major determinant of processivity in walking kinesins[47].

Nonprocessive kinesins cannot walk as single molecules, either because they only have a single head, or because they have head-pairs but lack the necessary interhead coordination. Instead, nonprocessive kinesins (such as KIFC1 (kinesin-14 family); FIG. 2a) need to operate in teams so that the team can move continuously whilst holding force, even though its individual members cannot. Members of the team can then release their grip on the microtubule but remain tethered to the cargo and use tethered diffusion to find a new binding site without the team as a whole losing traction. We refer to this as 'hopping' (FIG. 3b). Many kinesins can also use untethered one-dimensional diffusion to scan the microtubule surface over distances of a few microns and rapidly find a target site, for example the microtubule tip[48, 49].

In summary, both processive and nonprocessive kinesins generate force and movement by coupling a chemical cycle of ATP turnover in their active sites to a mechanical cycle of tethered diffusion, microtubule binding, conformational change(s) and microtubule release. This mechanochemical cycle enables the kinesins to function as tractors (cargo-transporters), winches (microtubule-sliders) or assemblers (drivers of microtubule dynamics) (FIG. 1 and 4). Combinations of these activities are possible, either within one motor or within teams of different motors.

Understanding the ways in which spindle self-organisation and dynamics emerge from the interactions of kinesins with dynamic microtubules demands that we understand the special force-generating characteristics of each type of mitotic kinesin. For a discussion of the functional diversity contributed by the tail domains of mitotic kinesins, see[50]. We now describe kinesin subfamily specialisations in the context of specific mitotic processes.

Centrosome separation

The key motor-dependent event during prophase **[G]** is the movement of the duplicated centrosomes (future spindle poles) to opposite sides of the nucleus (for a review see[51]). This motion (green arrows in FIG. 1a) absolutely requires KIF11[52], which is the only member of the kinesin-5 family in humans[53]. KIF11 is built from two homodimers arranged in an antiparallel manner so that identical pairs of heads project from each end of the tetramer, forming a microtubule cross-linker[52, 54, 55]. The C-terminal tip of the KIF11 tail also contributes to microtubule binding[56]. KIF11 can therefore crosslink the microtubules that project from one centrosome towards the other. *In vitro* KIF11 drives the sliding apart of microtubules that are orientated in such an anti-parallel configuration, thus explaining how the motor drives centrosome separation (FIG. 1a, *inset 1*, FIG. 4c)[52, 57]. Moreover, experiments in *S. cerevisiae* show that tetramerisation of kinesin-5 (Cin8p) – and presumably the anti-parallel sliding activity - is required for the *in vivo* function of the motor[58].

At zero-load, single tetramers of KIF11 move processively along single microtubules for ~100 nm at ~35 nm per second, towards the plus-end of the microtubule[59]. Experiments in *X. laevis* extracts show that the rate of spindle assembly is limited by this rate of KIF11-driven microtubule sliding[60]. Crosslinking between neighbouring microtubules mediated by KIF11 switches its motor activity from predominantly diffusional to predominantly directional[52, 57]. Recently Cin8p was shown to convert to minus-end directionality at low motor occupancy[61] and/or high ionic strength and/or when not crosslinking microtubules[62, 63]. The molecular mechanism for this directional reversal is as yet unclear, nor is it clear how many other members of the kinesin-5 family can reverse direction. Optical trapping experiments **[G]** show that truncated KIF11 dimers are mildly processive[64] and tend to dissociate from the microtubule at higher loads (>4pN), rather than slowing and stalling like kinesin-1. The detachment rate increases with load but is insensitive to the direction of the load[65]. Trapping experiments with full-length *X. laevis* KIF11 tetramers show that the motor tends to detach above ~2 pN applied load and so usually dissociates before it stalls[66].

KIF11 detaches relatively slowly from microtubules, which causes it to exert drag force in situations where faster motors are trying to drive higher-speed microtubule sliding in bundles[67]. The tendency for KIF11 proteins to dissociate under load indicates that the kinesin-5 family members rely predominantly on strain gating, rather than ATP gating (FIG. 3) to control stepping. Strain gating pauses ATP turnover until trailing head detachment occurs. The biological significance is unclear but we speculate that relying on strain-gating might serve to minimise ATP turnover in forced-walking situations in which KIF11 is being pulled forwards by faster motors and exerting drag. The emphasis on strain-gating causes the step that immediately follows landing of the KIF11 head on the microtubule to have different kinetics from subsequent steps[68], because immediately on touching down, the motor is unloaded, whereas subsequent steps are influenced by internal tension developed between the two attached heads[65] and by any external load. Single-headed KIF11 constructs can drive

unloaded microtubule sliding *in vitro* at almost the same speed as dimers[69]. In summary, the stepping mechanisms of KIF11 tetramers allow them firstly to drive the slow sliding apart of antiparallel microtubules, plus ends outwards, and secondly to resist any forces that tend to speed up sliding. This braking aspect of KIF11 mechanochemistry might be biologically important to limit the effects on the spindle of variable outward forces, for example the pulling forces on centrosomal microtubules exerted by dyneins tethered at the nuclear envelope and cell cortex **[G]** (FIG1a, inset 2-3; see[51]).

Self-organization of the bipolar mitotic spindle

Nuclear envelope breakdown marks the end of prophase in humans and the time when the bipolar spindle self-organizes via the nucleation, capture, sliding and re-orientation of microtubules from both asters **[G]**. This results in a structure (FIG.1b, lower panel) in which microtubules nucleated from opposite poles either form antiparallel overlaps that become stabilized, or engage kinetochores **[G]** and form parallel bundles of kinetochore-microtubules known as K-fibres. Other non-kinetochore microtubules remain highly dynamic. Depolymerization at the microtubule minus-end driving poleward microtubule flux[70] **[G]** within the spindle – this activity alone can turn over the entire human mitotic spindle in ~10 mins. Multiple protein classes, including microtubule-associated proteins and kinesin motors, are involved in spindle assembly[71]. The early stages of self-organization involve the continued separation of centrosomes (if not already completed in prophase) and require the anti-parallel sliding activity of KIF11 as well as pushing forces from kinetochores and pulling forces from cortical myosin[72-74] (termed the prometaphase pathway; FIG. 1b, upper panel - green arrows). Overexpression of the kinesin-12 family motor KIF15 (also known as hKLP2) can compensate for loss of KIF11 in the prometaphase pathway[75]. This functional redundancy has led to a model in which KIF15 (when bound to the microtubule-associated protein TPX2), similarly to KIF11, can slide apart anti-parallel microtubules[76]. Consistently, KIF11 and KIF15 motors are redundant in terms of maintaining spindle bipolarity, presumably by contributing to the outward sliding of overlapping non-kinetochore microtubules within the spindle. In line with this, *X. laevis* KIF15 (xKlp2) is a plus-end directed motor (~50 nm per second)[77] and the human KIF15 ATPase is similar to that of KIF11, although with weaker microtubule activation[78]. Simple models in which KIF15 and KIF11 both slide antiparallel microtubules apart are however called into question by recent experiments showing that KIF15 preferentially associates with parallel microtubules in K-fibres and generates forces that can counteract KIF11-derived forces[75, 79].

Once the bipolar spindle assembles, maintaining spindle length requires that the outward sliding forces generated by KIF11 and/or KIF15 be balanced against inward forces from the kinesin-14 family motor KIFC1 (also known as HSET (FIG1b, Inset 5-6)[76, 80, 81]. In contrast to most other motors, kinesin-14 family members are specialized to move towards microtubule minus ends and have their motor domain at the C-terminus. For *D. melanogaster* Ncd, one of

the best studied family members, single heads can drive minus end-directed microtubule sliding, but only if they are tethered to a surface via a stiff, two stranded coil that functions as a lever[82]. Individual motor molecules are non-processive under load. Optical trapping experiments have recorded individual strokes[83], but a force-velocity curve, which defines the stepping rate at different loads, has not yet been measured. A microtubule-binding domain in the tail of Ncd promotes microtubule assembly and stability[84].

Both Ncd and *S. pombe* Klp2 crosslink and sort microtubules *in vitro* by sliding apart anti-parallel microtubules, and stabilizing those that are parallel[85-87] (FIG. 4c). Sliding is in the reverse direction to that driven by kinesin-5 family motors such as KIF11, consistent with the antagonistic behaviour of these kinesins during spindle formation[6, 80, 88, 89]. Parallel crosslinking and sliding might represent one role for KIFC1 in the centrosome-independent focusing of microtubule minus-ends into spindle poles[90] (Fig1b, inset 4). Indeed, *D. melanogaster* Ncd is sufficient to self-organize microtubules into aster-like structures *in vitro*[2]. *D. melanogaster* Ncd also has a depolymerase activity[91], although the Ncd motor domain can also accelerate the assembly of GTP microtubules[92]. Similarly to kinesin-5 motors, and despite their opposite directionality, kinesin-14 motors have been proposed to function as brakes to oppose outwards forces in the spindle. *S. cerevisae* Kar3 is a kinesin-14 family member that heterodimerises with a nonmotor partner head (Cik1 or Vik1). Kar3 dimerised with Cik1 targets the plus-ends of GDP–taxol **[G]** microtubules *in vitro* and depolymerises them slowly[91]. Kar3 dimerised with Vik1, which has a kinesin-like fold but no active site, is motile[93], with the Vik1 domain showing interactions between its N and C termini reminiscent of neck linker–cover strand interactions in the motor domain[94]. Mutations in the Vik1 C-terminus inhibit the partner Kar3 head. It is clear that the nonmotor heads of Kar3 heterodimers modify and to some extent gate the mechanochemical cycle of the motor head. It will be important to discover how many other kinesins use this mechanical control strategy to adjust their activities.

Chromosome capture and congression

Coincident with bipolar spindle formation is the capture of chromosomes by microtubules and their congression **[G]** to the spindle equator to form the metaphase **[G]** plate (FIG. 1b)[95]. This process is dependent on multiple kinesin motors (KIF10, KIF18A, KIF2B, KIF2C, KIF4 and KIF10) that contribute to (at least) three key mechanisms:

Lateral sliding of kinetochores on microtubules.

The initial capture of kinetochores by the microtubule lattice and their subsequent transport to microtubule plus ends can be mediated by dynein[96] and the kinesin-7 family motor KIF10[97-99] (see Fig. 1b, chromosome v; FIG. 4b). The KIF10-dependent movement of chromosomes to the spindle equator is consistent with the finding that KIF10 is a plus-end directed walking kinesin with mechanochemistry similar to kinesin-1[100, 101]. Truncated *X. laevis* KIF10 dimers

walk processively under load, take 8nm steps, have a stall force of ~6pN and can drive microtubule sliding at up to ~340 nm per second unloaded[100]. The kinetic cycle of KIF10 uses one ATP turnover per step[102]. The ATP gate (FIG. 3a) of KIF10 may be less effective than that of kinesin-1, with ADP release from the tethered head of KIF10 being only relatively weakly coupled to ATP binding to the microtubule-bound head. There is a correspondingly greater tendency for KIF10 to develop a two heads-attached state, with both heads in the apo state[102]. Neck linker docking is also slower than in kinesin-1[102]. Differing conclusions were reached using a truncated human KIF10 that moves slowly, and binds microtubules slowly, but shows tight ATP-gating behavior[101]. In summary, KIF10 is a processive kinesin with a mechanism that enables it to transport kinetochores to microtubule plus ends (FIG. 1b, *inset 8* and Fig. 4b). There is also evidence that laterally attached kinetochores can be moved away from the plate by the minus-end directed activity of KIFC1, and that this counteracts KIF10 activity[103]. The underlying mechanism is unclear as KIFC1 is not localized to kinetochores in mammalian cells.

Kinetochore-mediated pushing and pulling.

Once sister kinetochores biorientate (meaning that each kinetochore is end-on attached to microtubules nucleated from opposite spindle poles; see Fig. 1b, chromosome iv) they are able to make movements both towards and away from their attached pole. These movements are possible because kinetochores can maintain attachment to dynamic microtubules and regulate their switching between growth and shrinkage (FIG1, *inset 7*). At least three classes of mitotic kinesin (KIF18A, KIF2B/C and KIF10) and dynein are implicated in this process.

KIF18 motors (members of the kinesin-8 family) are multitalented[104] plus-end directed kinesins that are implicated in the control of microtubule plus-end dynamics and microtubule sliding[104, 105]. KIF18A operates at kinetochores[106], whereas KIF18B is found on astral-microtubules[107, 108]. Some of the members of the kinesin-8 family are highly processive, and initial work on *S. cerevisiae* Kip3 indicated that it has depolymerase activity[49, 109]. However a depolymerase activity for KIF18 from non-budding yeast is controversial (for review see[110]). Recent work indicates that human KIF18A reduces the dynamicity of the microtubule plus-end rather than functioning as a depolymerase[111, 112]. KIF18A can also make tubulin rings[113] and has an extended loop-2 reminiscent of the kinesin-13 family (BOX 2), which supports the idea that it can bend tubulin to drive depolymerization. These effects on microtubule dynamics likely cause the reported changes in the speed and amplitude of kinetochore oscillations following depletion of KIF18A, and the resulting severe chromosome congression defect[106, 114, 115]. The first single molecule mechanics data for KIF18A were recently reported, showing that the motor stalls at very low load (~1 pN)[116]. Kip3 moves around the microtubule axis as it moves towards plus ends, with a leftwards bias[117], and can form traffic jams of piled-up motors at microtubule plus ends[118]. A microtubule-binding site at the C-terminus of the tail of KIF18A is required for mitotic function and is proposed to increase processivity[119, 120]. It is

possible that KIF18A contributes to kinetochore movement by this processive stepping, although this may be more relevant to the delivery of cargo and/or accumulation of KIF18A at kinetochores, perhaps acting as a read-out of microtubule length, which may explain congression[112]. Kip3 can also slide microtubules, thus balancing microtubule depolymerase activity in pre-anaphase **[G]**[105]. How this relates to chromosome movement is unknown.

There are three members of the KIF2 motor family (kinesin-13 family) in humans (KIF2A, KIF2B and KIF2C (also known as mitotic centromere associated kinesin (MCAK)), which interact at both ends of microtubules to remove subunits of the GTP-tubulin cap **[G]** and induce microtubule catastrophes (BOX 2; FIG. 4d). Depolymerization at kinetochore microtubule minus-ends (spindle poles) generates poleward microtubule flux, which can exert a pulling force on kinetochores[121, 122] (FIG1b,c; *inset 10*)**.** At the plus-end of microtubules these motors are implicated in correcting erroneous kinetochore attachments[123-5] and controlling the speed of kinetochore motility[115, 124]. Recent optical trapping experiments show that kinesin-13 family members can resist an applied load of around 1 pN whilst engaged with both ends of a slowly-depolymerizing GMPCPP microtubule[126], although it is not clear if this is true of a single KIF2C molecule or if the effect requires multiple KIF2C molecules[127]. Nevertheless, this raises the interesting idea that KIF2C may have a role in holding force between kinetochores and spindle poles.

Recent cell biological experiments indicate that KIF10 motors (kinesin 7 family) also contribute to the ability of kinetochores to track depolymerizing microtubules[128]. Indeed, full length and truncated KIF10 dimers can track growing microtubule tips for several seconds[128, 129] and full length KIF10 can track shrinking tips, requiring both motor activity and a C-terminal nonmotor microtubule binding site to do so[128]. There are also data suggesting that KIF10 may influence the dynamics of kinetochore-attached microtubules: a truncated human KIF10 construct accelerates microtubule growth in the presence of low concentrations of taxol[130]. This is likely to be due to stabilization of the lattice, because full length KIF10 does not interact with free tubulin[128].

Polar ejection forces:

The polar ejection force propels chromosome arms away from the spindle pole and towards the metaphase plate (FIG1b, inset 9[131])**.** This force is thought to be mediated by the polymerization of microtubules against the chromosome and by chromosome-bound motors known as chromokinesins **[G]** (for review see[132]). Of these, KIF22 (also known as NOD or KID), a member of the kinesin-10 family, is the most likely mediator of the polar ejection force[133], although the extent to which KIF22 contributes to chromosome congression is unclear[112, 133-138]. Optical trapping microscopy shows KIF22 to be a non-processive motor that steps towards the microtubule plus-end, consistent with a role in mediating the ejection force[139]. More detailed biochemical kinetics come from studies of *D. melanogaster* NOD,

which is required for meiosis. This work establishes that the NOD mechanism is unusual amongst the kinesins. NOD does not seem to be motile or to drive microtubule motility; instead, it tracks microtubule plus ends, linking them to chromosome arms and enabling microtubule assembly to push chromosomes towards the spindle equator. NOD binds tightly to microtubules in its apo state, but dissociates from microtubules in its ATP-bound state, hydrolyses ATP, releases phosphate, and then rebinds in its ADP state to allow microtubules to accelerate ADP release[27]. ATP binding to NOD is proposed to be blocked at the very tips of the microtubules, but to be sanctioned as the tip is internalized by microtubule growth[27]. An end-tracking mechanism for generating the polar ejection force is supported by recent experiments in mitotic cells[140]. It is not yet clear if the unusual kinetic cycle of NOD, with detachment in the ATP state, occurs in other, as yet uncharacterized, kinesins.

Members of the kinesin-4 family (such as mammalian KIF4[29]) are also plus-end directed chromokinesins that can regulate microtubule dynamic instability (and thereby microtubule length) as well as drive microtubule sliding and chromosome compaction[112, 134, 141-144]. However, there is no strong evidence that these motors contribute to the polar ejection force[133, 134]. In fact, it is has been proposed that they may counteract this force by suppressing spindle microtubule dynamics[112]. KIF15 has also been considered a chromokinesin as it is targeted to chromosome arms by binding Ki-67[81]. Loss of this chromosome-bound pool of KIF15 is associated with some congression problems[81].

Anaphase chromosome movement

Anaphase A — the shortening of the kinetochore to pole distance — can be driven by depolymerization of kinetochore-attached microtubules[145] (termed “Pacman”) or by poleward flux[146] (FIG. 1d, insets 10 and 11). Experiments in *D. melanogaster* show that Kinesin-13-driven microtubule depolymerization (see above and BOX 2) is crucial for a combined “Pacman–flux” mechanism[147]. It is suggested that the end-tracking properties of kinesin-7 and kinesin-8 family members (KIF10 and KIF18A, respectively) may assist kinetochores in maintaining attachment to depolymerizing microtubules[148]. However, the motor requirements for anaphase A in human cells are untested. Completion of chromosome segregation also involves the elongation of the spindle – a process known as anaphase B. Again, the motor requirements in human cells are not known, but experiments in flies[149] and yeast[150] reveal an important role for kinesin-5 family members. Current models require that these motors slide apart anti-parallel spindle microtubules, generating an outward pushing force on the spindle poles[8]. However, in worms the kinesin-5 operates as a brake to limit spindle elongation (which is driven by astral-microtubule pulling at the cell cortex)[151]. As for centrosome separation, kinesin-5 family members clearly generate both driving and braking forces during anaphase to modulate the rate at which microtubules slide apart.

Central spindle mechanics

As anaphase progresses, the mitotic spindle re-self-organizes to generate an anti-parallel microtubule bundle structure between segregating chromosomes known as the central spindle **[G]** (FIG. 1d, *insets 3* and 12; for review see[152]). The central spindle functions chiefly as a signaling platform to control the positioning of the cytokinetic cleavage furrow, rather than itself having a mechanical role in furrow ingression. However, the central spindle mechanically counters the pulling forces from the astral microtubules, limiting the extent of anaphase B spindle elongation[153, 154]. The structure and length of the central spindle are determined by the mechanochemical interactions of multiple kinesin motors[155]. KIF23 (MKLP1), a kinesin-6 family member, is required for assembly/stabilization of the antiparallel microtubule bundles during anaphase and cytokinesis **[G]**[152] and is specialised in several ways. First, it does not have a recognizable neck linker. Second, for efficient microtubule bundling both *in vitro* and *in vivo*, it needs to be in a stable complex, known as centralspindlin, with a second nonmotor subunit[21]. Third, centralspindlin further assembles into small polymers known as clusters[156]. The clustering and crosslinking activities of centralspindlin enable it to assemble cooperatively and maintain the central spindle array of antiparallel microtubules[157, 158]. KIF4 (kinesin-4 family) is recruited to the central spindle via its interaction with PRC1, a cross-linker that stabilizes antiparallel microtubule overlaps[159]. In addition to their function in pre-anaphase, KIF4 motors also have a key role in controlling the size of the central spindle through regulation of microtubule dynamics[160, 161] KIF4 recruitment to the central spindle is controlled by the phosphorylation of its C-terminal tail by a KIF20A (kinesin-6)-dependent pool of the Aurora B mitotic kinase[162]. KIF2A depolymerase activity is also targeted to distal (minus) ends of central spindle microtubules and drives their shrinkage in order to control the size of the central spindle[163]. Meanwhile, PRC1 also recruits KIF10, KIF14, KIF23, KIF20A (MKLP2) and KIF20B (MPP1)(for review see[155]).

Conclusions and future prospects

The central question in the field is how robust spindle self-organization and faithful chromosome segregation emerge from the interactions of mitotic motors with dynamic microtubules[7]. To answer this question we need to understand the local actions of the individual motors. Each mitotic motor has a mechanism for generating force and this mechanism responds to external forces in a predictable, measurable way, defined by its force-velocity curve. To understand how the interactions of these various mechanisms with dynamic microtubules produce robust structure and function at the level of the entire spindle, *in vitro* biophysical approaches and live cell microscopy approaches are both required. *In vitro* reconstitution approaches are needed to measure local forces in simplified subsystems and describe the intrinsic mechanisms of individual force generators, whilst live cell experiments are needed to dissect the positions and motions of individual kinesin molecules and teams of kinesin molecules in the spindle. *In vivo* force sensors[164] may allow local forces to be measured in the intact spindle. Studying the mechanisms of force-integration in the spindle poses technical challenges, but will be possible. If we can determine the characteristic

mechanical behaviour of each mitotic kinesin, we can make explicit models that predict collective behavior. These models can then be tested and refined by mutating the motors to alter (but not abrogate) their performance, measuring their altered performance curves *in vitro* and inserting them into living cells to assess their influence on spindle dynamics. With careful experimentation, ensuring that *in vitro* reconstitution is done under realistic conditions and live cell microscopy with non-perturbing tags, it should be possible to expand on our understanding of how the molecular mechanisms of individual spindle motors interact with one another to produce robust spindle self-organization and chromosome segregation.

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BOX 1 | Kinesins and Anticancer Drugs: Microtubule-based transport is a key target for cancer therapy[165]. Drugs that interfere with the function of microtubules (such as taxanes) are established, front-line chemotherapeutics but suffer from side-effects and drug resistance[166]. A new generation of anti-mitotic drugs that target the mitotic kinesins KIF11, KIFC1 and KIF10 are becoming available[167, 168]. Monastrol **[G]** and related molecules[168] inhibit KIF11 by binding to an allosteric site on loop 5 that results in the inhibition of ADP release from the active site, leaving the motor in a weakly bound state[169, 170]. ATP competitive inhibitors (FCPT; Merck) that leave KIF11 in a rigor state are also available[171]. KIF10 is inhibited by the ATP competitive inhibitor GSK-923295 (Cytokinetics) [172], which binds in a position similar to monastrol but functions very differently, in that it blocks phosphate release and locks the motor in a rigor-like tight binding state[172]. In the clinic, KIF11 inhibitors are the best studied, but their use as a monotherapy in patients with cancer has proven largely disappointing[168]. However, promising new trials using combinatorial therapies in myeloma are underway[173]. The functional redundancy between KIF11 and KIF15 means that KIF15 is now emerging as a potentially important therapeutic target[174]. As well as being of potential therapeutic use, these small molecule kinesin inhibitors will be powerful tools to dissect the cell biological function of each motor.

BOX 2 | How do kinesin-13 motors drive microtubule depolymerization? Kinesin-13 family (KIF2) motor heads have a substantial N-terminal extension called the neck, which is required, together with the C-terminal tail, for dimerization. Loop-2 of the head (FIG. 2e) is extended to form an antiparallel pair of beta sheets carrying a triplet KVD (Lys, Val, Asp) sequence at its tip. The KVD finger is required to induce microtubule catastrophe[175]. Dimerisation increases catastrophase activity but is not required for it[125, 176]; as KIF2 monomers are effective. The proximal part of the N-terminal neck is positively charged and this accelerates the initial recruitment of the motor to the microtubule[54, 176]. The next part of the N-terminal neck folds around and makes a helix that anneals to this KVD finger[175]. The mechanochemical cycle begins with landing of the kinesin dimer in its Mg•ATP state, either

directly at the microtubule tips or on the microtubule lattice. ATP hydrolysis is rate-limiting in the absence of microtubules[25] so that the Mg•ATP state (state 1; see figure) dominates in solution at low tubulin concentrations. This state binds preferentially to tubulin[177], but does also bind the microtubule lattice. Contact with the lattice activates Mg•ATP hydrolysis (producing state 2) and phosphate release (producing state 3) from the kinesin. However unlike for other kinesins (FIG. 3), lattice-mediated activation of Mg•ADP release is suppressed, allowing the motor to diffuse along the lattice in its Mg•ADP state (state 3) to the microtubule tips, where Mg•ADP release is activated, producing state 4 (the apo state). This mechanism ensures that KIF2 enzymatic activity is focused to the microtubule tip. There, Mg•ATP binds. KIF2•ATP (state 4) is thought to stabilize a curved conformation of tubulin, because KIF2•AMPPNP (an analogue of KIF2•ATP) binds tightly to curved protofilaments **[G]** and to highly curved tubulin rings formed by the drug dolastatin **[G]**[178]. Electron microscopy shows that KIF2 proteins bind between neighbouring tubulin heterodimers[179] in a protofilament, using three contacts (extended L2, L8 and alpha 4-L12; FIG.2)) to bend and stretch the[179] heterodimers[179]. KIF2 dimers probably bridge neighbouring protofilaments[178]. KIF2 removes tubulin heterodimers from (otherwise highly stable) GMPCPP microtubules. Futile cycles of ATP turnover occur at the tips of microtubules that are stabilized by both GMPCPP and taxol, indicating that the tip-activated ATPase activity of kinesins is only loosely coupled to the removal of tubulin subunits. Consistent with this, a hydrolysis-incompetent mutant of KIF2C has slow microtubule depolymerase activity in the presence of ADP[180], showing that the Mg•ADP form of KIF2C binds more tightly to microtubule tips than to free tubulin (whereas the Mg•ATP form prefers free tubulin[177]). Recycling of the KIF2 enzyme occurs via ADP release, which only takes a few seconds even in the absence of tubulin or microtubules[25].

Figure 1 | Motorization of mitosis in human cells.

a | Movement of duplicated centrosomes towards opposite sides of the nucleus during prophase absolutely requires KIF11 (#1), which drives outwards microtubule sliding. Dyneins tethered at the nuclear envelope (#2) and cell cortex (#3) also contribute **b** | Prometaphase starts with nuclear envelope breakdown and completion of chromosome condensation. A bipolar spindle then forms via the capture, sliding and re-orientation of microtubules (#4) by multiple motors. Dynein and KIFC1 focus microtubule ends to form the spindle poles (#5). Spindle length is maintained by balancing outward and inward sliding forces (#6). Kinetochores are initially unattached (type I) before forming biorientated (amplhilelic; type IV) attachments; which may be preceded by monotelic (II), syntelic (III) or lateral (V) intermediates. Bioriented chromosomes (#7) move to the metaphase plate by depolymerization-coupled pulling (Pacman) at the leading sister kinetochore. KIF10 drives lattice-bound kinetochores towards the spindle equator, opposed by dynein (#8). KIF2B and KIF2C promote microtubule depolymerization, whereas KIF18A dampens microtubule dynamics. KIF10 also contributes to these kinetochore movements. Chromokinesins (KIF22)

generate a polar ejection force (#9) that propels chromosome arms away from the poles, and minus-end depolymerization (mediated by KIF2A and KIF2C) (#10) drives poleward microtubule flux that exerts further force on kinetochores. **c** | During metaphase, the pushing/pulling by kinetochores (#7) and the polar ejection force and poleward flux (#9,10) lead to chromosome oscillations at the spindle equator. **d** | During anaphase A, sister kinetochores segregate polewards driven by depolymerization-coupled movement (#11) and poleward microtubule flux (#10). Conicidently, the spindle elongates (Anaphase B) via anti-parallel sliding of microtubules in anti-parallel overlaps (#6) and cortical pulling (#3). The central spindle (#12) assembles and modulates spindle elongation as anaphase progresses.

Figure 2 | Topology of mitotic kinesins. a | Homologous heads **(**motor domains) are coupled to subfamily-specific tails. **b** | Views of an individual head (here HQD.pdb, the head of KIF11, a kinesin-5 family member, with AMPPNP in the active site), showing the main elements of the active site. The active site is flanked by switch 1 and 2. A salt bridge links these elements during catalysis, forming the phosphate tube. The nucleotide docks against the highly-conserved P-loop. The Mg2+ ion holds the nucleotide in place. **c** | The same head rotated 90* around its long axis to show the beta sheet backbone flanked by two sets of alpha helices. **d** | A further 90* rotation, showing the microtubule binding interface, with a zoom view of the C-terminal neck linker and N-terminal cover strand docked against the main part of the head. **e** | Side-on view of the same head docked on to a tubulin heterodimer (4HNA.pdb[182]). Key landmarks are the alpha 4 helix, which sits into the slot between the two tubulin monomers, the loop 2 (L2) and the loop 8 (L8). L12 sits alongside alpha 4 in the centre of the interface.

Figure 3 | Force generation by mitotic kinesins: ATP-gating and strain-gating.
a | Mechanical processivity of kinesins, in which two coupled kinesin heads alternately generate force, is coordinated by two 'gates': the ATP gate and the strain gate. Kinesins pause between steps in an 'ATP waiting state', with one head bound to the microtubule and the other, (tethered) head detached and parked in some way[44, 182](step 1). ATP binding to the microtubule-bound head in the ATP-waiting state releases the tethered head from its parked state and effectively starts a race between the two heads, with the tethered head needing to find its next binding site and undergo microtubule-activated ADP release before hydrolysis and phosphate release complete on the bound head. We refer to this as the ATP gate (step 2). Once the tethered head finds its next site, it generates a kick, coupled to MgADP release (step 3). Bridging between two binding sites 8nm apart along the microtubule requires the neck linker on the leading head to undock, and places both neck linkers under strain. Backwards strain on the neck linker of the leading head inhibits ATP binding to that head until the trailing head detaches. We refer to this as the strain gate (step 4). Release of the trailing head from the microtubule (step 5) then allows the ATP waiting state to regenerate (step 6). **b** | The mechanism by which nonprocessive kinesins generate force is poorly understood but

may be a subset of the processive walking mechanism, shown here as a generic diffuse-bind-kick-release cycle.

Figure 4 | Elements of kinesin-driven spindle self-organisation. Kinesins can move by **a** | directional stepping, biased diffusion or unbiased diffusion and use these modes to function as **b** | tractors to haul cargo, **c** | winches that slide microtubules or **d** | assemblers (modifiers of tubulin exchange at microtubule tips). Singly and in combination, these elemental activities can produce large scale self-organisation and re-organisation of the spindle. Specific kinesin subfamily members tend to deliver a subset of these activities, though some (for example, members of the kinesin-8 family (see text)) may be capable of all three major classes of activity. Colours scheme as in FIG. 2a.

Glossary

Self-organisation In a cell biological context, self-organisation is distinct from molecular self-assembly in that it requires an energy source. Self-organisation reactions are typically dynamic and reversible. Self-assembly reactions are spontaneous and generate a stable product.

Plus ends of microtubules The fast-growing, relatively unstable ends of microtubules.

Minus ends of microtubules The slow-growing, more stable ends of microtubules.

Microtubule catastrophes Microtubules undergo dynamic instability, whereby episodes of steady growth end in a sudden transition to rapid shrinkage called a catastrophe.

Stall force The stall force of a motor is the force that stops it making net progress.

Unloaded velocity The velocity with which a motor moves along its track slows down with increasing inhibitory (backwards) load. The unloaded velocity is the velocity with which the motor moves without a load.

ATP-gate Mechanism whereby microtubule binding by one head of a 2-headed (walking) kinesin depends on ATP binding to the other head.

Strain-gate Mechanism by which the unbinding of kinesin heads from microtubules is inhibited by backwards strain and promoted by forwards strain.

Prophase The earliest stage of mitosis, in which the centrosomes separate and migrate.

Optical trapping experiments A laser is used to trap a micron-sized transparent bead with a single kinesin molecule attached and then steered to bring the kinesin–bead complex into contact with a microtubule. The walking action of the kinesin can then be tracked by tracking the bead.

Cell cortex Region of the cell that lies beneath the plasma membrane; often actin-rich.

Asters Radial array of microtubules nucleated around a centrosome.

Kinetochores A multi-protein complex that assembles on the centromere of each chromosome forming an attachment site for the plus-ends of spindle microtubules.

Poleward microtubule flux The translocation (flux) of tubulin towards the spindle pole that

requires microtubule minus-end depolymerization. Concurrent polymerization at plus-ends will result in treadmilling. .

Taxol Drug that binds to and stabilizes microtubules.

Congression the migration of chromosomes to the spindle equator to form the metaphase plate.

Metaphase The phase of mitosis when all chromosomes are positioned on the spindle equator.

Dynamicity The frequency of switching between growth and shrinkage and back again at microtubule tips

Pre-anaphase All stages of mitosis prior to anaphase, when the chromosomes begin to segregate to opposite poles of the spindle.

GTP-tubulin cap the region at the growing end of a microtubule that is built from tubulins that are in a GTP-bound state.

Chromokinesins Kinesins that bind chromosomes.

Central spindle During anaphase, the mitotic spindle reorganizes in preparation for cytokinesis. Kinesin motor proteins and microtubule-associated proteins bundle the plus ends of interpolar microtubules into antiparallel microtubules and generate the central spindle, which regulates cleavage furrow initiation and the completion of cytokinesis.

Anaphase The last stage of mitosis when sister chromatids are segregated to opposite ends of the mitotic spindle.

Cytokinesis physical process after mitosis when cytoplasm is divided into two new daughter cells.

Monastrol Drug that binds to kinesin-5 family motors and locks them in a weak-binding (low friction) state.

Protofilaments Microtubule protofilaments are built from tubulin dimers, assembled head-to-tail. Protofilaments assemble side-by-side to form a sheet and the sheet then curls up into a tube.

Dolastatin Drug that binds tubulin and inhibits microtubule assembly.

Online summary

1. Crosslinking kinesins support the self assembly and sliding of parallel or anti-parallel microtubule bundles to develop the bipolar symmetry of the spindle. These kinesins work in large teams, moving slowly and detaching slowly, which enables them to exert braking force as well as driving force, and thereby control microtubule sliding rates.

2. Kinesin-13 family members (KIF2A, KIF2B and KIF2C) are targeted to microtubule ends, provoking depolymerisation by stabilising a curved conformation of tubulin in the cap. In the central spindle and elsewhere, this activity is balanced against microtubule polymerisation and the sliding of microtubules in bundles.

3. One kinesin-14 family member, Kar3 (*Saccharomyces cerevisiae*), converts from a motor into a capping protein by heterodimerising with Cik1 or Vik1, both of which have kinesin-like folds but no ATPase activity. This theme of mechanical control of the motor head by a nonmotor partner might turn out to be a more general mechanism.

4. Several mitotic kinesins are processive (walking) dimers similar to kinesin-1. These are involved in the long-range transport of chromosomes.

5. Kinesin-8 (KIF18) morors are multi-talented, with various isoforms reported to have depolymerase, polymerase, transporter and microtubule sliding functions. This should caution us that the properties of kinesins need to be measured, not inferred from homology searches.

6. Kinesin-5 (KIF11) in yeast reverses direction *in vitro* depending on occupancy. It will be important to understand the molecular mechanism of this, to discover how many other kinesins do it and to understand the consequences for spindle self-organisation.

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