Microtubule organization in mitosis: the role of kinesin-5

Introduction

Mitosis, or the division of one mother cell into two daughter cells, is one of the basics of life, whether it is in a unicellular organism or in a single cell of a larger organism. Without cell division, growth of an organism would be limited to cell growth only. In mitosis, the DNA that is duplicated in an earlier phase of the cell cycle is condensed and separated, after which cytokinesis takes place in which the cell divides (Alberts et al., 2008). The importance of this process for the propagation of the genetic information of the cell and the growth of the organism implies tight regulation of this process. Indeed, deregulation of cell division and checkpoints involved in the cell division can lead to sustained growth of a tissue, one of the conditions necessary for cancer to arise (Hanahan and Weinberg, 2011). Conversely, deregulation of the cell cycle can also lead to cell death through extensive DNA damage, if one of the key effectors of the cell cycle is lost. Thus, targeting the cell cycle with inhibitors could lead to cell death in both healthy and diseased cells. The advantage of this method to target tumour cells is that tumour cells by definition divide more often compared to normal somatic cells, making cell cycle targeting a relatively specific treatment (Alberts et al., 2008; Hanahan and Weinberg, 2011). Therefore, it is interesting to elucidate specific players in the cell cycle, to help us understand how different mutations can give rise to cancer and to find new methods by which cancer can be treated.

In this review, we will look at the kinesin-5 family, a class of proteins important for the establishment of a proper mitotic arrangement of the cytoskeleton and the separation of the chromosomes.

In order to get an overview of the role of this protein, it is important to have an overview of mitosis in higher eukaryotes. There are some differences in yeast mitosis which will be discussed afterwards. Then, some information about motor proteins in general, and model systems that can be used to examine these proteins, will be discussed before the review focuses entirely on kinesin-5.

Overview of mitosis

In order to enter mitosis, cdk1 (cyclin-dependent kinase) needs to associate with cyclin B. This process is regulated by an inhibitory phosphorylation of cdk1. This is controlled by two proteins working towards opposite ends: Wee1 kinase phosphorylates the cdk protein to keep it inhibited, while cdc25 displays phosphatase activity to remove the inhibitory phosphate group. The cyclin B/cdk1 complex can phosphorylate cdc25 to increase its activity and at the same time inhibit Wee1, leading to an explosive increase of cyclin B/cdk1 complex in the cell and the beginning of mitosis (Alberts et al., 2008)

Mitosis starts after the DNA G2/M checkpoint has been satisfied. This means that the cell has successfully duplicated its DNA and that the Cyclin B/cdk1 complex is activated and starts phosphorylating its targets.

There are five distinct phases of mitosis: Prophase, prometaphase, metaphase, anaphase and telophase (Alberts et al., 2008). In prophase, the DNA starts to condense and the centrosomes start moving apart to form a mitotic spindle. The centrosomes serve as microtubule organising centres for the mitotic spindle. In prometaphase, the nuclear envelope breaks down and the microtubules

begin attaching to the chromosomes at their centromeres. This attachment is mediated by the kinetochore, a protein structure that is built upon the centromeres that can bind to microtubules. There are also microtubules that crosslink to other microtubules originating from the other centrosome, and microtubules that connect to the cell membrane to position the centrosomes. At this point, the attachment of microtubules starts to organize the chromosomes in the middle of the cell, which is called the metaphase plate. In the next phase of mitosis, metaphase, this alignment is eventually completed and the cell can process to the next phase. In anaphase, the chromosomes are pulled apart towards the centrosomes. This is mediated by the reduction in length of the microtubules attached to the kinetochore (anaphase A) and centrosomes that are pushed further apart from each other (anaphase B). Both parts of anaphase can occur at the same time. The separation is complete in telophase, in which the now separated chromosomes decondense, the nucleus reforms and the cell itself starts to divide. In cytokinesis, this is completed, leaving two daughter cells at the place of the mother cell (Alberts et al., 2008).

While the above explains the situation of mitosis in higher eukaryotes, the yeast mitosis has some differences. The most important difference is that in yeast, the nuclear envelope does not break down, so the mitosis is considered 'closed' (Biggins, 2013). Furthermore, yeast does not have centrosomes; instead they have spindle pole bodies which are embedded into the nuclear envelope. The microtubules that connect the centrosomes to the cell membrane in the mitosis of higher eukaryotes are used to position the nucleus properly in this closed mitosis (Biggins, 2013). Despite these differences, the cell cycle is regulated in a manner similar to eukaryotes.

While there are a lot of proteins involved in this process, many of these are conserved across evolution, such as kinesin-5. There are kinesin-5 proteins in model organisms including but not limited to *Drosophila (*Klp61f), Human (hsEG5) and *Xenopus (*Eg5), with the name of the kinesin-5 protein in brackets. *S. cerevisiae*, or budding yeast, has not one but two kinesin-5 proteins: Cin8p and Kip1. Budding yeast is a very good model to study cell division, as many important proteins found in yeast also have a similar function in higher eukaryotes. Differences such as the amount of kinesin-5 proteins need to be taken into account when translating these results.

Motor proteins

In order to get a proper mitosis, a lot of force generation has to occur. Pulling apart the chromosomes and the movement of the chromosomes towards the metaphase plate both require force generation (Alberts et al., 2008). Furthermore, in order for the cell to progress into anaphase, the cell should satisfy the spindle assembly checkpoint, which requires tension on the kinetochore (Liu et al., 2009). Thus, the cell needs to have a mechanism to generate force, and the cell needs a spindle that can withstand this force.

The mitotic spindle consists of microtubules. These are polymers of alpha- and beta tubulin subunits which forms protofilaments. These protofilaments associate together resulting in a hollow tube that is the microtubule. Microtubules are polar, and the microtubule polymerises and depolymerises at higher speed at the plus end. This is a continuous process of shrinking and growing, which occurs due to the inherent instability of the microtubules. Microtubules are known for numerous roles outside of mitosis, but here the focus lies on the microtubules during

mitosis. The microtubules protrude from the centrosome during prophase, with the minus end extruding from the centrosome and the plus end binding the kinetochore. The dynamic instability of the microtubules increases quite a lot during mitosis, creating an environment in which it is easier for the chromosomes to be captured by the spindle. When anaphase proceeds, the microtubules attached to the kinetochore depolymerise and pull the chromosomes apart (Alberts et al., 2008). This is one manner by which force is generated.

Another way by which mechanical forces are generated is by motor proteins such as kinesin and dynein. Motor proteins generally have a motor domain which allows them to move over a substrate such as actin or microtubules, and a cargo binding domain which allows for transport. The proteins use ATP to move across the substrate in a single direction. The cargo of motor proteins can vary wildly, ranging from cell organelles to chromosomes. In mitosis, one of the functions of motor proteins is to exert force on the spindle and the centrosomes during anaphase to help chromosome segregation (Alberts et al., 2008).

Model systems

As briefly mentioned before, there are quite a number of model systems for studying mitosis and the processes involved in it. These models range from *in vitro* models such as *Xenopus* egg extract, which can be used to study aspects like the movement of a specific motor protein under different conditions, to *in vivo* models with live imaging of cells. These methods all have their disadvantages and advantages, specifically due to the fact that a lot of proteins have multiple roles at different times during mitosis. Kinesin-5 for example performs functions during prophase and during anaphase, but knocking it out would lead to the cell being stuck in prophase and never reaching anaphase. Finally, although kinesin-5 is evolutionary well conserved, there are distinct differences between organisms. It has been mentioned before that budding yeast has 2 kinesin-5 proteins, Cin8 and Kip1. In *C. elegans,* knockdown of the kinesin-5 protein does not lead to completely impaired chromosome segregation. Therefore, a combination of model systems has been used to elucidate the function of kinesin-5 proteins to allow for these differences, which has lead to some very interesting insights in the protein.

In the following paragraphs, the structure of kinesin-5 and the characteristics that it infers will be discussed. Subsequently, the function and properties of kinesin-5, and abnormalities when compared with other motor proteins will be discussed. In the next paragraphs, the structure of kinesin-5 and its characteristics will be discussed, as well as the manner by which the protein is regulated. Finally, conclusions drawn during the description of kinesin-5 will be revisited and expanded upon, to look for possible future directions for kinesin-5 research and to discuss the implications of some of kinesin-5's properties.

Kinesin-5 functions

Kinesin-5 is well known for its ability to crosslink microtubules and slide them apart. It is able to achieve this by binding to two microtubules that are in an anti-parallel configuration and pushing away the minus ends of the microtubules (Figure 1A) (Kapitein et al., 2005). In mitosis, these minus ends terminate at the spindle poles, or the spindle pole bodies in yeast. The motor action then pushes the two spindle poles away from each other. This leads to the formation of a bipolar spindle, an essential step in proper chromosome segregation (Figure 1B). The localization of kinesin-5 is very dynamic, with

the localization changing according to the phase of mitosis. During metaphase, kinesin-5 is most prominently found close to the spindle poles, with only trace amounts found near the chromosomes (Figure 1C). The protein can be found on the interpolar microtubules (iMT), the kinetochore microtubules (kMT) and surprisingly, also on the astral microtubules (aMT) (Gable et al., 2012). The localization on the aMT is surprising due to the fact that there are less microtubules in the aMT zones, meaning that there is less chance for a successful binding of two microtubules.

Loss of kinesin-5 during the onset of mitosis leads to the formation of a monopolar spindle (Figure 1E), preventing segregation of the sister chromatids. It has also been shown that kinesin-5 plays a role in anaphase by pushing the spindle poles even more apart, thus generating more force on the sister chromatids. The protein appears more prominently in the midzone during late anaphase, which is in accordance with this role(Figure 1D) (Gable et al., 2012). The part of the anaphase where the force generated by kinesin-5 proteins is most important is anaphase B, which is the primary anaphase in yeast due to shorter kMTs and less condensation of DNA (Roostalu et al., 2010). It has been shown that treating cells with an inhibitor of kinesin-5 at the onset of anaphase leads to a longer spindle, which is counterproductive for the separation of chromosomes: If the spindle becomes longer,

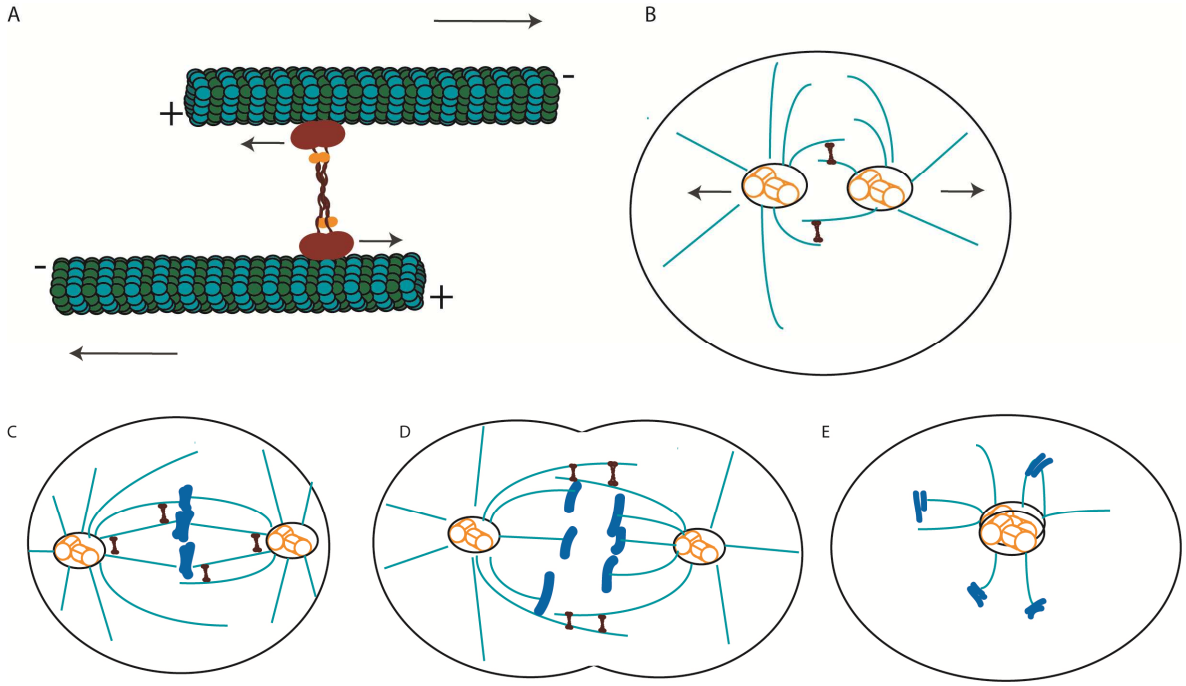

Figure 1: Schematic overview of kinesin-5 functions. (A) Basic principle of kinesin-5 microtubule sliding. The kinesin-5 molecule will bind to two microtubules with anti-parallel orientation and start 'walking' towards the plus end on both microtubules. This will exert force on the microtubules, sliding them away from each other. **(B,C,D) schematic overview of kinesin-5 activity during mitosis.** Kinesin-5 proteins on astral microtubules are not visualised because the function of kinesin-5 on these microtubules remains unclear. **(B) Kinesin-5 activity during prophase**. During prophase, kinesin-5 will bind to anti-parallel microtubules originating from the two centrosomes and push these apart, which will assist in formation of a bipolar spindle. The chromosomes are condensing and have not been visualised to provide a clearer schematic. **(C) Kinesin-5 activity during prometaphase and metaphase.** At this point in mitosis, the microtubule binding activity is primarily for crosslinking of microtubules, while the motor functions seem to be less important. **(D) Kinesin-5 activity during anaphase.** In anaphase the microtubule sliding activity of kinesin-5 contributes to the centrosome movement away from the middle, thus generating force for the separation of the chromosomes. The crosslinking that was important in earlier phases may still play a role, but this is difficult to study. **(E) Schematic representation of a cell which has no kinesin-5.** A cell with no kinesin-5 present is unable to separate the 2 centrosomes, resulting in a monopolar spindle. The microtubules will be able to capture chromosomes, but bipolar attachment is very rare, and if it occurs, the tension required to satisfy the checkpoint is not present.

this would counter the movement of the spindle poles that is characteristic of anaphase B. This occurs when the activity of kinesin-5 is inhibited using a small molecule inhibitor, and is consistent with results found in yeast (Collins et al., 2014; Gardner et al., 2008).

However, the motor function may not be the only important role kinesin-5 has in the process of mitosis. In the example given above, concerning longer spindle microtubules, this may also be due to an indirect effect of kinesin-5, as small molecule inhibitors also reduce the affinity for binding to the microtubules (Maliga et al., 2002). A well established function apart from the motor activity is that, due to the two distinct microtubule binding sites the protein possesses, it can also crosslink microtubules (Kapitein et al., 2008; Vladimirou et al., 2013; Weinger et al., 2011)(Figure 1C). This can lead to some interesting phenomena, without making use of the motor function. It has been shown that kinesin-5 plays a role in connecting the kMTs, indirectly connecting chromosomes. This may explain part of the observed chromosome oscillation that is seen in cells in metaphase during microtubule flux (Vladimirou et al., 2013). Why this microtubule flux occurs remains elusive.

Kinesin 5 does not necessarily have to bind to two antiparallel microtubules. It can also bind to parallel microtubules, which has an entirely different effect. The fact that kinesin can bind microtubules in a parallel fashion is illustrated by the enrichment of kinesin-5 at the spindle bodies. However, it has also been shown that kinesins preferably bind in an antiparallel fashion (Wildenberg et al., 2008) (as visualized in figure 1A), so this explanation cannot fully clarify the enrichment at the spindle poles that is observed in mammalian cells and yeast alike. The budding yeast kinesin-5 Cin8 has been shown to be able to switch to a minus-end-directed motor protein under certain conditions (Gerson-Gurwitz et al., 2011; Roostalu et al., 2011). This abnormality in motility will be discussed later in this review. A possible mechanism by which the higher eukaryotes might achieve a stable enrichment of kinesin-5 near the spindle poles is by binding to parallel microtubules. If the motor domains start walking, this would be just enough movement to counter the movement of the microtubules itself due to microtubule flux and microtubule growth (Weinger et al., 2011). As this still leaves the issue that kinesin-5 prefers to bind to anti-parallel microtubules (Wildenberg et al., 2008), it is likely that there is another level of regulation which thus far remains elusive.

A study done by Gardner *et al* has shown that kinesin-5 can also affect the microtubules itself. They showed that the binding of kinesin-5 to the microtubules in higher numbers had a destabilizing effect on the microtubules (Gardner et al., 2008). These experiments were performed in budding yeast, which has two kinesin-5 proteins instead of one. A knockdown of one of these kinesins, Cin8, lead to longer microtubules. This was seen looking at kMTs, and also at aMTs. The model suggested by the authors says that an increased amount of Cin8 motors can bind to longer kMTs, leading to a destabilizing effect on the plus ends, thus preventing the kMTs from becoming too long (Collins et al., 2014; Gardner et al., 2008). Interestingly, the other kinesin-5 protein, Kip1, appears to be modulating the stability of the iMTs during anaphase B, which is necessary for proper depolymerization of the iMTs and for proper spindle breakdown (Fridman et al., 2013). It remains to be elucidated whether this can also be found in higher eukaryotes.

Kinesin-5 functions in a non-mitotic cell

Besides the above described functions of kinesin-5 proteins in mitosis, there are some reported functions of kinesin-5 in other

processes. It has been shown that kinesin-5 plays a role in the migration of neurons, with overexpression of kinesin-5 leading to an abrogation of migration. Inhibition of kinesin-5 leads to more extensive movement of the neuron, but loss of clear directionality. It is thought that in this case, the forces generated by kinesin-5 antagonize those generated by the minus-end-directed motor dynein (Falnikar et al., 2011). Furthermore, a study has reported that inhibition of kinesin-5 by a small molecule inhibitor leads to a decrease in certain neurotransmitter receptors on the cell surface. It is worth noting that the Alzheimer-related protein amyloid beta seems to perform this inhibitory role *in vivo*, which would be one of the reasons that neuronal function degrades in Alzheimer patients (Ari et al., 2014).

Finally, there has been a report of a non-mitotic function of kinesin-5 in *Drosophila* cells which is not specific neuronal. This report shows a reduction in the release of proteins contained in carriers called CARTS if kinesin-5 is inhibited (Wakana et al., 2013). It remains to be elucidated whether these are true effects of kinesin-5 or an artefact, as it has thus far not yet been reported that kinesin-5 proteins can bind cargo other than microtubules.

Motility of kinesin-5

The motility of kinesin-5 proteins is essential for its function, because generating movement requires motility. Thus, the plus-end-directed movement that is necessary for the microtubules to slide is an important part of its motility. In this paragraph, some of the specifics of this movement will be clarified, after which some peculiarities in different model systems will be discussed. However, it should be realized that despite these peculiarities, the plus-end-directed movement described earlier is the most prominent motile function of kinesin-5.

An important part of the function of kinesin-5 proteins is performed by being at the proper location to exert force. For instance, in anaphase B, kinesin-5 becomes more prominent in the midzone (Gable et al., 2012), allowing for the movement of the spindle poles that is inherent to this phase (Alberts et al., 2008). Kinesin-5 is able to transport itself using active movement and by diffusion over the microtubules. This diffusion is ATP-independent movement (Kapitein et al., 2008). Research has shown that at low ionic strength, the movement of vertebrate kinesin-5 over single microtubules is primarily through ATP-dependent movement; increasing the ionic strength to physiological level switches the protein to move primarily via diffusion over a single microtubule. The binding of a second microtubule is enough for the protein to switch to ATP dependent movement again (Kapitein et al., 2008). Enzymatically, kinesin-5 is one of the slower kinesins, with an average unloaded speed of 100nm/s. As a comparison, kinesin-1 can reach 5 times that speed (Hesse et al., 2013).

Minus-end-directed movement

Kinesin-5 is a protein with the motor domain on the N-terminus, which leads to plus-end-directed motility. Most of the other members of the kinesin superfamily also have the motor domain on the N-terminus. Exceptions are the kinesin-14 protein, which has the motor domain on the C-terminus and is minus-end-directed, and kinesin-13, which has the motor domain in the middle of the protein and does not display motility (Alberts et al., 2008). However, in 2011 studies showed that one of the budding yeast kinesin-5 proteins, Cin8, has the ability to switch directionality to minus-end-directed motility under certain circumstances (Gerson-Gurwitz et al., 2011; Roostalu et al., 2011). Interestingly, recent studies have shown that the other kinesin-5 protein in budding yeast, Kip1, exhibits similar

behavior (Fridman et al., 2013), as well as the fission yeast kinesin-5 (Edamatsu, 2014). Elucidating the manner by which this directionality switch in yeast occurs can provide valuable insights on the mechanism by which kinesin-5 is regulated, warranting extensive research into this property.

It was shown that single Cin8 motors display minus-end-directed motility on single microtubules, but also in the presence of anti-parallel microtubules; even though there was microtubule sliding, there was still an accumulation of Cin8 motors at the minus end of the microtubule. Further experiments show that the amount of motors on a microtubule control this switch. If there are more motors present, the switch towards plus-end-directed movement is made.

It was also shown that in the presence of ADP the minus-end-directed motion was not occurring, meaning that this motion could not be explained by diffusion (Roostalu et al., 2011). These results were expanded upon to show the involvement of an insert in Loop 8 of the motor domain, which, if deleted, allows the protein to switch direction at lower salt concentrations when compared to wild-type (Gerson-Gurwitz et al., 2011). The involvement of ionic strength in the switching of directionality implies that binding of cargo

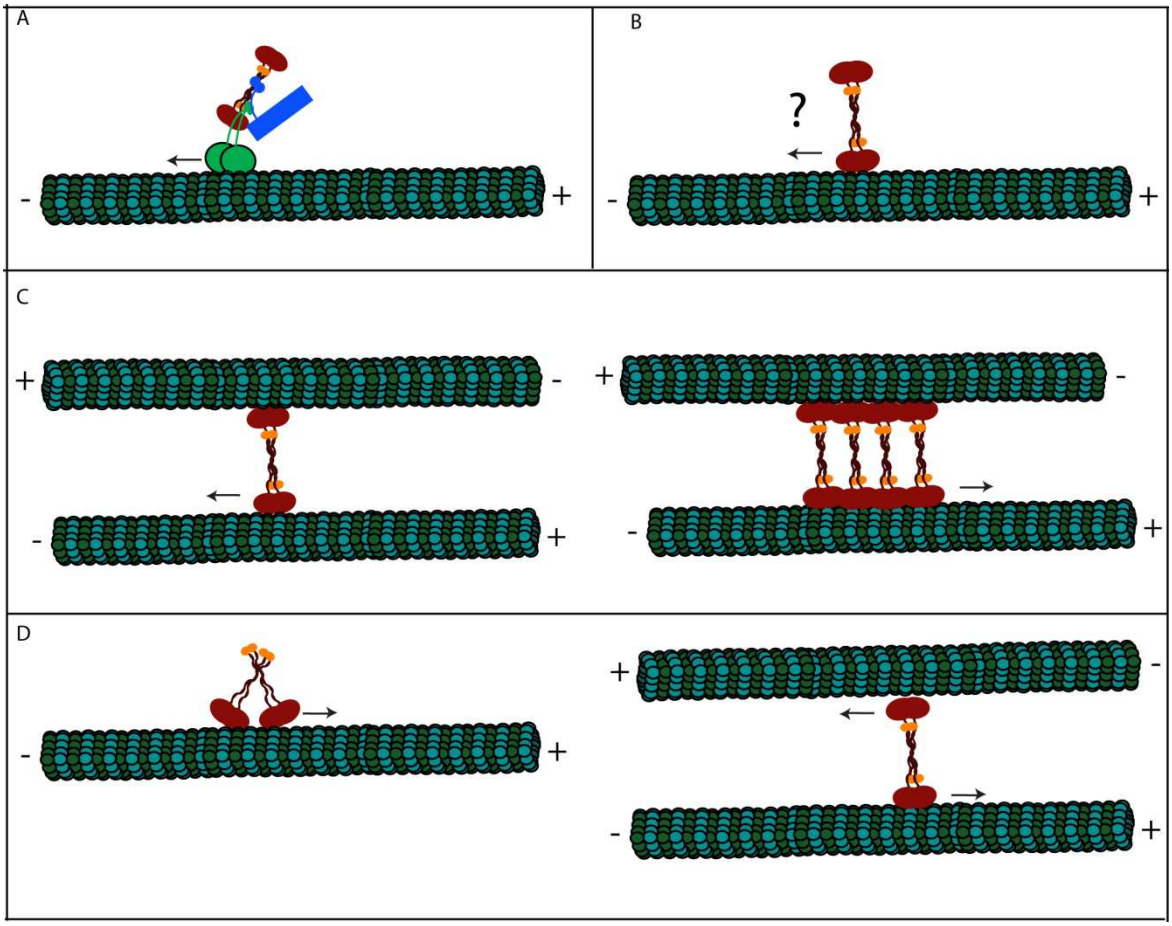

Figure 2: Schematic representation of minus-end-directed motility in mammalian cells (A) and yeast (B). (**A)** The minus-end directed motor dynein (green) can associate with the p150 subunit of dynactin (blue), which in turn can associate with the kinesin-5 stalk (Blangy et al., 1997). This coupling allows kinesin-5 to be transported towards the minus end of the microtubule. In yeast (**B),** there is no dynein present, and minus-end-directed movement is observed without the assistance of other proteins (Roostalu et al., 2011). The results supply two possible interpretations, assuming the minus-end-directed movement is standard for yeast kinesin-5. (**C) Clustering of kinesin-5 proteins lead to plus-end-directed movement.** Additional kinesin-5 proteins associate together, allowing for a subtle change in the motor domain, which then allows the kinesin to switch to plus-end-directed motility. Electrostatic interactions modulate this change, allowing the protein to switch autonomously if the ionic strength drops. (**D) Binding of cargo to both motor domains of kinesin-5 allows for plus-end-directed movement.** If the ionic strength is reduced, the stalk region becomes flexible, allowing both motor domains to bind the same microtubule. When both motor domains are bound, plus-end-directed motility is achieved. Similarly, this is how sliding and motility on two microtubules occurs.

or intra-molecule interactions might regulate the directional switch.

It is clear that there are electrostatic interactions present in Cin8 which can alter the directionality, as is evidenced by the directional switch at varying ionic strengths (Gerson-Gurwitz et al., 2011; Roostalu et al., 2011; Thiede et al., 2012). An interesting hypothesis has been put forward that proposes that when the ionic strength is low, the protein is more likely to lose its bipolar orientation due to a flexible neck linker, and to bind one microtubule with both motor domains. In this hypothesis, occupation of both motor domains is inherent to the switch (Roostalu et al., 2011; Thiede et al., 2012). Another possible hypothesis is that the kinesin-5 protein needs to associate with another kinesin-5 protein before it can in fact display plus-end-directed motility. This would explain why a higher concentration of motors would lead to plus-end-directed motion (Roostalu et al., 2011). The question this model does not immediately present a solution for is as to why lower ionic strength allows plus-end-directed motility. This could perhaps be explained if the protein undergo a structural alteration due to the association with other kinesin-5 proteins. The decreased ionic strength would then allow the protein to make this structural switch more easily, granting plus-end-directed motility more easily. These two hypotheses are visually represented in Figure 2. In reality, it is most likely a compromise of both hypotheses, with a conformational change in the motor domain and cargo binding to both motor domains being important.

A study towards the bidirectionality of Kip1, the other kinesin-5 protein in fission yeast, has shown that this protein has the same penchant for bidirectionality as Cin8, and that bidirectionality is retained if the L8 loops of the proteins are exchanged (Fridman et al., 2013), hinting at similar regulatory mechanisms.

The Cut7 kinesin-5 of fission yeast has most recently been shown to display bidirectionality. This study also attempted to determine whether the N-terminal microtubule binding extension which is found in the kinesin-5 proteins of lower eukaryotes, such as yeast, is involved in this switch, but no evidence for this was found (Edamatsu, 2014).

In summary, while the yeast kinesin-5 proteins show a very interesting minus-end-directed motility, it has to be stressed that the plus-end-directed motility remains the most important part of kinesin-5 movement. The minus-end-directed movement can give important information of structural peculiarities and specifics in regulation of kinesin-5. As regulation is often achieved via modifications on the protein itself, the structure has to be understood, to fully grasp regulation.

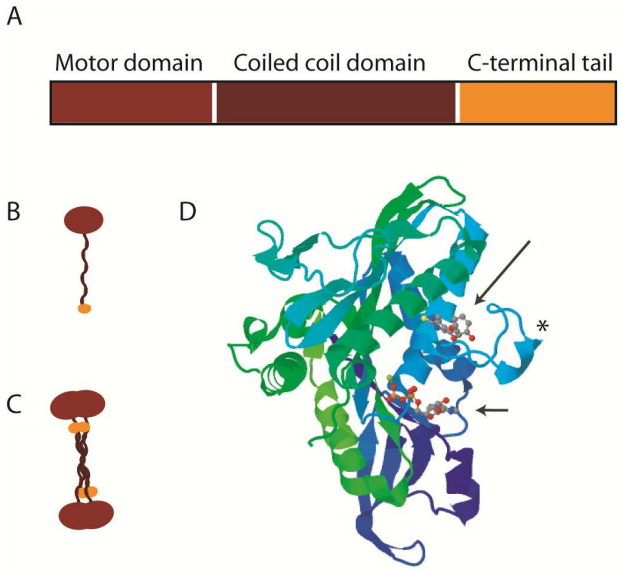

Figure 3: General structural composition of kinesin-5 proteins. (A) Domain structure of kinesin-5. Both the motor domain and the C-terminal tail have microtubule binding domains, while the coiled coil domain is necessary for trimerization. (**B and C) Schematic overview of a single kinesin-5 protein (B) and the homotetramer in which it organizes (C)**. The structure of the homotetramer illustrates the function of the protein, with microtubule binding sites on either side of the protein. This allows for the sliding of microtubules that is the hallmark of kinesin-5 activity**.(D) A ribbon structure of a single kinesin-5 motor domain (**PDB code 1Q0B (Yan et al., 2004)) **in complex with ADP (short arrow) and a small molecule inhibitor (long arrow).** The binding of ADP shows the ATP binding site, while the location of the inhibitor shows loop 5, which is an α-helix important for inhibition studies. (*) shows the insert in loop 5 to which small molecule inhibitors bind.

Kinesin-5 structure

The structure of kinesin-5 is quite striking, as it gives a good indication of the function that the kinesin exhibits. A single kinesin-5 molecule consists of 4 identical kinesin subunits, making the protein a homotetramer (Figure 3). The kinesin-5 subunit consists of a N-terminal motor domain, a coiled coil domain and a C-terminal tail that can also bind microtubules (Figure 3A). Upon assembly into the homo tetramer, the coiled coil domains intertwine, forming a stalk, leading to a molecule that has two motor domains opposite each other, a construction similar to a dumbbell (Figure 3C) (Acar et al., 2013; Scholey et al., 2014; Weinger et al., 2011). This means that kinesin-5 has microtubules as its cargo, with both motor domains having the other motor domain as cargo site. The structure obtained after tetramerization leads to a protein which has two motor domains with each two microtubule binding sites, plus two C-terminal domains which can also associate with microtubules (Figure 3C) (Acar et al., 2013; Wildenberg et al., 2008). Interestingly, it seems that the extra microtubule associating C-terminal domains are required for proper functioning of the motor domains. Presumably, the additional attachments to the microtubules allow the kinesin to exert its motor function, while loss of this tail leads to interactions that are too transient for proper force generation (Weinger et al., 2011). Experiments in *Drosophila* have shown that a construct with a deleted motor domain can still bind to microtubules via these C-terminal tails (Wildenberg et al., 2008).

The stalk, which consists of the 4 coiled coil domains, connects the two motor domains to each other (Scholey et al., 2014). It has to be sturdy enough to be able to transmit the forces that are generated when both motor domains bind cargo and start exerting force. Finally, it has to be flexible as well, to allow binding of both parallel and antiparallel microtubules. In order to achieve this, the motor domains have to be able to rotate in a 180° arc (Scholey et al., 2014; Wildenberg et al., 2008). The combination of these properties imply that the stalk has to have a specific structure that is well adjusted to these functions.

The structure of the motor domain has been resolved, but resolving the stalk section has proven to be more challenging. The minimal required residues of the coiled coil necessary to assemble into a tetramer have been determined (640-802), but crystallization of the entire stalk has not yet been achieved (Acar et al., 2013; Scholey et al., 2014). Studying the motor domain has shown the different states of the protein during the cycle

of ATP binding and hydrolysis (Figure 2D) (Goulet et al., 2014). It has also given important information about structural properties that are unique for this kinesin family compared to other kinesins. Kinesin-5 has a very characteristic loop close to the nucleotide binding pocket called the L5 loop (Figure 3D,*) (Larson et al., 2010).

Regulation of kinesin-5

There are several structural elements which can be conserved through evolution that play an important role in the regulation of kinesin-5 function. Examples of these are two loops found in the motor domain of kinesins, the aforementioned Loop 5 and also Loop 8. Loop 5 is a loop which is conserved in all members of the kinesin superfamily and is thought to be involved in binding to inhibitors such as S-trityl-L-cysteine (STLC) (Larson et al., 2010). Loop 8 is a loop found in kinesin proteins which has an insert of considerable and variable length in eukaryotes (Gerson-Gurwitz et al., 2011).

Loop 8 has been shown to be important in regulating the directionality of kinesin-5. There are two phosphorylation sites present in this loop for cdk1 which, when deleted, increase the time that the protein is attached to the spindle in budding yeast (Gerson-Gurwitz et al., 2011). This loop is also involved in the binding of microtubules in lower and higher eukaryotes (Chee and Haase, 2010; Goulet et al., 2014). In Cin8, the insert in the loop is significantly longer compared to the insert in other kinesins (Chee and Haase, 2010). A mutant in which the insert in loop 8 from Cin8 was changed to the insert of Kip1 showed that the Cin8 mutant was still viable (Gerson-Gurwitz et al., 2011).

Small molecule inhibitors of kinesin-5

The function of loop 5 has been studied more extensively. It is known that inhibitors such as STLC can bind to this loop, which prevents the motor from completing its ATP cycle by blocking ATPase activity and reducing the affinity of kinesin-5 for the microtubules (Goulet et al., 2014; Larson et al., 2010; Maliga et al., 2002). Kinesin-5 is the only member of the kinesin superfamily that can be inhibited using a small molecule inhibitor. This makes it possible to research functions of kinesin-5 with more temporal control. This has allowed studying the function of kinesin-5 in anaphase by blocking cells that are specifically in anaphase, where knockdown of kinesin-5 would have caused a monopolar spindle in prophase, never reaching a proper anaphase. Thus, looking at the target of these inhibitors can show how the inhibitor works, which can then be translated to other kinesin proteins. High resolution elucidation of the structure of ATP binding cycle has shown that loop 5 is close to the ATP binding site and that this loop folds away from the binding site during ATP hydrolysis (Goulet et al., 2014). Binding of STLC prevents this conformational change, capturing the protein into a state similar to the ADP release structure (Larson et al., 2010).

Another very interesting implication of the inhibitors for kinesin-5 is the use of these inhibitors for the treatment of patients. Kinesin-5 is primarily active in mitosis, meaning that inhibition of these proteins in cancer patients would have less side effects than for instance microtubule stabilizing agents, which are among the most successful classical chemotherapies used in the clinic. As such, several inhibitors of kinesin-5 have entered clinical trials for both solid and lymphoid tumours. In phase 1 and 2 trials, the primary factor in dose limiting toxicity was found to be neuropathy. This is in accordance with reports that kinesin-5 plays a role in mediating proper neuronal functioning through transport of neurotransmitter receptors to the cell surface (Ari et al., 2014). However, despite good results in the pre-

clinical studies and dose limiting toxicity results, indicating that the drugs were well tolerated, there have been no promising results concerning tumour regression in patients (Gerecitano et al., 2013; Jones et al., 2013; Khoury et al., 2012).

Recently a report has been compiled on another inhibitor of kinesin-5 that seems to have a different mechanism of inhibiting (Tarui et al., 2014). An assay to check for microtubule binding affinity after inhibition showed that this compound, terpendolin E, reduced the affinity of kinesin-5 for the microtubule to a lesser degree than STLC (Maliga et al., 2002; Tarui et al., 2014). Finally, cells that were resistant to STLC were still inhibited when treated with terpendolin E, implying a different binding site. (Tarui et al., 2014).This implies that this compound could potentially be used as a complementary treatment together with STLC in patients. Furthermore, this drug will allow for more studies into the motor activity of kinesin-5 proteins, because the binding affinity to microtubules is reduced less compared to kinesin-5 treated with STLC.

Alternative pathways

The results from the clinical trials imply that the inhibition of kinesin-5 can easily be overcome by tumours. The question that needs to be answered is whether the resistance occurs due to a mutation of kinesin-5 that abrogates the effect of the inhibitor, or whether there is an alternative pathway which is used to overcome the inhibition. There have been studies towards possible alternative pathways which have identified a few candidates that are also important for the assembly of a bipolar spindle. For instance, kinesin-12 motors seem to be important in generating a bipolar spindle. Inhibition of kinesin-12 motors strongly sensitizes cells to inhibitors of kinesin-5, but does not abrogate the establishment of a bipolar spindle (Tanenbaum and Medema, 2010). There is also some overlap in binding partners between both kinesin families, as both kinesin-5 and kinesin-12 proteins have been shown to interact with TPX2 (Ma et al., 2010, 2011). Finally, it seems there is also some connection in function, as depletion of both kinesin-5 and kinesin-12 can rescue some of the phenotype of a kinesin-5 only knockout (Vladimirou et al., 2013). Other protein families which seem to be important in bipolar spindle assembly are kinesin-4 and kinesin-10, which are both chromokinesins, meaning that they are found to be associated with mitotic chromosomes. Finally, it has been reported that the minus-end-directed motor dynein is also involved in the establishment of a bipolar spindle (Tanenbaum and Medema, 2010). It remains to be elucidated whether these alternative pathways are important in development of resistance or if this is mainly achieved by mutations in the drug binding loop.

Table 1: The different phosphosites of kinesin-5 currently described. Other post translational modifications have not yet been published, although there are a few mass spectrometry hits which seem to indicate acetlyation or ubitiquination, but these have not sufficiently been described to confirm their presence*. (*) Data recovered from phosphosite database, 25-6-2014, uniprot code P52732.

No.	Phosphosite	Kinase	Organism	Effect	Comments	Reference
1	Tyr23	Wee1	*Drosophila melanogaster*	Alters activity of kinesin-5		(Garcia et al., 2009)
2	Tyr152	Wee1	*Drosophila melanogaster*	Alters activity of kinesin-5		(Garcia et al., 2009)
3	Tyr207	Wee1	*Drosophila melanogaster*	Alters activity of kinesin-5		(Garcia et al., 2009)
4	Tyr399	Unknown	*Homo sapiens*	Unknown	Seen in various MS approaches in Jurkat cells	*
5	Ser543	Aurora A	*Xenopus laevis*	Unknown	Seems to have no discernable effect thus far	(Cahu et al., 2008)
6	Thr926	Cdk1	*Xenopus laevis* *Homo sapiens*	Localisation of kinesin-5		(Bertran et al., 2011; Cahu et al., 2008; Rapley et al., 2008)
7	Ser1033	NiMA	*Xenopus laevis* *Homo sapiens*	Localisation of kinesin-5	Very low efficiency of phosphorylation	(Bertran et al., 2011; Rapley et al., 2008)

Post translational modifications of kinesin-5

An important part of protein regulation is based on the post translational modifications that a protein can undergo. The best described post translational modification is phosphorylation, and kinesin-5 has a few phosphorylation sites which play an important role in regulating the protein. These are summarized in Table 1. For a visual respresentation, see Figure 4 (top). It has been found that cdk can phosphorylate the protein in both the motor domain and the C-terminal tail. It has been shown that the cdk phosphorylation site in the C-terminal tail (THR927) affects localization of the protein. This phosphosite is localized inside the BimC box, and phosphorylation leads to association with the mitotic spindle (Blangy et al., 1995). The mechanism by which this is achieved has yet to be elucidated, as the negatively charged phosphogroup should theoretically repulse the protein from the negatively charged microtubules. It seems likely that a conformational change some distance from the phosphosite is the cause of this increased association, in order to prevent this repulsion. An interesting difference between budding yeast and higher eukaryotes is found in the phosphorylation by cdk1, where mutation of phosphosites leads to impaired segregation of the chromosomes, but not to impaired localization of the protein on the mitotic spindle (Chee and Haase, 2010).

There are also reports of phosphorylation in the motor domain by Wee1 in *Drosophila*, a kinase that in itself is important in entry into mitosis, as it inhibits this process. The phosphorylation of kinesin-5 activates the protein, with mutation of the phosphosites leading to a defective spindle (Garcia et al., 2009). This leads to interesting connotations, with Wee1 itself being a kinase that inhibits progression into mitosis by an inhibiting phosphorylation of cdk1 (Alberts et al., 2008), which, as discussed earlier, is also necessary for proper kinesin-5 functioning.

It has been reported that Aurora A can phosphorylate kinesin-5 in *Xenopus,* but this did not alter the localization of the protein, as the cdk phosphorylations did (Cahu et al., 2008). The physiological relevance of this phosphorylation has yet to be established.

The kinase NEK, a NIMA (Never in Mitosis A) family member, has been shown to phosphorylate a small amount of kinesin-5 at ser1033, with a mutation study showing that this small amount of phosphorylation plays an

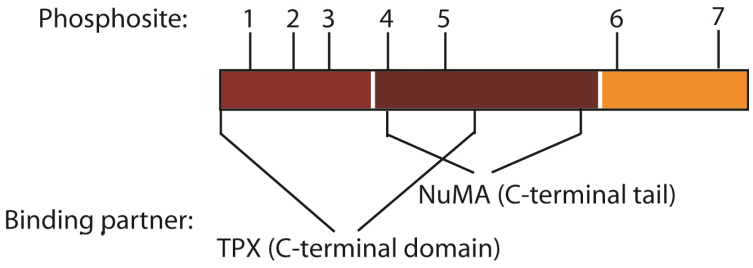

Figure 4: Schematic representation of sites necessary to associate with binding partners and phosphosites. The schematic representation of kinesin-5 is colour-coded as in figure 3A. The shown binding partners are those whose binding site has been elucidated, with the part of the protein they use to interact with kinesin-5 in brackets. The phosphosite numbers correspond with those given in table 1.

important role during mitosis. The kinesin-5 that is phosphorylated by this kinase localizes primarily near the spindle poles, suggesting a potential different role in mitosis for this fraction of the protein, compared to the fraction of the protein that is used to position the centrosomes in metaphase or the pushing away of the centrosomes in anaphase (Rapley et al., 2008).

Thus far, no other post translational modifications have been described in detail. Some data have been generated using a bulk approach that identifies some acetylation and some ubiquitination sites. However, these sites have not yet been verified using additional experiments and as such, it remains elusive whether these sites actually are modified.

Kinesin-5 binding partners

Finally, another important determinant for the function and regulation of proteins is found in their interactions with other proteins. Thus, in order to elucidate the effect of kinesin-5 on mitosis, extensive studies have been performed to search for binding partners. A very important binding partner of kinesin-5 is TPX2, a Ran regulated spindle assembly protein. TPX2 has multiple important roles in spindle assembly, such as recruitment of Aurora A kinase and localization of the kinesin-12 protein family member Xklp2 (*Xenopus kinesin like protein 2).* Furthermore, TPX2 also has the ability to nucleate microtubules. Due to the plethora of different functions performed by this protein, it is quite difficult to determine the separate functions of TPX2, as knockdown studies lead to impaired chromosome segregation, which is incompatible with life. A recent study has suggested that TPX2 binding to both dynein and kinesin-5 is essential for coupling these two proteins so that kinesin-5 can be transported poleward. A knockdown of TPX2 leads to plus-end-directed motion of kinesin-5 on astral microtubules, while in control cells this movement is primarily minus end directed. This movement is caused by the activity of dynein, a minus-end-directed motor. The coupling of kinesin-5 and TPX2 to dynein allows both proteins to move to the minus-end of the microtubule, which is necessary for the localization (Gable et al., 2012). Furthermore, mutation analysis with a TPX2 protein lacking the kinesin-5 binding domain has shown that kinesin-5 needs TPX2 to be located on the spindle microtubules, but not to be located near the spindle poles. An interesting experiment performed on the interaction between kinesin-5 and TPX2 has demonstrated that the addition of TPX2 in an *in vitro* sliding assay with kinesin-5 leads to abrogation of sliding, indicating an effect of TPX2 on the activity of kinesin-5 (Ma et al., 2010, 2011). This is further supported by phenotypes observed with the overexpression of the part of the protein that binds to kinesin-5, a large part of the TPX2 C-terminus (Figure 4, bottom). This overexpression shows cells which arrest in mitosis and cells with collapsed spindles. These phenotypes can be rescued with an excess of kinesin-5 (Eckerdt et al., 2008). These provide powerful indications that TPX2 can in fact regulate the activity of kinesin-5 via a hitherto unknown mechanism.

Another binding partner for kinesin-5 appears to be NuMA (Nuclear Mitotic Apparatus

protein), a protein that is necessary in spindle assembly and chromosome segregation. NuMA is usually only found in the nucleus, while kinesin-5 is almost exclusively found in the cytoplasm. Thus, the only point at which these proteins can interact is after nuclear envelope breakdown. Several biochemical analyses have been performed on this interaction. It was shown that the interaction was still possible in mitotic cells that were treated with the pan-kinase inhibitor staurosporin, indicating that there is no phosphorylation required for the interaction between the proteins. The proteins bind between the stalk of kinesin-5 and the C-terminal tail of NuMA (Figure 4, bottom). The interaction between these proteins is primarily important for the localization of NuMA towards the spindle poles, as depletion of kinesin-5 leads to severely reduced levels of NuMA at the spindle poles. Finally, an experiment in which the level of kinesin-5 was reduced, but not depleted, showed a phenotype similar to that of NuMA knockdown in mitotic cells. It was necessary to maintain a baseline level of kinesin-5 for the formation of a bipolar spindle, as NuMA activity is only present after a bipolar spindle has been formed (Iwakiri et al., 2013).

A report from 2012 has shown that there is an interaction between kinesin-5 and XPF. XPF is an endonuclease which plays a role in nucleotide excision DNA damage repair. Loss of the function of this protein is the cause of *xeroderma pigmentosum*, a syndrome which can either lead to a predisposition to skin cancer or to a more severe phenotype with neurological disorders and accelerated aging. A knockdown of this protein *in vitro* leads to an increase in abnormal mitosis and there was overlap found in expression levels of these proteins (Tan et al., 2012). The specifics of this interaction remain to be elucidated; it is not known whether the binding is necessary for the function of either protein, as the proteins have very distinct roles. As such, this interaction deserves closer scrutiny.

Discussion

There is a lot of exciting research still possible in the field of kinesin-5, which can help elucidate its function and regulation. In this part, a few questions that were raised shall be examined and discussed, in a similar order as to how they were posed in the review.

Microtubule dynamics modified by kinesin-5

As described earlier, it has been shown in budding yeast that the kinesin-5 proteins can have an effect on the stability of microtubules, with Cin8 destabilizing microtubules and Kip1 stabilizing microtubules (Collins et al., 2014; Fridman et al., 2013; Gardner et al., 2008). It is interesting that similar functions for kinesin-5 have not yet been found in other organisms, even though the proteins are well conserved through evolution. The main reason that this function is not found in higher eukaryotes is probably due to the differences between yeast and mammalian cell division. The property itself remains interesting, especially since both proteins have opposing effects on the microtubules. It would be interesting to do an evolutionary screen to see whether the microtubule affecting properties appear after the speciation, or if it can be traced backwards. Furthermore, this could also elucidate if there are specific mutations that cause the microtubule modulating effects. This would be a challenge due to the numerous differences in sequence that no doubt exist, but it would give increased insight in the kinesin-5 function throughout evolution.

Bidirectionality of kinesin-5 in yeast

An evolutionary screen could also be interesting to study the bidirectionality that was observed in the yeast kinesin-5 proteins.

This property came as a complete surprise to the field; the fact that a kinesin with an N-terminal motor domain was able to move towards a minus end had not yet been reported (Edamatsu, 2014; Gerson-Gurwitz et al., 2011; Roostalu et al., 2011). But what are the implications of this finding for kinesin-5 proteins in higher eukaryotes? It seems that part of the reason that the kinesin proteins would need this minus-end-directed movement would be for proper localisation. In *Xenopus,* the minus-end-directed motor dynein seems to be responsible for the minus-end-directed motility, via interactions with kinesin-5 and dynactin, which would render any minus-end-directed movement of the kinesin-5 protein itself unnecessary (Kapoor and Mitchison, 2001; Uteng et al., 2008). Conversely, in yeast, dynein has no nuclear function, which makes the switching to minus-end-directed movement an attractive candidate for proper localisation (Gerson-Gurwitz et al., 2011; Roostalu et al., 2011; Thiede et al., 2012). It would be interesting to elucidate the manner by which this switch is achieved in a structural manner, as this would provide a wealth of information on how kinesin motor proteins function. It could also be used with mutation analysis to discern what processes specifically need the minus-end-directed movement.

Tip tracking and minus-end-directed motion of kinesin-5 in yeast

Another hypothesis to explain the minus-end-directed movement of the yeast kinesin-5 proteins has been that this movement allows the proteins to track the tips of polymerising and depolymerising microtubules. Experiments in budding yeast have shown that Kip1 has the ability to track the tips of the microtubules (Fridman et al., 2013). In order to track the depolymerising microtubules, minus end activity would be very beneficial, if not necessary, for the protein to accurately follow the microtubule end. As such, it might be expected that the higher eukaryotes could use the minus-end-directed movement to track the tips of the plus end. However, currently only Kip1 has been shown to demonstrate this tip tracking activity (Fridman et al., 2013). This leads to the question what this tip tracking activity is for and whether Kip1 is the only member of the kinesin-5 family that demonstrates tip tracking activity. A possible hypothesis involves the 2-micron plasmid which is found in budding yeast. This plasmid is a DNA element in yeast that seems to have no functional advantage in evolution, but is propagated nonetheless (Cui et al., 2009). Kip1 interacts with this plasmid, and a knockdown of Kip1 abrogates segregation. It is hypothesized that the tip tracking ability of Kip1 is necessary for the proper segregation of this plasmid (Fridman et al., 2013). Since this is only relevant for yeast and not for mammalian cells, this also seems to suggest that the minus-end-directed movement is specific for yeast and unlikely to be found in higher eukaryotes.

Tetramerization of kinesin-5

The lack of a full structure of kinesin-5 remains an important caveat in our understanding of the protein. Solving the entire structure would allow more in depth comparison of the protein in different organisms, and as such, might give structural clues as to why the yeast kinesin-5 can demonstrate minus-end-directed motility.

However, in order to solve a full structure, it would be beneficial to learn more about the coiled coil domain. Until recently, there were barely any structural studies published concerning the coiled coil domain. Partly, this is due to the motor domain (Figure 3D) being considered the more interesting part, and partly due to difficulties in obtaining a structure of the coiled coil domains. Recent advances in techniques combined with

research that showed the intricacy of the tetramerisation have been strong counterpoints to the belief that the coiled coil is a less interesting part of the protein. This year some fundamental experiments on the bare minimum of residues required to tetramerize were performed, which showed a very intricate mechanism of tetramerisation, with the N-terminal domains assembling into a parallel coiled coil while minimal domain associating in an anti-parallel coiled coil. Mutations in the coiled coil also showed residues important for the tetramerisation (Scholey et al., 2014). However, this only shows the bare minimal domain assembling, without the C-terminal tail that is known to assist in microtubule binding. It would be interesting to study the association of the entire coiled coil domain which, until recently, proved impossible. With the recent determination of the stalk structure necessary for tetramerisation (Acar et al., 2013; Scholey et al., 2014), it should soon be possible to determine the entire coiled coil domain of the kinesin-5 holoenzyme. This knowledge can then be applied to develop small molecule inhibitors that can disrupt tetramerisation, thus inhibiting kinesin-5 activity. This would be especially useful if the current mechanism of drug resistance would be due to mutations in the drug binding pocket, as most current small molecule inhibitors affect the same part of the motor domain. This is also an interesting subject, as this might interject with the relevance of kinesin-5 inhibition in the clinic, so this warrants a closer look.

Clinical usefulness of kinesin-5 inhibitors

The kinesin-5 inhibitors have yet to demonstrate clinical relevance: preclinical studies show effectiveness, but the clinical trials do not show the desired cytostatic effects. While this is often the case with the development of drugs, it does give rise to speculation as to why this treatment does not work. The fact that there is toxicity at a certain level of the drugs (Gerecitano et al., 2013; Jones et al., 2013; Khoury et al., 2012) seems to indicate that the compounds are not overly metabolized. The most simple answer would be the activation of an alternative pathway due to evolutionary pressure, or mutation of the drug-interaction site. But why was this not spotted in preclinical studies? Presumably this could be due to insufficient penetration into the tumour, leading to a suboptimal dose of the compound. There are reports of mutations in tumour cell lines that confer resistance to the compound (Tcherniuk et al., 2010). It would be interesting to try and replicate this *in vitro,* and then use RNA sequencing to find upregulated pathways that are prevalent in these cells, to examine whether the patients develop a mutation in the drug binding loop or whether an alternative pathway is upregulated. Another possible method of inhibiting kinesin-5 would be by using a combination treatment of two inhibitors that bind at different locations. Recent research has shown that Terpendole E can inhibit STLC-resistant kinesin-5 (Tarui et al., 2014). A combined treatment of these two compounds might increase the pressure on kinesin-5 to such a level that mutation to escape the treatment is not possible, if the escape mechanism is by mutation of the drug binding pocket. Another possible solution in fighting resistance might be an agent which can prevent the protein from arranging into a tetramer, as has been discussed above.

Another intriguing avenue of thought that is currently being pursued is whether the inhibition of mitosis alone can be enough to be harmful to tumours in patients. This is due to some key differences between cell lines and patients, most notably the frequency of mitosis. *In vitro,* cells divide on average every 24 hours, while tumour cells in patients may

have a dividing frequency which is far less than this. If the cells divide sporadically, the question has to be posed whether simply targeting mitosis is enough to establish tumour control in patients. It implies that it is necessary to use either combination therapy with agents that target other aspects of the tumours, or forego mitotic targeting altogether. Due to the relative specificity of drugs that target mitosis, it remains attractive to use these drugs, but it would be necessary to use these drugs in combination treatment for them to be of any use. As such, it would be wise to look at the effect of kinesin-5 inhibitors on highly proliferative tumours, which should be more sensitive to the inhibitor according to this theory. Regardless, it would seem that there is much knowledge to be gained in kinesin-5 inhibition and tumour response in general.

While this part only describes the non-physiological inhibition of kinesin-5 proteins, it should be noted that an important part of regulation is inhibition, for instance due to phosphorylation. It is difficult to use these mechanisms as therapy when they are not fully understood, so understanding regulation is essential for the development of new therapies. In the next paragraphs, some interesting, seemingly contradicting results, will be discussed to illustrate the possible extent of this regulation. In order to grasp this paradox however, a short retread of mitosis is beneficial, so some information discussed earlier will be briefly discussed again.

Phosphorylation by Wee1 in tandem with cdk1 phosphorylation

In order to enter mitosis, cdk1 has to be activated and start signalling by associating with cyclin B. In order to prevent sudden entry into mitosis, this activation is tightly regulated by an inactivating phosphate group. The kinase responsible for this inactivating phosphate is Wee1, and when the cell is ready to enter mitosis, cdk1 becomes active and deactivates Wee1, thus releasing the break imposed by the deactivating phosphorylation (Alberts et al., 2008). Interestingly, both Wee1 and cdk1 have phosphosites on kinesin-5. Cdk1 can phosphorylate the stalk of kinesin-5, which helps the localisation of kinesin-5 to the mitotic spindle (Blangy et al., 1995; Chee and Haase, 2010). Wee1 has been shown to phosphorylate the motor domain of the *Drosophila* kinesin-5 protein, which is called Klp61F. These phosphorylation sites seem to be important for the functioning of Klp61F, as loss of the phosphosites leads to an inability to rescue cells with a low expression of native Klp61F (Garcia et al., 2009).

This means that both proteins are necessary for a proper functioning kinesin-5 protein, and as such, for a proper mitosis. However, for the mitosis to be initiated, one of these kinases, Wee1, has to be deactivated. So how is this apparent contradiction overcome? One of the possibilities is that *Drosophila* Wee1 has no function in the entry into mitosis, and as such is not necessarily downregulated before mitosis can occur. This is supported by the fact that Wee1 mutants can in fact survive into adulthood (Garcia et al., 2009), and there has been a report that the Myt1 kinase can in fact perform the cdk1 inhibitory role in *Drosophila* wing development (Jin et al., 2008). This would mean that there has been a strong evolutionary divergence in Wee1 function in *Drosophila,* while it is very well conserved in other organisms.

Another possibility is that the phosphorylation of the motor domain is very stable, with the phosphorylation persisting long enough after the disappearance of Wee1 for Klp61f to perform its function. This raises the question as to why this form of regulation would be necessary, but it might include a phosphatase that becomes active after separation of the

chromosomes, which would limit Klp61F function to mitosis.

Finally, the most simple explanation for this apparent enigma is that *in vivo* another kinase is responsible for the phosphorylation. This would mean that the preferred phosphorylation site of this kinase is similar enough to that of Wee1 to have a crossreaction *in vitro.* This would ensure that there are no problems with the deactivation of Wee1 with regards to kinesin-5.

Regardless of which hypothesis is correct, it should be investigated whether this phosphorylation persists throughout evolution or if this phosphorylation is only found in *Drosophila*. If the latter, it seems logical that the evolution of Klp61F has diverged from the other kinesin-5 proteins. If the former, it means that some exciting research can be performed in this field. It would be interesting to investigate the questions posed above. For instance, determining the phosphorylation state of Klp61F throughout the cell cycle and mitosis could show whether the phosphorylation is an on-off switch which is only used during mitosis.

Binding partners of kinesin-5

Finally, despite the importance of kinesin-5, only a handful of binding partners are known, such as those described in the review. Kinesin-5 has also been shown to interact with dynein in order to be transported towards the centrosome (Uteng et al., 2008). However, most of these interactions have been found using the protein that kinesin-5 interacts with as a basis. No reports of interaction studies with kinesin-5 as a basis have been described to the knowledge of the author. This is partly due to the conformational changes that kinesin proteins undergo when they bind microtubules. This conformational change is distinct enough that most kinesin binding proteins only bind when the kinesin is bound to a microtubule. This complicates studies to find binding partners, as it is necessary to capture kinesin-5 bound to the microtubule.

Finding proteins that transiently associate with kinesin-5 would also lead to interesting research questions, as this could help with finding possible regulatory proteins, such as kinases and phosphatases. In order for these regulatory proteins to perform their function, they need an association. Thus, it would seem that a study of protein-protein interactions, for instance via a bioinformatics approach, could give answers to several questions, for instance whether there is a phosphatase that removes phosphates as the cell leaves mitosis.

Elucidating more binding partners of kinesin-5 will help find more information about the regulation of kinesin-5 during mitosis and tell more about how the protein localizes. It may also lead to more therapeutic targets which may be even more specific than inhibition of kinesin-5 is in treating tumours.

Concluding remarks

The microtubule sliding properties of kinesin-5 proteins are essential for a proper mitosis, while there only a few non-mitotic functions of the protein that are specific for neurons. As such, kinesin-5 makes an excellent drug target for cancer treatment. While quite a bit is already known about the function of kinesin-5, there are still some caveats in our knowledge about the regulatory aspects and the assembly of four kinesin-5 subunits into a homotetramer. Furthermore, there seems to be a drug resistance aspect which remains to be fully understood, and there are alternative pathways (including kinesin-12 and chromokinesins) present which can take over some of the roles of kinesin-5. As such, it would be interesting to expand the knowledge we have already gained on kinesin-5 and its roles, both for increasing the knowledge about mitosis, and for the potential cancer

treatment that underlies kinesin-5's specificity for mitosis.

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