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Mechanism and Regulation of Kinesin-5, an essential motor for the mitotic spindle

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Abstract

Mitotic cell division is the most fundamental task of all living cells. Cells have intricate and tightly regulated machinery to ensure that mitosis occurs with appropriate frequency and high fidelity. A core element of this machinery is the kinesin-5 motor protein, which plays essential roles in spindle formation and maintenance. In this review, we discuss how the structural and mechanical properties of kinesin-5 motors uniquely suit them to their mitotic role. We describe some of the small molecule inhibitors and regulatory proteins that act on kinesin-5, and discuss how these regulators may influence the process of cell division. Finally, we touch on some more recently described functions of kinesin-5 motors in non-dividing cells. Throughout, we highlight a number of open questions that impede our understanding of both this motor's function and the potential utility of kinesin-5 inhibitors.

Keywords

kinesin; mitosis; microtubule; tpx2; phosphorylation

I. Kinesin-5 plays a critical role in the mitotic spindle

The mitotic spindle is a complex multi-protein machine made up of microtubule (MT) filaments, motor proteins that walk along and organize these filaments, nonmotor microtubule-associated proteins (MAPs) and various non-structural signaling molecules. MTs act as the major structural scaffold of the mitotic spindle, while the dynamic properties of the mitotic spindle depend upon the variety of proteins that attach to MTs in different regions of the mitotic spindle.

While hundreds of components have been shown to associate with the mitotic spindle (Sauer *et al.*, 2005), theoretical and experimental evidence both point toward a central role for kinesin-5. In simulations, only four mechanical activities are required to establish and maintain a stable MT-based spindle: 1) extension and retraction of MTs, 2) a pole cohesion factor that pulls the minus-ends of MTs together, 3) the MT cross-linking force of a minus-

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end directed motor protein, dynein, and 4) an outward-directed force between interpolar MTs, generated by kinesin-5 motors (Loughlin *et al.*, 2010) **(Figure 1A)**. An RNAi screen of all the MT-based motor proteins identified only three that were absolutely required for completion of mitosis: kinesin-5, kinesin-6 (which is involved in separating the two daughter cells) and kinesin-8 (which acts to shorten MTs) (Goshima , Vale, 2003). Thus, determining how kinesin-5 functions is a critical task as we seek to understand how mitosis works and how it can be altered for therapeutic interventions.

Despite the core function shared by all eukaryotic kinesin-5 homologs, each protein seems to play a somewhat different role in its organism's mitotic process. In *S. cerevisiae* and *Drosophila* embryos, overexpression of kinesin-5 increases the metaphase spindle length (Brust-Mascher *et al.*, 2009; Saunders *et al.*, 1997; Straight *et al.*, 1997); while in other cell types, including *Drosphila* S2 cells, spindle length is unaffected by overexpression of kinesin-5 (Goshima, Vale, 2005). In both *S. cerevisiae* and *D. melanogaster* embryos, kinesin-5 is responsible for pushing spindle poles apart in anaphase (Brust-Mascher et al., 2009; Straight et al., 1997). In contrast, mammalian cells require kinesin-5 in early mitosis for centrosome separation (Blangy *et al.*, 1995; Tanenbaum *et al.*, 2008). Unlike the other kinesin-5 family members, which are essential for mitosis and generate outward force to power spindle separation, the *C. elegans* kinesin-5 BMK-1 does not appear to be required for mitosis and acts to slow the rate of spindle extension (Saunders *et al.*, 2007). The kinesin-5 motor homologs in these organisms are generally similar, based on primary sequence. Determining how these motors fulfill their basic role of pushing mitotic spindle poles apart and identifying what variations on this theme occur in different organisms remain major outstanding questions for the field. In our view, addressing these questions requires a focus on the mechanism and regulation of kinesin-5 motors, as well as on their interplay with other mitotic proteins.

II. Molecular anatomy of kinesin-5

Kinesin-5 is a homotetrameric protein, with each subunit containing an N-terminal kinesin motor domain, a central stalk domain and a C-terminal tail domain (**Figure 1B**. These subunits are thought to arrange themselves into bipolar homotetramers, with a pair of motor domains on either end. While only the *D. melanogaster* kinesin-5 Klp61F and the *S. cerevisiae* kinesin-5 Kip1 have been directly observed to form bipolar homotetramers, vertebrate kinesin-5s slide two MTs antiparallel to each other in microscopy assays, consistent with such an organization (*Acar *et al.*, 2013; Gordon , Roof, 1999; *Kapitein *et al.*, 2005; Kashina *et al.*, 1996).

The N-terminal kinesin-5 motor domain is ~350 residues long and has all of the conserved structural elements that form the engine of every kinesin (Vale , Milligan, 2000). All kinesin motor domains, myosin motors and small GTPases have a central beta-sheet flanked by alpha helices. These proteins also share several critical structural elements that translate nucleotide hydrolysis into conformational changes. Several of these structural features are of particular interest for kinesin-5 motors and are discussed in detail in this review. They are identified here from the N-terminus to the C-terminus of the motor domain and are shown in **Figure 1C**

- **Cover strand**: The cover strand is a short, ~ 5-10 residue segment at the N-terminus of the motor domain. This element is not often visualized in kinesin crystal structures, but has been shown to be critical for forward movement in kinesin-1 motors (Khalil *et al.,* 2008). The cover strand forms a short beta-sheet segment, referred to as the cover neck bundle, with the neck linker during forward movement by both kinesin-1 and the human kinesin-5, Eg5 (*Goulet *et al.*, 2012; Hesse *et al.,* 2013).
- **Loop 5**: The alpha 2 helix of all kinesin motors breaks into a short loop, called loop 5, near the nucleotide pocket. The sequence of this loop is kinesin family-specific, and it is unclear whether loop 5 has a conserved function for all kinesins. In kinesin 5 motors, loop 5 has been implicated in ADP release (Waitzman *et al.* 2011) and in communicating changes in nucleotide state to the neck linker and microtubule-binding regions of the motor domain (*Goulet et al., 2012).
- **Switch I and switch II**: Switch I and Switch II are conserved nucleotide-sensing elements found in all kinesins, myosins, and small GTPases. These elements have been shown to play a role in nucleotide-induced conformational changes. Switch I plays a large role in mediating nucleotide release, which is stimulated by exchange factors in all of these enzymes (Kull , Endow, 2002; Vale , Fletterick, 1997). Several allosteric inhibitors of Eg5, like the STLC molecule shown in **Figure 1C**, bind in a groove between loop 5, alpha 2, alpha 3, and switch I and strongly affect microtubule-stimulated ADP release (Cochran *et al.* 2005). Switch II plays a role in binding to exchange factors, namely MTs for kinesins. Switch II communicates changes in motor nucleotide state to the relay helix within the MT-binding site and to neck linker, which drives forward motility (Kull , Endow, 2002; Sindelar , Downing, 2010).
- **Neck linker**: The neck linker element undergoes nucleotide- and microtubule-dependent conformational changes to result in the directed movement of all kinesin motors (Endres *et al.,* 2006; *Goulet et al., 2012; Rice *et al.,* , 1999; Vinogradova , 2004). This directed movement is thought to be a coordinated, hand-over-hand movement of the motor domains for both kinesin-1 and the human kinesin-5 motor Eg5 (Vale *et al.,* 1996; *Valentine *et al.,* 2006), and the neck linker is critical to this coordination between motor heads (Jiang *et al.,* 1997; Yildiz *et al.,* 2008).

Given the importance of the human kinesin-5, Eg5 as a drug target, the kinesin-5 motor domain has been the subject of many crystallization screens and its mechanochemical mechanism has been intensively studied. The useful trove of structural and mechanistic data on kinesin-5 has informed our understanding of both its mechanism in particular and the enzymology of kinesins and ATPases in general. An X-ray crystal structure of Eg5 bound to AMPPNP (PDB ID#3HQD) revealed that kinesins, and possibly several other ATPases as well, hydrolyze nucleotide by coordinating the positions of two critical water molecules (*Parke *et al.,* 2010). Structure-based molecular dynamics studies and cryo-EM studies on kinesin-5 motors have demonstrated that many kinesin families are likely to initiate forward movement by a similar mechanism (*Goulet et al., 2012; Hesse et al., 2013). While these reports enriched our general understanding of the kinesin superfamily, others have revealed

significant differences between the enzymatic and kinematic properties of kinesin-5s vs. other kinesin families, suiting kinesin-5 family motors to their unique role in mitosis. These properties are discussed in detail in the “Mechanism of kinesin-5” section below and in **Table 1**.

Remarkably, kinesin-5 requires not just its enzymatic heads, but also its nonmotor stalk and tail domains to cross-link and slide microtubules. The central stalk domain of kinesin-5 orients the motor subunits relative to one other. Robust dimers of motor domains can be formed using the N-terminal motor domain and half of the stalk (residues 1-513 in human Eg5; **Figure 1B**). Using Electron Microscopy and electron paramagnetic resonance (EPR) spectroscopy on *Drosophila* Klp61F, Acar et al. identified *a bipolar* assembly (“BASS”) domain toward the C-terminal end of the stalk (residues 671-791) that allows two dimers to associate in an antiparallel fashion to form bipolar homotetramers (*Acar et al., 2013).

The C-terminal tail of kinesin-5 appears to have two major functions: to aid the motor's localization in mitosis and to increase the motor's affinity for MTs (Weinger *et al.,* 2011). In early work on kinesin-5, it was noted that the motor localized to the mitotic spindle. This localization depends upon the BimC box, a 20-residue stretch in the C-terminal tail of the motor, which contains a consensus site for the cell-cycle kinase M-CDK at residue Thr926 of human Eg5/Thr937 of *Xenopus* Eg5 (Blangy et al., 1995; Sawin, Mitchison, 1995) (**Figure 1B**). The tails of all known kinesin-5 motors except for *S. pombe* Cut7 appear to be phosphoregulated by M-CDK (Drummond , Hagan, 1998). This is discussed in detail below, along with phosphoregulation of kinesin-5 heads.

III. Mechanism of Kinesin-5

While vertebrate Eg5 has been the primary focus of *in vitro* studies to determine the mechanism of kinesin-5 motors, recent work on yeast kinesin-5 motors suggests remarkably divergent behavior that challenges the field's thinking on how directionality is determined for kinesin superfamily motors.

In general, kinesins whose motor domain is located at the protein's N-terminus move toward the microtubule's plus end, while those whose motor domain is located at the C-terminus move toward the microtubule's minus end. This notion is supported by structural and computational data showing that the repositioning of the neck linker and alpha 6 helices in C-terminal vs. N-terminal motors accounts for the directionality difference (Jana *et al.,* 2012; Vinogradova et al., 2004). However, new results on the directionality of the *S. cerevisiae* kinesin-5 Cin8 have called this idea into question.

Two groups have observed Cin8 walking toward the minus-end of the MT; as the motor contains an N-terminal motor domain, this result was entirely unexpected (Gerson-Gurwitz *et al.,* 2011; Roostalu *et al.,* 2011). In Roostalu et al, the authors observe that tetramers of Cin8 walk toward the minus-end of the MT when attached to a single MT, but switch direction when bound to two MTs such that both pairs of motor domains walk toward the plus-ends of the MTs to which they are attached. The authors attribute this switch to a collective behavior of the motor, as the motor acts with a minus-end-directionality at very low concentrations. Gerson-Gurwitz and colleagues note that this directionality preference is

biased by the presence of M-CDK phosphorylation sites in Loop 8 and the ionic strength of buffers used. Recently the same lab has reported similar direction-switching behavior in Kip1, another *S. cerevisiae* kinesin-5 motor (Fridman *et al.*, 2013). The biophysical basis of this switching behavior and the role that it plays in mitosis are not yet established, and it clearly stands in contrast to the “N-terminal motors walk toward the plus-end” paradigm.

Unlike the yeast kinesin-5 motors mentioned above, human Eg5 does follow the kinesin directionality paradigm. Eg5 tetramers walk toward the plus-ends of both MTs to which they are attached, allowing the motor protein to generate an outward force on the mitotic spindle. This point was elegantly demonstrated in the work of Kapitein et al. (Kapitein *et al.*, 2008; *Kapitein et al., 2005). The authors observed that, when attached to a surface-bound MT bundle, fluorescent Eg5 diffused randomly along the MT's length; when additional fluorescent MTs were flowed in, the Eg5 acted to crosslink between the surface and free MTs and slide them antiparallel to each other by walking processively to the MT plus ends. Recent work by Thiede et al on a kinesin-1/Eg5 chimera shows that the diffusive and processive behaviors of the motor are separated by an energetic barrier, and propose that this switch is mediated by an interaction between the heads and tails of Eg5 (Thiede *et al.*, 2013). While a head-tail interaction in Eg5 has never been directly observed, understanding the nature of the diffusive/processive switching behavior of this motor would greatly inform the field's view of its role in mitosis and provide a potential window for the development of specific inhibitors.

Given the size of the kinesin-5 tetramer and the difficulty of studying four identical but non-synchronized catalytic sites, much effort has focused on determining the mechanism of monomers and dimers of motor domains. The amino acid sequence of the human Eg5 motor domain is 45% identical to that of the well-studied kinesin-1, and the use of stable dimers of human Eg5 in a majority of these studies allows for a direct comparison of Eg5 activity to that of kinesin-1. While both motors hydrolyze ATP to perform mechanical work, there are several features of the Eg5 mechanism that distinguish it from kinesin-1 (**Table 1**).

While the core structural elements that enable ATP hydrolysis and MT motility are conserved between kinesin-5 and kinesin-1, there are substantial differences between the motors’ sequences that translate to differences in enzymatic properties and function. The largest difference between kinesin-1 and Eg5 is an 8-residue insertion in Loop 5 of Eg5 (residues 125-132; see **Figure 1c**). Loop 5 is a break in the α2 helix that occurs in all kinesin family members; however, the length of the loop is longer in kinesin-5 than any other family member. Eg5 also has a noticeably longer Loop 1 and neck linker than kinesin-1.

Within a single Eg5 head, allosteric communication between Loop 5, the nucleotide-sensing elements (the P-loop, Switch I and Switch II), and the neck linker accounts for the motor's distinct kinetics and functional properties. This communication has been revealed by kinetic studies of monomeric kinesin motor heads, which show substantial, and at least to some extent, unexplained differences between Eg5 and kinesin-1. The ADP release rate and ATPase rates of wild-type Eg5 are far less activated by the addition of MTs than kinesin-1 (25-fold v. 3000-fold; **Table 1**). Deletion of seven residues in Loop 5 in Eg5 abolishes the

MT stimulation of ADP release upon initial engagement, but has only modest effects on the ATPase activity and motility of Eg5 along MTs after this slow engagement step (Waitzman et al., 2011). The reason why Loop 5 is critical for Eg5 ADP release is unclear. Whether Loop 5, which is found in all kinesins, has a conserved function in the superfamily is also unknown.

Eg5 dimers have curious properties as well. Eg5 dimers release ADP from one head quickly (28 s^{-1}), but then release their second ADP after a slow, ~ s^{-1} isomerization event. This isomerization event appears to be unique to Eg5, and this isomerization event only occurs during Eg5's initial engagement with the MT. Subsequent steps and coupled ATP hydrolysis events occur much more rapidly than this first step, at about 13 s^1 **1**). (Krzysiak *et al.*, 2008; *Valentine et al., 2006). These data suggest that the Eg5 dimer releases ADP very slowly as it takes its first step on the MT. However in taking subsequent steps coupled to ATP hydrolysis events, Eg5 releases both ADP and phosphate simultaneously and far more repidly than the initial microtubule-stimulated ADP release event (Wiatzman et al., 2011). This mechanism may help establish an energy barrier kinesin-5's diffusing and walking states, or create a rate-limiting step in the motor's engagement that can be targeted by exogenous regulators. The structural features that are responsible for this unique property of Eg5 are unknown. However, a clue has emerged from recent cryo-EM data suggesting that binding to two adjacent sites on the microtubule places constraints on the neck linker conformations of the two heads. These constraints may influence Loop 5 conformation and the ADP•Pi or ADP release properties of Eg5 through allosteric connections (*Goulet et al., 2012).

Like dimers of kinesin-1 heads, Eg5 dimers move towards the plus ends of microtubules, alternating their heads in a hand-over-hand fashion (*Valentine et al., 2006). Both kinesin-1 and Eg5 dimers are processive, meaning that they can take multiple coupled mechanical and enzymatic steps along microtubules. However, Eg5 dimers are much more weakly processive than kinesin-1. Eg5 dimers are capable of taking 8-10 processive steps along a single MT (*Valentine et al., 2006). In contrast, kinesin-1 can take one hundred or more steps along the MT without detaching (Vale et al., 1996). The specific mechanical and kinetic properties responsible for Eg5's low processivity are not yet known, but several ideas have been proposed. Kinetic studies on Eg5 dimers indicate that the dimer initiates a processive run when one of its heads releases ADP and engages the MT (Krzysiak et al., 2008). The same head then binds ATP, sending the second head forward to its binding site. If the second head remains ADP-bound, or prematurely binds ADP from the APO state, it will not be tightly bound to the MT when the first head hydrolyzes ATP and detaches (Valentine , Block, 2009). Eg5 has a longer neck linker than kinesin-1, but studies differ as to whether Eg5's longer neck linker may be responsible for its reduced processivity (Duselder *et al.*, 2012; Shastry, Hancock, 2011). Another possible explanation is that Eg5 remains MT-bound during processive stepping as long as it releases ADP from the ADP †Pi state, but if it releases Pi before ADP or binds ADP with its other head, it detaches. In this manner, Eg5's mechanochemical properties may be the result of its unique kinetics, which certainly involve Loop 5.

IV. Loop 5 of the human kinesin-5, Eg5 is a target for inhibitor development

Aside from its significant role in Eg5's mechanochemical cycle, Loop 5 is the target of a structurally diverse class of chemical inhibitors that shut down the activity of human Eg5. The first of the Eg5 inhibitors was identified in a screen performed by Tim Mitchison's group in 1999, and named “monastrol” for the mono-astral spindle phenotype it induced in treated U2OS cells (Kapoor *et al.*, 2000; *Mayer *et al.*, 1999). Further studies have identified a number of drugs that inhibit Eg5 activity, including ispinesib, enastron and S-Trityl-L-Cysteine (STLC). These agents require residues in Loop 5 and the α3 helix to bind the motor and inhibit it (Brier *et al.*, 2006; Maliga , Mitchison, 2006) (**Figure 1C** Kinetic characterization of these agents indicates that they inhibit the motor via an allosteric mechanism that reduces the affinity of the motor for MTs and inhibits its ATPase activity. Interestingly, these agents appear to bind to Eg5 primarily while the motor is in solution, as MT-bound Eg5 has low affinity for monastrol, and monastrol-bound Eg5 has low affinity for the MT (Cochran et al., 2005; Maliga , 2002). These properties of Loop 5-directed inhibitors are consistent with the kinetic data described above, which suggest that Loop 5 is required for Eg5 engagement on MTs.

Loop 5 is clearly a “hot spot” for Eg5 inhibitor binding. For much of the history of Eg5 drug development, nearly all of the agents targeted Loop 5 (Brier *et al.*, 2004). However, new classes of Eg5 inhibitors have been developed that appear to bind to other parts of the motor or have alternate catalytic effects. The chemical FCPT (2-(1-(4-fluorophenyl)cyclopropyl)-4-(pyridin-4-yl)thiazole) appears to bind at a site near Loop 5, but has ATP-competitive like effects on the kinetics of Eg5 (Groen*et al.*, 2008). Additionally new work by the Kozielski group has identified BI8 (2-(3-fluoro-4-methoxyphenylamino)-1-((2-trifluoromethylbenzyl)-1H-benzo[d]imidazole-5-carboxylic acid), an agent that binds a novel pocket of Eg5 (Ulaganathan *et al.*, 2013). While these agents may yet enter clinical trials and demonstrate efficacy, determining the role played by Loop 5 in the Eg5 motor mechanism remains a question of high basic and clinical interest.

Eg5 inhibitors targeting Loop 5 have been tested in several different Phase I and II clinical trials, where their efficacy has been poor. A good summary of these can be found in (Sarli , Giannis, 2008). This poor efficacy has been a disappointing development, as Eg5 inhibition very robustly arrests cell division in cultured cells. Eg5 inhibitors did work in selective conditions, such as xenograft tumor models (Sakowicz *et al.*, 2004). There are several possible reasons for the failure of Eg5 inhibitors in these early trials. One reason is common to all antimitotics; human tumor cells undergo mitosis much less frequently than cells within tumor xenografts in mice, and cells have to enter mitosis for Eg5 inhibitors to cause apoptosis (Chan *et al.*, 2012). Secondly, mutations in Eg5 can confer drug resistance to Eg5 inhibitors via either direct or allosteric mechanisms (Brier et al., 2006). Lastly, mitosis is an extremely robust process, and alterations in regulatory factors or other mitotic prote may potentially overcome Eg5 inhibition (Raaijmakers *et al.*, 2012). It is therefore possible that agents acting on Eg5 or other mitotic target proteins may be more effective in combination than when used as monotherapies. That said, before we can understand what combination therapies with Eg5 would be effective, we must know how Eg5 is regulated and how it interacts with other potential drug targets in mitosis. What other targets would be effective

in conjunction with Eg5? Are there kinases or other regulatory binding partnersupstream of Eg5 that might be better targets with similar cytotoxic effects?

V. Regulation of Kinesin-5 by binding partners

As one would expect given their critical role in mitosis, kinesin-5 motors are regulated by binding partners as well as by phosphorylation. Kinesin-5 localization changes throughout mitosis in a tightly regulated manner. During metaphase, kinesin-5 localizes along spindle microtubules and at spindle poles. The motor relocalizes to the spindle midzone in anaphase (Gable *et al.,* 2012). Kinesin-5 has been reported to associate directly with NuMa (**Nu**clear **M**itotic **A**pparatus protein). which is involved in spindle pole assembly. NuMa appears to require both kinesin-5 and dynein, but kinesin-5 does not require NuMA for localization to the spindle pole (Iwakiri *et al.,* 2013).

While kinesin-5 does not require NuMA, it does require the minus-end directed motor dynein for proper targeting throughout mitosis. The human Eg5 tail interacts with dynein through the $p150_{glued}$ subunit of dynactin, and this interaction is regulated by a conserved phosphorylation site, Thr926, which is discussed in more detail below (Blangy *et al.,* 1997). Nuclear envelope-associated dynein cooperates with Eg5 to separate spindle poles during prophase, and in fact, this dynein can separate spindle poles in cell lines that completely lack functional Eg5 (Raaijmakers et al., 2012). During metaphase, a fraction of Eg5 remains stationary at the spindle midzone, while the remainder is transported poleward. Inhibition of dynein by injection of the CC1 fragment of p150 blocks this movement, resulting in altered spindle length and organization (Gable et al., 2012).

Proper Eg5 targeting also requires the MAP TPX2 (**T**argeting **P**rotein for ***X****enopus kinesin-like protein* ***2*** *(Xklp2))*. Xklp2 is a kinesin-15 family motor protein that localizes to spindle poles and is essential for their separation. Because TPX2 is required for targeting both Eg5 and Xklp2 kinesins, it appears to play multiple roles in mitotic spindle assembly. TPX2 resides in the nucleus in interphase, and undergoes dyneindepenent relocalization to MTs near the spindle poles in early mitosis.

Wittmann, et al. showed that TPX2 is critical for establishing the localization of the Xklp2 motor protein during spindle pole separation. They also noted that adding an excess of TPX2 resulted in the formation of monopolar spindle structures (Wittmann *et al.,* 2000). This finding was not followed up on until nearly a decade late, when Eckerdt et al showed that injection of *Xenopus* embryos with either the full-length or the C-terminal half of TPX2 led to cleavage arrest (Eckerdt *et al.,* 2008). Based on an observation that both TPX2 and Eg5 had been isolated in a complex together (Koffa *et a;.*, 2006), Eckerdt et al performed pull-downs between Eg5 and a variety of TPX2 constructs. The authors noted that TPX2 interacted with Eg5, and that this interaction required the C-terminal 35 residues of TPX2. could cause cleavage arrest in *Xenopus* Additionally, the only TPX2 proteins that embryos contained this C-terminal Eg5-interacting region (Eckerdt et al., 2008). Using LLC-Pk1 cells, Ma and colleagues demonstrated similarly that disrupting the TPX2-Eg5 interaction by deleting the C-terminal 35 residues of TPX2 led to mislocalization of TPX2 and poorly organized mitotic spindles (Ma *et al.,* 2010). The findings that TPX2 appeared to directly

interact with Eg5 and that excess amounts of the Eg5-interacting region of TPX2 induced monopolar spindle formation suggested that TPX2 might regulate Eg5 activity. Supporting this notion, TPX2 helps localize Eg5 to the mitotic spindle (*Ma *et al.*, 2011), and TPX2 is required for Eg5 to transition from spindle pole localization to midbody (Gable et al., 2012).*In vitro*, TPX2 slows down the MT-sliding activity of Eg5 but not kinesin-1 (Ma et al., 2011). These data indicate that TPX2 may facilitate slower, but perhaps more persistent movement of Eg5 to the spindle midzone.

Previous studies proposed mechanisms by which MAPs could regulate kinesin motors through local MT lattice alteration and/or competition for MT binding sites (McVicker *et al.*, 2011; Seitz *et al.*, 2002). The TPX2/Eg5 interaction is certainly different, because the effects of TPX2 are specific to Eg5 and are not seen on kinesin-1 (*Ma et al., 2011). This suggests higher specificity than could be explained by either MAPs locally altering the MT lattice or competing for binding sites. It is intriguing that similar high-specificity regulation of kinesin-1 by the MAP ensconsin has been proposed (Barlan *et al.*, 2013; Metzger *et al.*, 2012). Therefore, TPX2 regulation of kinesin-5 may typify a new class of mechanisms for kinesin motor regulation that we do not yet fully understand.

VI. Phosphoregulation of Kinesin-5

Phosphorylation of almost all kinesin-5 homologs at a co within the BimC box (Thr926 in human Eg5, Thr937 in *Xenopus*) is essential for targeting the motor to the mitotic spindle (Blangy et al., 1995; Sawin , Mitchison, 1995). The only known exception is the kinesin-5, Cut7 (Drummond , Hagan, 1998). A second phosphorylation site at residue Ser1033 the Eg5 tail, phosphorylated by Nek6/7, was shown to help localize around 3% of the total Eg5 protein to the spindle pole (Rapley *et al.*, 2008). Phosphorylation of the BimC box threonine (Thr926 in human Eg5) also regulates the interaction of Eg5 with dynactin, suggesting that association with dynein and dynactin may help target Eg5 to MTs (Blangy et al., 1997). However,*in* Cahu, et al. showed that phosphorylation of the *Xenopus* Eg5 tail by M-CDK increases Eg5 affinity for MTs ~10 fold, and also increases the amount of time the motor spends on the MT, with no additional factors in the assay (Cahu *et al.*, 2008). This suggests that phospho-regulation of the kinesin-5 tail exerts a direct effect on motor activity that is not mediated exclusively through binding partners. This increased MT affinity upon phosphorylation is counter-intuitive, given that the microtubule surface is negatively charged. It is not known how phosphorylation alters the structure of the Eg5 tail or the Eg5 holoenzyme to enhance MT binding.

In addition to work described above that identified phosphoregulatory mechanisms that control kinesin-5 localization through the motor's tail, recent studies have shown that phosphorylation of kinesin-5 enzymatic heads may alter their activity. In a 2010 paper, Chee and Haase identified three sites in the *S. cerevisiae* Cin8 motor domain and four sites in the Kip1 motor domain that met the minimal consensus sequence for the checkpoint kinase M-CDK (Chee , Haase, 2010). One of these sites (Ser388 in Kip1/Ser455 in Cin8) is conserved between the two motors and many other kinesin-5 family members. The authors found that deleting Cin8 and mutating Ser388 in Kip1 to alanine or deleting Kip1 and mutating Ser455 in Cin8 to alanine resulted in defects in spindle pole body separation.

Subsequently, Avunie-Masala et al found that the consensus sites for M-CDK in the Cin8 motor domain are phosphorylated and that phosphorylation of these sites alters Cin8'S motility. Cin8 was phosphorylated in anaphase, but mutation of the three consensus M-CDK sites described above abolished this phosphorylation (*Avunie-Masala *et al.*, 2011). Two of the three identified sites, Ser239 and Thr247, are located in the yeast-specific Loop 8 insertion of the motor, while the third, Ser455, is the conserved site identified above. Cin8 with all three phosphorylated residues mutated to alanine localized primarily to the spindle poles in anaphase, as opposed to wild type motors that primarily occupy the midzone. A second study performed by the same group found that Cin8 proteins containing Ser239Ala and Thr247Ala mutations exhibited spindle-pole directed (or toward the minus-end of the MT) movements, suggesting that Loop 8 may help determine the directionality of the motor (Gerson-Gurwitz et al., 2011). In contrast, Cin8 with all three phosphorylated residues mutated to aspartate failed to localize to the spindle apparatus, and cells expressing this Cin8-3D mutant were not viable.

Garcia, et al. showed that the *D. melanogaster* kinesin-5 motor Klp61F is phosphorylated on up to three tyrosines in its motor domain by the cell cycle kinase dWee1 *in vitro* (Garcia *et al.*, 2009) To determine the effects of this phosphorylation event, Garcia et al expressed Klp61F with the phosphoacceptor tyrosines mutated to phenylalanine in *Drosophila* embryos, and observed increases in lethality and spindle defects. This work suggests an interesting interplay between M-CDK and Wee1 at the onset of mitosis. Wee1 phosphorylates and deactivates M-CDK until the onset of mitosis (Russell , Nurse, 1987). In turn, M-CDK activation negatively regulates Wee1 (McGowan , Russell, 1995). As described above, M-CDK also phosphorylates and activates kinesin-5 tails. It is intriguing that a kinase that directly activates Eg5 is antagonized by a kinase that directly inhibits it. One could speculate that Eg5 is acting as a mechanical conduit between two antagonistic signaling mechanisms mediating mitosis, Wee1 and M-CDK. Together, the phosphorylation data on kinesin-5 motors tell two stories: first, a highly conserved mechanism by which M-CDK phosphorylation recruits kinesin-5 to microtubules and second, multiple, possibly species-specific, phosphoregulatory mechanisms (M-CDK in yeast, Wee1 in *Drosophila*, and perhaps others?) that fine-tune the activity of the motor domains.

Much work remains to be done regarding phosphoregulation of kinesin-5 and kinesin motors in general. The PhosphoSitePlus database has identified phosphorylation sites in nearly every human kinesin family member (Hornbeck, 2012). Many of these sites have been reproduced by multiple MS techniques, but very few studies have probed the mechanistic effects of these phosphorylation events on kinesin motor activity *in vitro* and *in vivo*. It is difficult to assess how much of this sea of data is an opportunity to discover specific, poorly understood regulatory mechanisms governing kinesins, and how much of it is simply artifact. However, intriguing mechanistic studies on kinesin motor phosphoregulatory mechanisms continue to emerge, such as those referenced above and the work of Mennella et al, who mechanistically link phosphorylation of the *Drosophila* kinesin-13 Klp10A to changes in MT depolymerization (Mennella *et al.*, 2009).

VII. Kinesin-5 roles beyond mitosis

While kinesin-5 is considered a “mitotic” kinesin, several lines of evidence have suggested roles for the motor outside of mitosis. Peter Baas'S group has published several studies demonstrating that Eg5 regulates the length of axons and the migration of neurons in culture (Falnikar *et al.*, 2011; *Myers , Baas, 2007). Recent work by the Baas group suggests that phosphorylation of the BimC box of Eg5 may act as a switch that turns on the motor'S activity in axonal growth cones, as it does in mitosis (Nadar *et al.*, 2012).

Additionally, Bartoli et al have reported a global decrease in protein synthesis in cells treated with specific Eg5 inhibitors, and shown that Eg5 helps structure polysomes along MT scaffolds (Bartoli *et al.*, 2011).

VIII. Conclusions: Directions for future study

Investigations of the mechanism and the biological role of kinesin-5 motors have generated several emerging and controversial storylines that force the entire kinesin field to re-examine many of its assumptions. Having highlighted many of these storylines above, we conclude here with a series of questions that, in our view, would make excellent topics for future research.

- How and why is it that yeast kinesin-5s, which have N-terminal motor domains, switch directions? Is this direction switching the target of regulatory mechanisms that establish unique roles for yeast kinesin-5s?
- Does loop 5 have a conserved function for all kinesins that is amplified for the kinesin-5 family? Is this structural element a natural regulatory target region for kinesins, and/or a “hot spot” for inhibitors?
- How can a specific MAP, TPX2, regulate a specific kinesin, Eg5?
- Can combination drug therapies be developed with a knowledge of how Eg5 interacts with regulatory proteins and mitotic kinases?
- Does kinesin-5 require different binding partners to fulfill its conventional roles in mitosis vs. “moonlighting” roles in axonal length regulation and protein synthesis? Does kinesin-5 perform different mechanochemical activities in these settings?
- Does kinesin-5 have other “moonlighting” roles besides axonal length regulation and protein synthesis?

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Abbreviations

MT	microtubule
MAPs	Microtubule-associated proteins
EPR	electron paramagnetic resonance
“BASS” domain	*b*ipolar *ass*embly domain
STLC	S-trityl L-cysteine
TPX2	**T**argeting **P**rotein for *X*enopus *K*inesin-*l*ike *p*rotein *2*
MS	Mass spectrometry

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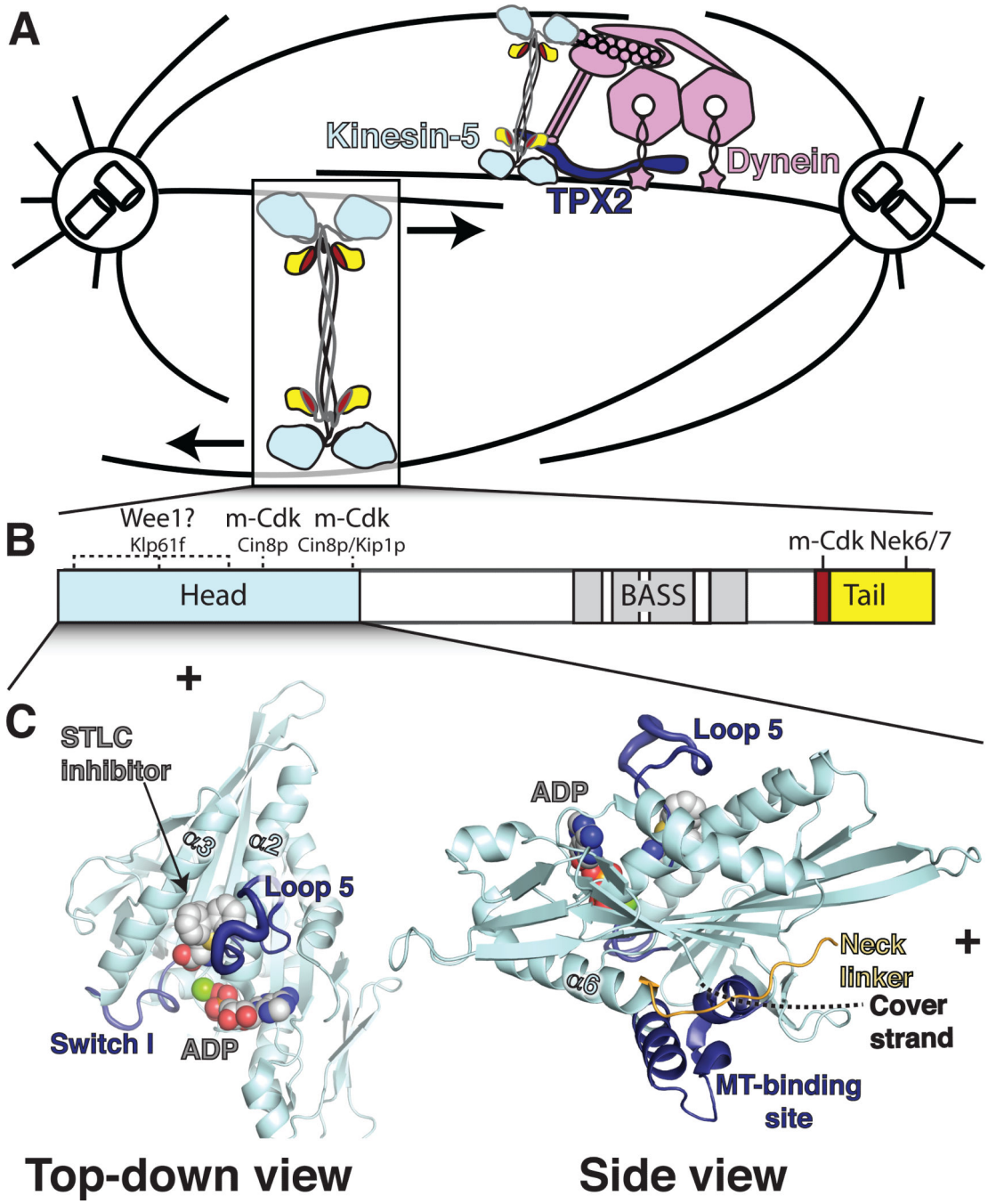

Figure 1. Kinesin-5 structure, function, and regulation

A. Kinesin-5 slides spindle poles apart during mitosis. The motor associates with dynein (pink) and TPX2 (dark blue) for proper localization. The Eg5 tail interacts with the p150 $_{glued}$ subunit of dynactin (Blangy et al., 1997). It is not which domain(s) of Eg5 interact with TPX2. **B.** Bar diagram of kinesin-5, showing conserved domains and known phosphorylation sites. Coloring is as in **A** with the BimC box in red. The BASS domain has only been identified in the *D. melanogaster* homolog Klp61F and is shown in gray and white (*Acar et al., 2013). Phosphorylation sites in the heads are only known to occur in

non-mammalian kinesin-5 homologs. These are indicated with dashed lines along with the homolog. Tail phosphorylation sites are conserved among metazoan kinesin-5 motors and are shown in solid lines. **C.** Left, Eg5 head structure (PDB ID#3KEN, (Kim *et al.*, 2010) showing Loop 5 and the binding site for allosteric inhibitors. This view would be top-down for a MT-bound Eg5 head, with the MT plus end up as indicated. Right, side view, with MT plus end to the right. The MT-binding site is at the bottom of the molecule. The neck linker is shown in orange. The cover strand is not ordered in this structure, but its approximate position is shown in dashed lines, based on the work of Goulet and Hesse (*Goulet et al., 2012; Hesse et al., 2013).

Table 1

ADP release, ATPase, and motility rates of human Eg5 motor proteins.

	Eg5-367	Kinesin-1 monomer	Eg5-513	Kinesin-1 dimer
Basal ADP release (s^{-1})	0.27	0.02		
Basal ATPase (s^{1} $site^{-1}$)	0.25	0.006		
Maximal MT-stimulated ADP release (s^{-1})	42.3	>100	28.2 *1.0*	95
MT-stimulated ATPase (s^{-1} $site^{-1}$)	6.25	60	0.48 (steady-state) 5-10 (while stepping)	31
Motility rate (nm/s)	20.5	88	32.9	450

Data are taken from these references: (Case *et al.*, 2000; Krzysiak , Gilbert, 2006; Ma , Taylor, 1995; *Valentine et al., 2006; Waitzman et al., 2011). Steady-state microtubule-stimulated ATPase of Eg5-513 includes both engagement onto MTs and active stepping. Engagement is rate-limiting in this case. The rate of active stepping after engagement was estimated based on optical trapping and calculation from other rate constants at 5-10 s^{-1}. At saturating MTs, Eg5-513 has two different rates for the MT-stimulated ADP release rates of the first and second heads; the second head rate is italicized.