The role of molecular motor Hklp2 in spindle assembly

David Vanneste

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Isabelle Vernos – Cell and Developmental Biology (CRG)

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ABSTRACT

During cell division, a macromolecular machine is assembled to segregate the two sets of chromosomes carrying the genetic material into the two daughter cells. This machine, the mitotic spindle, is a bipolar array composed of microtubules and proteins that control their dynamics and organization. Kinesins are molecular motors that can apply forces to the microtubules and arrange them in space to assemble a bipolar spindle.

Here we characterized the human kinesin Hklp2 as an important player in bipolar spindle assembly and maintenance.

Hklp2 is a kinesin that localizes to the spindle microtubules and the chromosomes in mitosis. Its localization to the microtubules depends on TPX2, a crucial factor in microtubule nucleation. Hklp2 localization to the chromosomes necessitates the presence of cell proliferation marker Ki67 whose function is currently unknown. Hklp2 helps the separation of the centrosomes during the establishment of spindle bipolarity and plays a role in the subsequent maintenance of bipolarity during metaphase. This role is probably achieved through a cross-linking of the microtubules. Interestingly, the populations of the kinesin present on the microtubules and the chromosomes have different functions.

RESUMEN

Durante la división celular, tiene lugar el ensamblaje de la maquinaria macromolecular encargada de segregar los dos conjuntos de cromosomas en las dos células hijas. Esta maquinaria, el huso mitótico, es una matriz bipolar compuesta de microtúbulos y de proteínas que controlan la dinámica y la organización. Las quinesinas son motores moleculares que pueden ejercer fuerzas sobre los microtúbulos y organizarlos en el espacio para dar lugar al huso bipolar.

Este estudio aporta evidencias del papel importante de la quinesina humana Hklp2 en el ensamblaje y en la estabilidad del huso mitótico.

Hklp2 es una quinesina que se localiza en los microtúbulos del huso y en los cromosomas durante la mitosis. Su localización en los microtúbulos depende de TPX2, un factor importante en la nucleación de microtúbulos. Para que Hklp2 se localice en los cromosomas, este necesita la presencia del marcador de proliferación Ki67, la función del cual se desconoce actualmente. Hklp2 participa en la separación de los centrosomas durante el establecimiento de la bipolaridad del huso mitótico y posteriormente juega un papel importante en el mantenimiento de esta bipolaridad durante la metafase. Esta función probablemente se consiga haciendo lazos entre los microtúbulos. Las poblaciones de la quinesina presente en los microtúbulos y en los cromosomas tienen funciones diferentes.

KEYWORDS

HeLa cells

Cell cycle

Cell division

Mitotic spindle

Microtubule

Centrosome

Metaphase

Motor

Kinesin

TPX2

Hklp2

Ki67

Eg5

PALABRAS CLAVES

Células HeLa

Ciclo celular

División celular

Huso mitótico

Microtúbulo

Centrosoma

Metafase

Motor

Quinesina

TPX2

Hklp2

Ki67

Eg5

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INTRODUCTION

Mitosis

Mitosis is the culmination of the process that generates two cells out of one and by doing so ensures the perpetuation and extension of life. During mitosis, the previously duplicated genome is compacted in discrete units, the chromosomes, made up of two identical pieces, the sister chromatids, that will be segregated equally to the two daughter cells. The process is carried on by a macromolecular machine that captures, aligns and segregates the chromosomes thus ensuring the proper distribution of the genetic material between the two daughter cells.

This machine, the mitotic spindle, is a bipolar array assembled from the microtubule cytoskeleton thanks to the concerted action of hundreds of proteins that control the dynamics of the microtubules (MTs) and their arrangement in space and time (Gadde and Heald, 2004; Karsenti and Vernos, 2001).

Mitosis is generally divided into five phases that correspond to different configurations of spindle and chromosomes and also different molecular cell cycle signals.

During prophase, the chromatin experiences a higher order of folding, condensing in discrete units called chromosomes. Simultaneously, outside of the nucleus, the two centrosomes separate from one another to opposite sides to define the future poles of the spindle. The break-down of the nuclear envelope marks the beginning of prometaphase, during which the MTs emanating from the centrosomes, the chromosomes and more MTs assembled close to the chromosomes will gain access to each other. These interactions lead progressively to the formation of a bipolar spindle with chromosomes aligned at

the equator. The firm attachment of the chromosomes to MTs focused at the centrosomes occurs at a specialized proteinaceous structure assembled on the centromeres called kinetochore. During prometaphase, each chromosome makes a connection at each of its two kinetochores with MTs bundles emanating from opposite spindle poles and become bioriented. Metaphase is the moment when all chromosomes are bioriented and aligned at equal distance from the two spindle poles to form what is named the "metaphase plate". The cell then makes a transition into anaphase during which the cohesion between the sister chromatids of each chromosome is lost. Chromatids are then free to separate and are pulled towards the two opposite poles of the spindle by the MTs. At the end of the process, during telophase, the two sets of chromosomes decondense and a nuclear envelope forms, surrounding them, to build up the nuclei of the daughter cells. Cytokinesis designates the constriction of the cytoplasm, due to a contractile actin ring that follows an axis perpendicular to the spindle, until complete partitioning and the individualization of the daughter cells.

The entry into and exit from mitosis are controlled by the cell cycle machinery. This machinery controls the dramatic changes in the cell and is remarkably conserved in all eukaryotes. Cyclins are synthesized and degraded during certain phases of the cell cycle and the cyclins in turn associate with and activate cyclin-dependent kinases that activate substrates through phosphorylation cascades. The master controller of mitosis is the association cyclinB/cdk1 (Gautier et al., 1990; Murray et al., 1989). The activation of cyclinB/cdk1 induces mitosis and once all the chromosomes are aligned, a large ubiquitin-ligase complex (anaphase promoting complex, APC) targets cyclin B for degradation allowing the cell to go to anaphase and mitosis exit (Clute and Pines, 1999; Morgan, 1999).

Microtubules

The MTs form a network inside the cell providing mechanical support but also intracellular transport and positioning of organelles. They reorganize into a bipolar array in mitosis to segregate the chromosomes between the two nascent daughter cells.

Each microtubule is a hollow cylinder composed of 13 protofilaments generated by the non-covalent polymerization of α and β-tubulin heterodimers. These heterodimers are associated in the same direction, conferring a polarity to the MT with different tubulin subunits exposed at each end of the tube. The α-tubulin end is called minus-end and is slower in polymerizing while the β-tubulin end, the plus-end, is characterized by a faster polymerization rate. This polarization is important for the movement of molecular motors that "walk" along the MT lattice in directional fashion, towards the plus or the minus-end, to position organelles, transport protein complexes or organize MTs (Desai and Mitchison, 1997). MTs have the ability to stochastically switch from a polymerization-state to a depolymerization-state (called catastrophe) and vice-versa (called rescue). Thus, the steady-state length of a MT population is defined by the rate of polymerization (or "growth"), the rate of depolymerization (or "shrinkage"), the frequency of rescue and catastrophe (Howard and Hyman, 2007; Mitchison and Kirschner, 1984a; Verde et al., 1992). For MTs to keep a constant length, there preferentially is a constant addition of tubulin dimers at one end and depolymerization at the other end. This phenomenon generates a movement of tubulin called flux or treadmilling (Margolis and Wilson, 1978). In cells, the nucleation of MTs and their dynamics are controlled by different microtubule-associated proteins (MAPs) and MT organizing center (MTOC) which is the centrosome in higher eukaryotes.

Centrosomes

The centrosome is a cellular organelle with a specialized and conserved function in MT nucleation and organization in most animal cells. During interphase, the polarized radial network of MTs extends away from the centrosome at the center, with the MT minus-ends focused at the centrosome and the plus-end reaching the rest of the cytoplasm. Consequently, the centrosome participates in all processes involving MTs, including cell shape and motility as well as intracellular transport. During S-phase, the centrosome duplicates. The two resulting centrosomes play an essential role in mitosis by defining the poles of the spindle and therefore its axis and orientation (Bettencourt-Dias and Glover, 2007; Nigg, 2002). A centrosome is composed of a pair of centrioles shrouded in amorphous pericentriolar material (PCM). Each centriole is composed of MTs arranged as nine sets of triplets to form a hollow cylinder. One of the two centrioles is dubbed the mother centriole as it served as template to generate the other one, the daughter centriole. The PCM is an electron dense material composed of core proteins and a number of non-permanent components including numerous structural and regulatory proteins. One of the core components is γ-tubulin that forms, together with other proteins, a large ring-shaped complex that is responsible for the nucleation of MTs at the centrosomes and also responsible for the polarity of the MTs array.

Spindle assembly

At the time the cell enters mitosis, the microtubule cytoskeleton undergoes a massive reorganization to generate the mitotic spindle. The reorganization is done on two levels: (1) MT dynamics, as they go from relatively stable to highly dynamic, and (2) MT geometry, as they change from broadly spread in the cell to precisely structured.

The MTs are organized into a bipolar array with the minus-ends focused and anchored at the spindle poles that contain a centrosome and the plus-ends contacting the chromosomes and interacting with the MTs emanating from the other pole in an antiparallel manner (Figure 1). Three different classes of MTs can be defined in the spindle: interpolar MTs, kinetochore MTs and astral MTs. Kinetochore MTs (or "K-fibers") are bundles of 10 to 40 MTs that connect each kinetochore to a pole. The interpolar MTs are all MTs located between the two spindle poles, they can emanate directly from the poles or not. The astral MTs extend away from the poles into the cytoplasm and establish connections with the cell cortex (Compton, 2000; Wittmann et al., 2001).

Two complementary mechanisms are responsible for the MT assembly and the formation of the spindle (Figure 2). The first, called "search and capture", postulates that the highly dynamic mitotic MTs emanating from the centrosomes are constantly growing and shrinking, randomly "searching" for chromosomes until they are stabilized and "captured" by an interaction with the kinetochores (Kirschner and Mitchison, 1986; Mitchison and Kirschner, 1984a; Mitchison and Kirschner, 1984b).

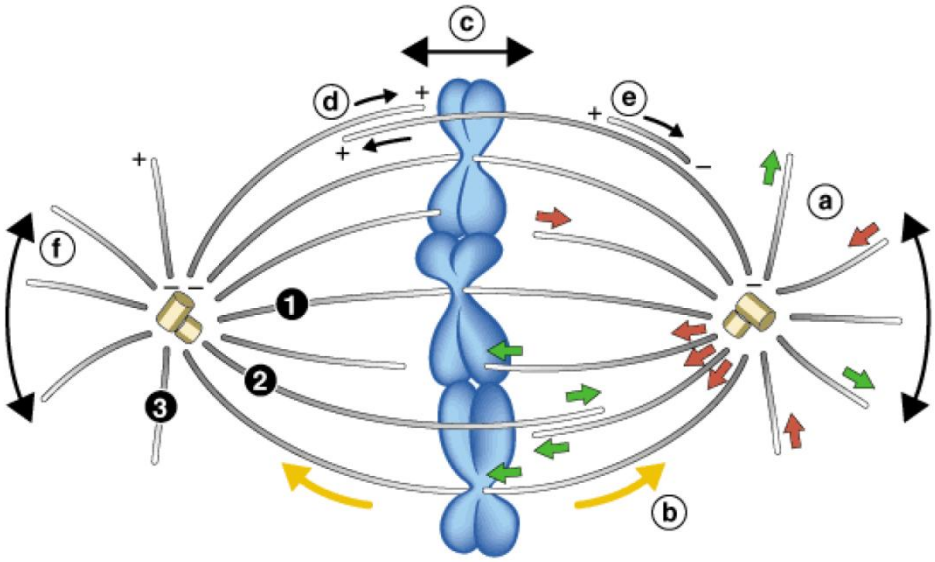


Figure 1: Model for the spindle structure and dynamics

The spindle is composed of three populations of MTs: (**1**) kinetochore-MTs, (**2**) interpolar MTs, (**3**) astral MTs. Different dynamic processes take place in the spindle: instability (**a**, green arrows for growing and red for shrinking), poleward flux (**b**, yellow arrows), chromosome movements (**c**), motor-driven antiparallel sliding of MTs (**d**), dynein-dependent transport of MTs (**e**), orientation of spindle poles (**f**). Adapted from Wittmann et al 2001 (Wittmann et al., 2001).

In time, the search and capture process results in the bioriented attachment of all kinetochores and the completion of spindle assembly. The second mechanism, called "self-organization", was hypothesized to explain the assembly of the spindle in systems where centrosomes are absent (e.g. plants, meiosis of some vertebrates) and confirmed by experiments showing the assembly of spindles around DNA-coated beads incubated in Xenopus egg extract (Heald et al., 1996) or in cells with inactivated centrosomes (Khodjakov et al., 2000).

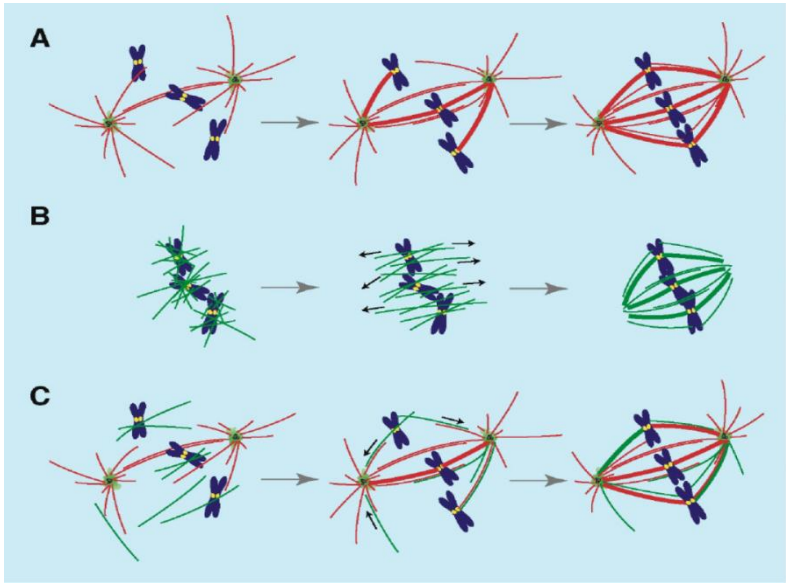


Figure 2: Different models of spindle assembly
A- "Search- and-Capture": MTs nucleate from centrosomes and contact chromosomes and kinetochores by chance to become stabilized and with the action of motors form the spindle.
B- "Self-Organization": randomly oriented MTs nucleate around the chromosomes and are organized into a bipolar array by motors.
C- Combined model: MTs emanating from the centrosomes and chromosomes all combine to form a spindle. MTs nucleated by the centrosomes are in red, the ones nucleated by the chromosomes in green. Adapted from Gadde and Heald 2004 (Gadde and Heald, 2004).

These experiments demonstrated that the chromatin has the property to favor the nucleation of MTs that "self-organize" into a bipolar spindle. It has also been described that some MTs can emanate directly from the kinetochores (Tulu et al., 2006). Currently, the accepted view is that a combination and merging of the two mechanisms allows the assembly of the spindle, with a different weight depending on the cell type and/or organism.

Microtubule assembly close to the chromatin

The capacity of the chromatin to promote MT assembly depends on the small GTPase Ran and the release of spindle assembly factors (SAF) that are inhibited due to their association with importins. Ran interacts with distinct sets of proteins depending on its nucleotide-state (GTP or GDP-bound). In interphase, the presence in the cytoplasm of a factor, RanGAP1, that stimulates the Ran GTPase activity and the presence in the nucleus of a Ran GTP exchange factor, RCC1, generates a clear distinction between the two compartments: Ran is mostly GTP-bound in the nucleus, and GDP-bound in the cytoplasm. This distinction determines the direction of the nucleo-cytoplasmic transport as proteins containing a nuclear localization signal (NLS) bind to importins in the cytoplasm, are imported in the nucleus and released from importins by Ran-GTP (Gorlich and Kutay, 1999; Kalab and Heald, 2008; Mattaj and Englmeier, 1998). In mitosis, the break-down of the nuclear membrane results in a mixing of the content of the nucleus and the cytoplasm. As the nucleotide exchange factor RCC1 is associated with chromatin, it generates a gradient of Ran-GTP spreading away from the chromatin. In this situation, proteins containing an NLS are still released by Ran-GTP close to the chromatin. Ran-GTP regulated SAFs identified so far include MT associated proteins (MAPs) and motors that induce the nucleation and elongation of MTs as well as their self-organization into a spindle (Ciciarello et al., 2007; Kalab and Heald, 2008).

TPX2 and its interactors

The targeting protein for motor Xklp2 (TPX2) was one of the first SAFs identified and is a key contributor to the Ran pathway (Gruss et al., 2001). TPX2 is nuclear in interphase and in mitosis, after nuclear breaks down, it triggers MT nucleation in the vicinity of the chromosomes.

TPX2 displays the behavior of a microtubule-associated protein (MAP) in every biochemical assays and colocalizes with spindle MTs in vivo and in vitro (Garrett et al., 2002; Gruss et al., 2002; Trieselmann et al., 2003; Wittmann et al., 2000). However, TPX2 does not stabilize MTs like other MAPs but probably plays a yet poorly understood role in the early steps of microtubule nucleation (Gruss et al., 2002). Recombinant full-length TPX2 has the ability to initiate MT polymerization in a pure tubulin solution, an activity regulated by importins (Schatz et al., 2003). Together, these data indicate strongly that an essential early role of TPX2 is to promote MT assembly in the vicinity of chromosomes. Accordingly, TPX2 has been found to be crucial for spindle formation in Xenopus egg extract and in tissue culture cells (Garrett et al., 2002; Gruss et al., 2001; Gruss et al., 2002; Wittmann et al., 2000).

In metaphase spindles, TPX2 accumulates at the poles in a dynein–dynactin-dependent way but a direct interaction between the two has not been described yet. The loss of spindle pole integrity when TPX2 is absent indicates that its pole localization has functional importance and suggests a possible MT bundling activity of TPX2 (Garrett et al., 2002; Wittmann et al., 2000). Also, the function of TPX2 at spindle poles could be related to its targeting activities. Three proteins have been shown to be targeted to the MTs by TPX2: the kinesin Xklp2 (Wittmann et al., 1998), RHAMM (Maxwell et al., 2005) and the kinase Aurora A (Kufer et al., 2002). RHAMM seems to be relevant for spindle pole structure and has a C-terminal structure very similar to Xklp2 (Maxwell et al., 2005; Maxwell et al., 2003). When released by Ran-GTP, TPX2 can interact and activate Aurora A that, in turn, phosphorylates TPX2 (Eyers et al., 2003; Kufer et al., 2002; Tsai et al., 2003). Structural studies showed that a short N-terminal sequence of TPX2 locks the kinase in an active conformation (Bayliss et al., 2003). Aurora A is a kinase that participates in cell cycle progression and spindle assembly through the phosphorylation of different substrates like

TACC3/Maskin that plays a crucial role in the stabilization of spindle MTs. During centrosome maturation in late G2 and prophase, some additional components are recruited to the PCM like TACC3/Maskin which localization and function is regulated by Aurora A (Peset et al., 2005). Thus, Ran-GTP signaling network near the chromatin could be translated into a phosphorylation network through the activation of Aurora A by TPX2.

Motor proteins in spindle assembly

Two classes of MT-based motors are implicated in the spatial arrangement of MTs during spindle assembly: kinesins and dynein.

The founding member of the kinesin protein superfamily, conventional kinesin-1, was first purified from giant squid axons in the 1980's (Vale et al., 1985). Abundant bidirectional particle transport had been observed in extruded axoplasm that stopped within minutes upon the addition of the nonhydrolyzable ATP-analog, AMP-PNP (Allen et al., 1982; Lasek and Brady, 1985). The discovery that MT motor proteins bind to MTs in a rigor state in the presence of the nonhydrolyzable AMP-PNP allowed the purification of motor proteins using taxol-stabilized MTs. Numerous kinesins have been identified since then, all sharing a highly conserved motor domain of about 350 amino acids that contains both ATP and a MT binding site. One approach for the identification of novel kinesins was PCR-based screens using primers in the highly conserved region of the motor domain (Nakagawa et al., 1997; Vernos et al., 1993). Recently, the crystal structure has been solved for a number of kinesin motor domains and also for the tubulin. However it is still not completely understood how the energy from ATP-hydrolysis is converted into movement along MTs lattice (Kozielski et al., 1997; Kull et al., 1996; Nogales et al., 1998; Vale and Fletterick, 1997). It is clear now that processive kinesins follow the path of a

protofilament along the MT, taking steps of 8 nm at a speed of up to 150 μm/min for the fungal kinesin (Ray et al., 1993). Most kinesins however are considerably slower, in particular mitotic ones.

The conventional kinesin-1 structure is composed of a globular motor domain at the N-terminus, a small "neck" region linking it to a long coiled-coil called "stalk" finishing in a globular "tail" domain that interacts with cargoes (Figure 3). Members of the kinesin superfamily can differ in the relative position of the motor domain and the neck in the polypeptide chain (at any of the ends or even in the middle) which correlates with their directionality (Bloom and Endow, 1995; Goldstein, 1993; Hirokawa, 1998). It was possible to reverse the directionality of a kinesin by placing the motor domain at the other end of the protein (Endow and Waligora, 1998; Henningsen and Schliwa, 1997). More than a hundred kinesins from different organisms have been identified and classified according to the degree of homology in the motor domain (Lawrence et al., 2004; Miki et al., 2001).

In 1990, results obtained in yeast and A. nidulans were the first to link kinesins to the cell division process (Enos and Morris, 1990; Meluh and Rose, 1990). Following this, the first evidence for the presence of multiple kinesins in mitotic spindles came from experiments using antibodies against conserved peptides in the kinesin motor domain (Sawin et al., 1992). Since then, numerous kinesins have been shown to be involved in three essential mechanisms in spindle assembly: the regulation of MT dynamics, the transport of specific mitotic cargoes along the spindle MTs, the cross-linking and sliding of MTs relative to adjacent MTs or other structures (Figure 4A).

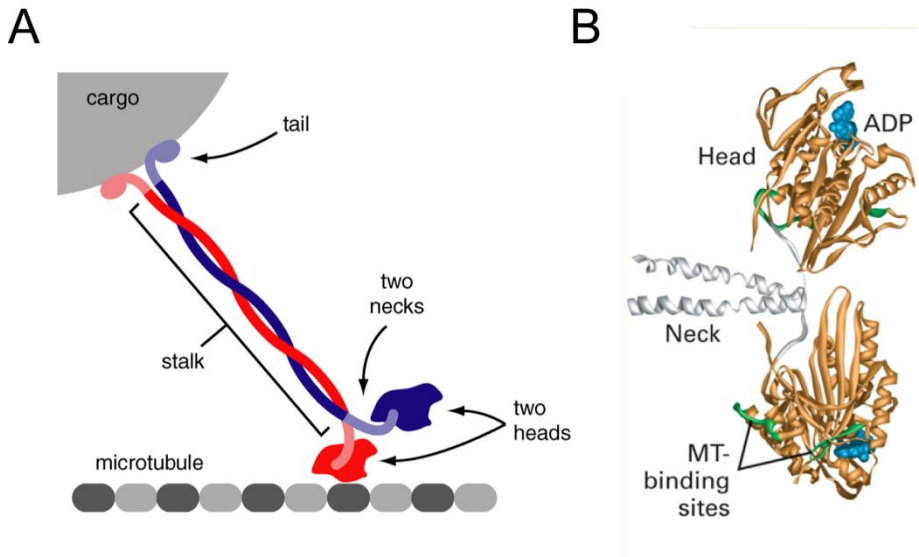


Figure 3: Kinesin structure
A- Cartoon structure of a kinesin dimer displaying the globular motor heads, with the long coiled-coil stalk ending as the globular tail that can interact with cargoes. From Asbury et al 2003 (Asbury et al., 2003).
B- 3D structure the two motor heads with the ATP and MT binding site. The two heads are linked by the coiled coils called "neck". Adapted from Kull et al 1996 (Kull et al., 1996).

Chromokinesins

The main structures that move relative to MTs are the chromosomes (Figure 4). Chromokinesins localize to the chromosomes and interact with MTs through their motor domains. Two chromokinesins have been identified with similar roles but with distinct mechanisms: Kid and Kif4 (Figure 4A). Kid is essential to generate what is called "polar ejection forces": being associated to chromosomes through its C-terminus, Kid walks towards the MT plus-end and pushes the chromosomes away from the poles, helping to align them on the metaphase plate (Antonio et al., 2000; Levesque and Compton, 2001; Rieder and Salmon, 1994). Kif 4 is also essential for the correct alignment of the chromosomes but its mechanism of action goes through a role in the organization of the spindle MTs rather than a polar ejection force (Castoldi and Vernos, 2006; Mazumdar et al., 2004; Vernos et al., 1995).

Eg5

The MTs generated by the centrosomes and around the chromatin need to be organized into a bipolar array. This is achieved using the ability of some motors to cross-link and slide MTs relative to adjacent MTs or other structures. One in particular, kinesin 5 (Eg5), has been shown in recent years to be the major factor in spindle bipolarity. Eg5 is present in cells as a homotetramer with motor domains at both ends of the complex, a characteristic that suits perfectly with the idea of cross-linking MTs (Kashina et al., 1996; Sharp et al., 1999). Moreover, Eg5 motor domain has a plus-end directionality which gives it the ability to organize MTs into an array and slide the antiparallel MTs apart, thus contributing to the creation of a bipolar array (Figure 4A). This function was confirmed by the injection of antibodies, using RNA interference in human cells and using of a specific inhibitor called monastrol. The impairment of Eg5 activity prevents the separation of the centrosomes, resulting in the formation of a monoastral spindle with chromosomes attached to MTs emanating from the center (Blangy et al., 1995; Kapoor et al., 2000; Mayer et al., 1999; Zhu et al., 2005). Monastrol has been a tool of great help to study bipolarity since its discovery (Figure 4B). Monastrol binds to Eg5 motor domain but does not compete directly with MT or ATP binding site (Maliga et al., 2002). It is rather an allosteric inhibitor that induces a conformational change at the MT-interaction site and also the neck linker, preventing the interaction between the kinesin and MTs (Krzysiak et al., 2006; Maliga et al., 2006). It is cell permeable, fast to act and can be washed out to restore Eg5 activity.

The accepted hypothesis about spindle bipolarity is that the establishment and maintenance of such bipolarity is due to a balance of outward and inward forces. When Eg5 activity is absent, one outward pushing force is missing and the global forces are imbalanced in favor of the inward forces resulting in the lack of separation of the centrosomes.

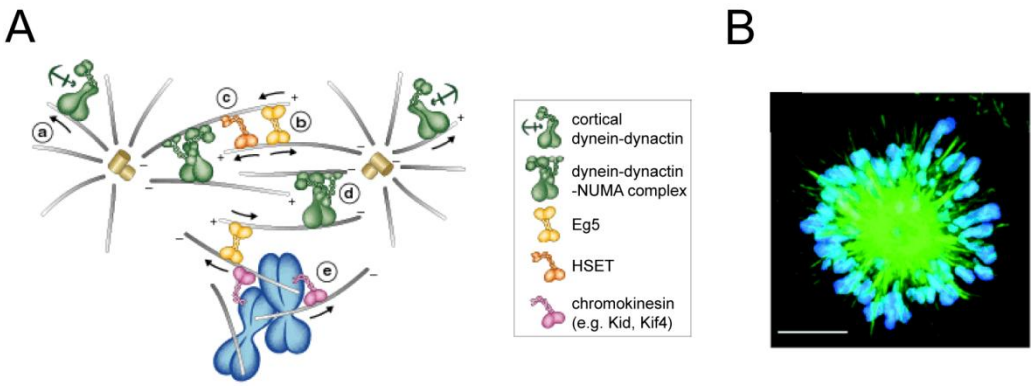


Figure 4: Motors and forces in the spindle
A- Minus-end directed cortical dynein plays a role in the positioning of the spindle by interacting from the cell cortex with the astral MTs (**a**); homotetrameric Eg5 is able, by walking towards the plus-end, to slide antiparallel MTs and separate the centrosomes (**b**); minus-end directed motor HSET applies a force that counteracts the separation of centrosomes (**c**); cytoplasmic dynein in complex with structural protein NuMA focuses the MTs at the poles (**d**); chromosome-associated kinesins have a plus-end directionality and mediate interactions between chromosomes and MTs (**e**). The forces applied by the motors on the MTs are indicated by arrows; note that these arrows point in the opposite direction to that of the motor directionality. Adapted from Wittmann et al 2001.
B- Monkey epithelial kidney cell (BS-C-1) treated with Eg5 inhibitor monastrol in mitosis. The centrosomes cannot separate and the spindle forms a monoastral structure. MTs are in green, DNA is in blue. Scale bar is 5 μm. Adapted from Mayer et al 1999.

In the monopolar situation, the spindle retains part of its organization as the MTs minus-ends are still focused at the centrosomes with the plus-ends pointing towards the chromosomes that are maintained at distance due to the polar ejection forces. The balance of forces model is supported by experiments performed in tissue culture cells (Tanenbaum et al., 2008) and in the Xenopus egg extract system (Mitchison et al., 2005). Impairing both dynein, a minus-end directed motor, and Eg5 functions results in the formation of bipolar spindles that, at least in tissue culture cells, can undergo anaphase and chromosome segregation (Tanenbaum et al., 2008). Bipolarity is also achieved when impairing the activity of antagonistic motor HSET together with Eg5 (Mountain et al., 1999). How bipolarity is established under these double inhibition

conditions is however unclear and these results suggest that other factors are involved in bipolarity.

Xklp2

Another motor that has been proposed to have a role in centrosome separation is the Xenopus kinesin-like protein 2 (Xklp2)(Boleti et al., 1996). Xklp2 is a plus-end directed motor with the highly conserved motor domain (around 350 amino acids) located at the N-terminus of the polypeptide chain (Figure 5A). The rest of the chain is a long coiled-coil, down to the C-terminus where it ends with a Leucine zipper. Thus, the protein lacks a globular C-terminus domain found in most kinesins. This motor has been classified in the Kinesin-12 family according to the standardized nomenclature (Lawrence et al., 2004). Xklp2 was found to localize to the centrosomes during the entirety of the cell cycle. It is present also on the spindle MTs in mitosis, more precisely to the minus-ends of the MTs (Figure 5B) (Boleti et al., 1996; Wittmann et al., 1998). This localization was shown to be due to the combined action of TPX2 that targets the kinesin to the MTs and dynein that transports the protein towards the minus-ends of MTs. The Leucine zipper at the C-terminus of Xklp2 is absolutely necessary for its TPX2-dependent binding to the mitotic MTs. All attempts of coimmunoprecipitation or pull-down of the three proteins (Xklp2, TPX2, dynein) have been unsuccessful and the exact mechanism of interaction and localization is still not completely understood.

Functional experiments in Xenopus egg extract indicated that Xklp2 may participate in centrosome separation during spindle assembly (Boleti et al., 1996). A recombinant protein encoding for the tail of Xklp2 fused to a GST tag was used as a dominant negative as it could localize to the minus-end of the mitotic MTs but could not have any activity as it lacked the motor domain.

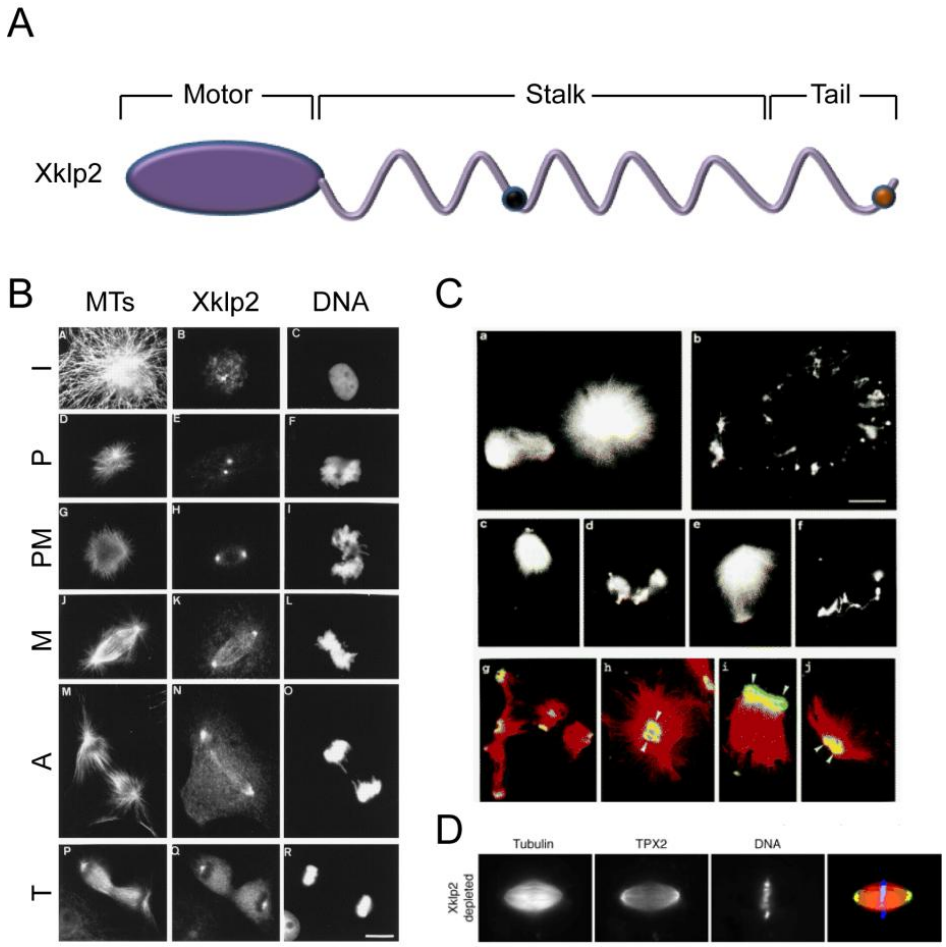


Figure 5: Xklp2 may be involved in spindle bipolarity
A- Cartoon of the full length Xklp2 showing the globular motor domain at the N-terminus followed by a coiled-coil down to the C-terminus. The Leucine zipper is indicated with a red dot, a predicted phosphorylation site by cdk1 is indicated by a black dot.
B- Immunofluorescence images of Xenopus XL177 cells stained with anti-Xklp2 and anti-αtubulin antibodies. The DNA was stained with Hoechst. Xklp2 is present on the centrosomes during the entirety of the cell cycle and is seen also on the MTs during mitosis. (I, interphase; P, prophase; PM, prometaphase; M, metaphase; A, anaphase; T, telophase;) (bar, 10 μm)
C- Structures observed when spindles are assembled in the Xenopus egg extract in presence of dominant negative GST-Xklp2 tail. In all cases the centrosomes could not separate. (a), (c) and (e) show the tubulin staining with (b), (d) and (f) show the corresponding DNA staining. (g)-(j) are overlays of confocal images of figures assembled with rhodamine tubulin (red) and stained with anti-Xklp2 (green). The GST-Xklp2 tail is localized close to the centrosomal area. (bar, 20 μm; except for (g), 50 μm)
C- Representative bipolar structure assembled in a Xklp2-depleted Xenopus extract (tubulin, red; TPX2, green; DNA, blue). The spindle assembles normally. Adapted from Boleti et al (1996) and Wittmann et al (2000).

The addition of this dominant negative fragment, GST-Xklp2-Tail, to mitotic Xenopus egg extract resulted in abnormal monopolar spindles with unseparated centrosomes, similar to what is obtained when inhibiting Eg5 (Figure 5C). The same results could be obtained by addition of specific antibodies against the endogenous Xklp2. These data strongly suggested that Xklp2 was involved in centrosome separation. As its localization is close to the poles, Xklp2 was proposed to function in a different way than Eg5. Xklp2 tethered to or close to one centrosome could move towards the plus-end of MTs emanating from the opposite centrosome, leading to their separation and stabilizing spindle bipolarity (Boleti et al., 1996).

However, additional experiments did not provide support to this hypothesis. Inhibition of Xklp2 function did not have a major effect on spindle assembly without centrosomes, around chromatin beads, suggesting that Xklp2 may be only required for pushing or holding centrosomes apart and not in the self-organization mechanism (Walczak et al., 1998). Also, when assembling spindles in cycled Xenopus egg extract that had been depleted of Xklp2, the percentage of normal bipolar spindles formed was indistinguishable from control (Figure 5D) (Wittmann et al., 2000). So the spindles did not show any difficulties to form in the absence of the protein.

Different homologues of this kinesin exist in mouse (Kif15), sea urchin (Krp180), C. elegans (KLP18) and human (Hklp2 or Kif15). The mouse homologue Kif15 has been shown to localize to the centrosomes and spindle MTs in mitosis in fibroblasts. In neurons, it decorates tight bundles of MTs in the dendrites (Buster et al., 2003). In sea urchin embryos, Krp180 also associates with the spindle MTs but in a region close to the chromosomes. Injection of a specific antibody or dominant negative fragment of the kinesin results in a shortening of the pole-to-pole distance but cell division is taking

place without any striking defect (Rogers et al., 2000). First data generated in C. elegans indicated that KLP18 was helping the organization of the MTs into a bipolar array during the meiotic divisions only (Segbert et al., 2003). A more detailed microscopy analysis demonstrated that acentrosomal spindles that assemble in meiotic oocytes depleted of KLP18 can organize but cannot establish bipolarity and remain in a monoastral configuration (Wignall and Villeneuve, 2009). Human kinesin Hklp2 shares a high percentage of homology with Xklp2 and the same secondary structural features (Sueishi et al., 2000). At the beginning of this project no functional data were available on this motor.

Overall, the results obtained for the different homologues pointed towards an involvement of this kinesin in spindle assembly and bipolarity, but the exact role and the importance of this motor as well as a mechanistic model were still missing.

OBJECTIVES

The aim of the thesis presented here was to clarify the functional role of the human homologue of the kinesin-like protein 2 (Hklp2, also named Kif15) using human tissue culture cells.

The main objectives were:

1. To perform a detailed analysis of Hklp2 localization in cells.
2. To examine the functional role of Hklp2 in spindle assembly and, more particularly, spindle bipolarity.
3. To study the regulation of Hklp2 localization and activity.

RESULTS

The role of kinesin Eg5 in bipolar spindle assembly

In the last years, evidences accumulated demonstrating that the role of homotetrameric kinesin Eg5 in bipolar spindle assembly is to bind antiparallel microtubules, align them and sort them apart, thanks to its motor activity towards the plus-end of the fibers; all of this resulting in an outward pushing force that tends to separate the poles (Kapitein et al., 2005). A number of results challenge this well-established model and raise some questions about the precise role of Eg5 and the involvement of other factors in spindle bipolarity.

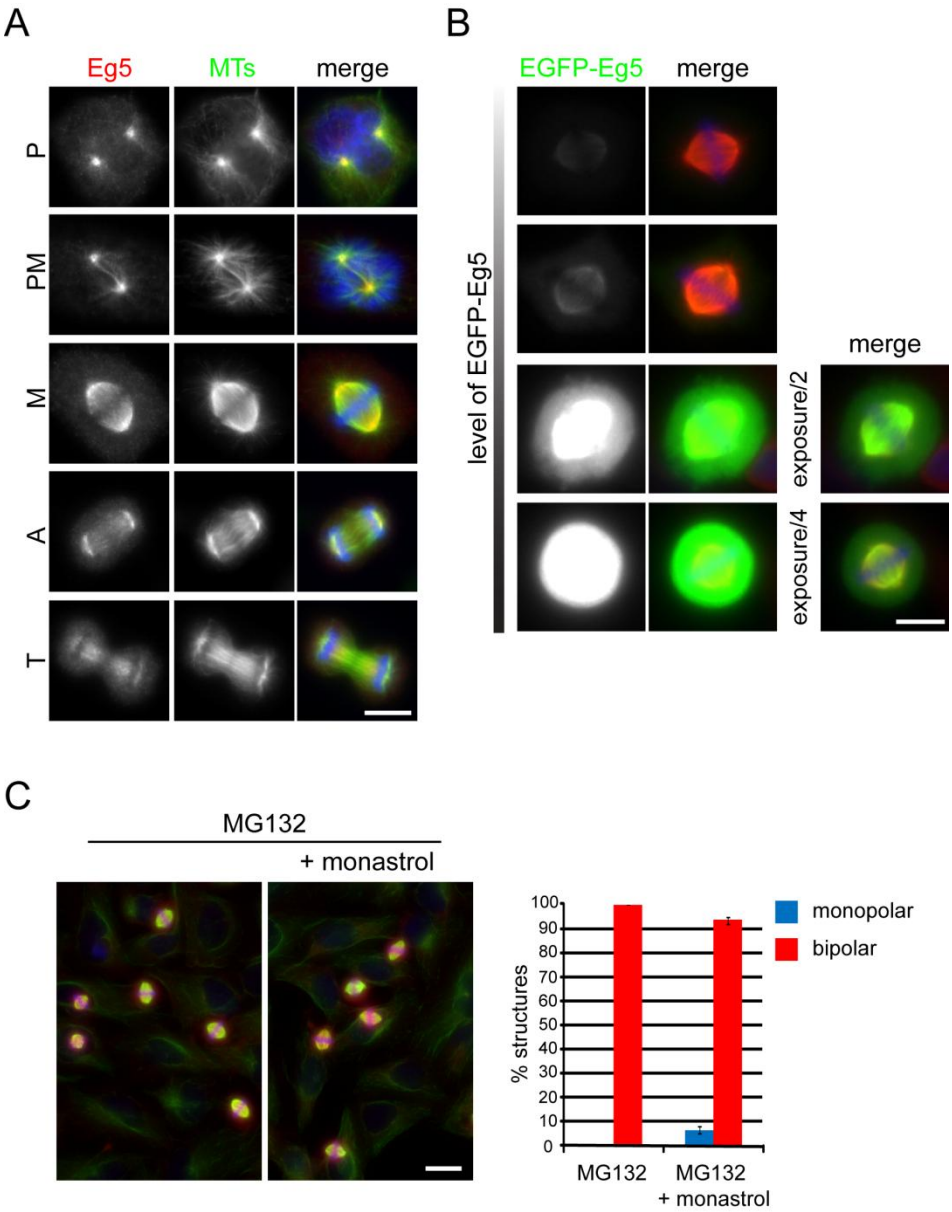
We first examined more closely Eg5 localization, the consequences of its overexpression on spindle assembly and whether it is required for bipolar spindle stability in metaphase HeLa cells.

First of all, the localization of the kinesin during metaphase, on the MTs and close to the poles, does not fit with its proposed position at the center of the spindle during metaphase pushing on the antiparallel MTs (Figure 6A). How can Eg5 play a role in pole separation in metaphase if it is not close to the antiparallel MTs? Second, impairing the activity of Eg5 generates an imbalance of forces that results in the lack of centrosome separation. Astonishingly, the overexpression of Eg5 in HeLa cells did not produce any imbalance of forces and spindle assembly occurred normally (Figure 6B). Third, the current model fails to explain why in some systems Eg5 inhibition does not promote bipolar spindle collapse (Cameron et al., 2006; Kapoor et al., 2000). Also, the C. elegans Eg5 orthologue is not required for bipolar spindle formation (Bishop et al., 2005). To examine whether Eg5 is required for the stability of the bipolar spindle once metaphase has been reached, HeLa cells were treated with proteasome inhibitor MG132 to prevent anaphase onset (Figure 6C). When the

proteasome is blocked, the spindle can perfectly assemble and satisfy the checkpoints controlling the correct attachment of the kinetochores to the microtubules of opposite poles. However the activation of the anaphase-promoting complex (APC) does not have any effect as the degradation of securin cannot occur due to the blocking of the proteasome and therefore the separation of the sister chromatids cannot take place. In these conditions, cells accumulate in metaphase with a fully formed bipolar spindle. These cells were then incubated with monastrol to block Eg5. Surprisingly, the spindles maintained their bipolarity with no visible change of shape. The quantification of the structures statistically confirmed this result. We conclude that once the spindle has reached bipolarity and chromosome alignment, the activity of Eg5 is not required anymore.

These results together suggest that bipolarity cannot be explained only by a balance of Eg5 and dynein activities. Spindle bipolarity probably involves numerous factors that need to be identified and characterized.

Figure 6: Eg5 role in spindle bipolarity is not completely understood
A- HeLa cells immunostained with anti-tubulin (green), anti-Eg5 (red). At prophase, Eg5 localizes to the centrosomes. In metaphase it extends on the MTs of the spindle, close to the poles. (bar, 10 μm)
B- HeLa cells transfected with EGFP-Eg5 and immunostained with anti-tubulin (red) antibodies. The EGFP-Eg5 localizes as endogenous Eg5 and even at high levels of expression does not interfere with spindle assembly. Pictures were taken with the same exposure. Reduced exposure was used to visualize the localization of EGFP-Eg5 where indicated. (bar, 10 μm)
C- Left panel shows HeLa cells treated with MG132 with or without the addition of monastrol. Immunostaining done with anti-tubulin (green), anti-Hklp2 (red). (bar, 25 μm)
Right panel shows the frequency of bipolar and monopolar spindles found in each condition. Eg5 activity is not necessary to maintain bipolarity once metaphase has been reached. The data were obtained from > 100 spindles from each of three independent experiments. Error bars correspond to the standard error of the mean done on proportions.
In all the cases, DNA was stained with DAPI (blue)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Hklp2 is a cytoplasmic kinesin whose expression is not cell cycle regulated

Xenopus kinesin-like protein 2 (Xklp2) is a plus-end directed kinesin that has the globular motor domain at the N-terminal end of the amino acid chain and that localizes at the minus-ends of MTs. Results obtained in Xenopus egg extracts showed that the kinesin Xklp2 could have a role in spindle bipolarity using a dominant negative approach (Boleti et al., 1996). However, additional experiments did not support this hypothesis as depletion of the protein did not generate any phenotype and spindles could assemble with the same shape and efficiency as control (Wittmann et al., 1998). Data on different homologues of Xklp2 in sea urchin (Rogers et al., 2000) and C. elegans (Segbert et al., 2003; Wignall and Villeneuve, 2009) also suggested a role in spindle pole separation.

We decided to investigate the role of the human homologue, Hklp2 (also named Kif15). This kinesin has the same predicted secondary feature than Xenopus Xklp2: a globular head composed of the motor domain at the N-terminal end, followed by a long coiled-coil down to the C-terminus with a Leucine zipper at the end of the polypeptide chain (Figure 7A)(Sueishi et al., 2000). The sequence identity between full-length Hklp2 and Xklp2 is 53% (88% in the motor domain).

We first generated a polyclonal antibody by injecting rabbits with a recombinant C-terminal fragment of Hklp2. Antibodies were purified from the serum by immunoaffinity. These antibodies gave a specific signal in Western blot against a total lysate of HeLa cells, recognizing a band of approximately 160 kDa corresponding to the predicted size of Hklp2 (Figure 7B). Also, as kinesins can be found in the cytoplasm (e.g. Eg5 (Blangy et al., 1995)) or in the nucleus (e.g. Kif4 (Mazumdar et al., 2004)), protein content was extracted from cells and separated in cytosolic and nuclear fraction to determine the global

distribution of Hklp2 (Figure 7C). Hklp2 was found exclusively in the cytoplasmic fraction, in contrast to TPX2, a microtubule associated protein and putative Hklp2 interactor (by analogy to Xklp2 (Wittmann et al., 1998; Wittmann et al., 2000)) that is present in the nuclear fraction. Some kinesins can be cell-cycle regulated by degradation (e.g. Kid (Funabiki and Murray, 2000)). To assess whether Hklp2 maybe cell-cycle regulated, HeLa cells were synchronized by a double thymidine block and collected at different time points after release (Figure 7D). The progression of the cells through the cell cycle was followed by FACS analysis and showed that the majority of cells was in M-phase 10 hours after the release (data not shown). The level of Hklp2 did not change substantially as monitored by Western blot analysis of cell extract made at different time points. In contrast, TPX2 which is known to be upregulated in mitosis showed a peak at 10 hours, when the majority of cells is in G2/M (Gruss et al., 2002). These results suggest that Hklp2 is a kinesin present in the cytoplasm of human cells and whose expression is not cell-cycle regulated.

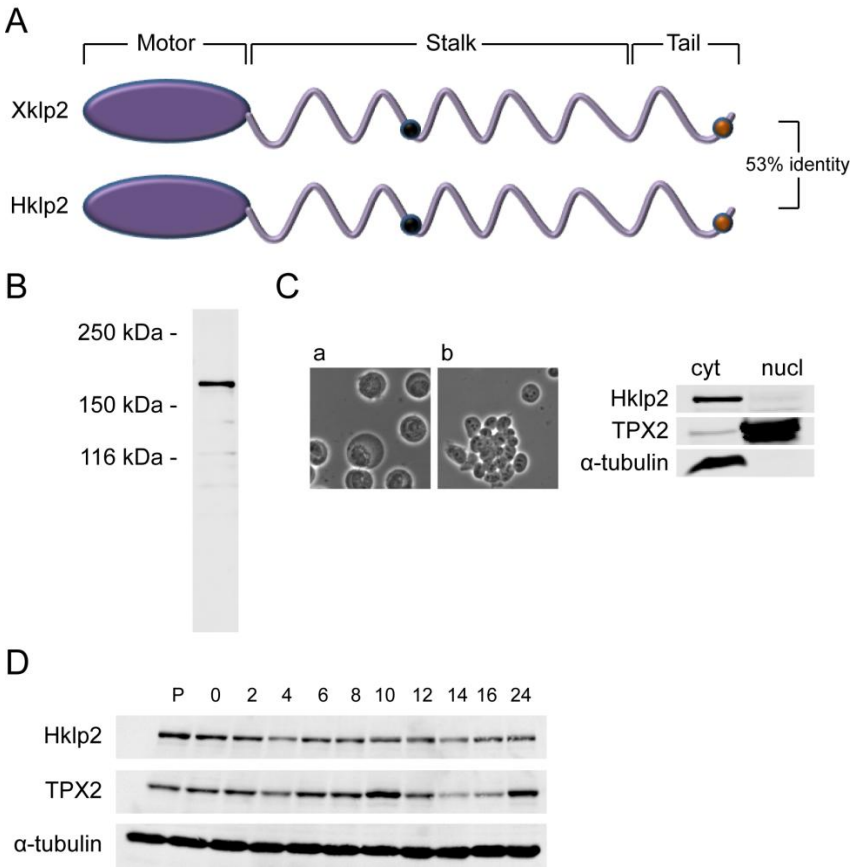


Figure 7: Hklp2 is a cytoplasmic protein whose level is not cell cycle regulated
A- Cartoon showing the domains of Hklp2 and Xenopus homologue Xklp2. Both proteins are 1388 amino acids long (53% identity) and share the same secondary structural features. Leucine zippers are represented by red dots. The black dots indicate potential cdk1 phosphorylation site interrupting the coiled-coil structure of the stalk.
B- Western blot of a total HeLa cell lysate showing the specificity of the affinity-purified anti-Hklp2 antibody that recognizes a single band at around 160 kDa .
C- Cytoplasmic and nuclear extracts were prepared from HeLa cells. Left: Brightfield pictures of proliferating Hela cells treated with hypotonic buffer before (a) and after (b) homogenization with a pestle, showing that the cytoplasm was broken while preserving the nucleus during the preparation. Right: Western blot of cytoplasmic and nuclear fractions probed with the anti-Hklp2, anti-TPX2 and anti-tubulin antibodies. Hklp2 is clearly enriched in the cytoplasmic fraction as α-tubulin whereas TPX2 is enriched in the nuclear fraction as expected.
D- Left: Western blot analysis of lysates of HeLa cells taken at the indicated time points after the release from a double thymidine block. Detection with anti-Hklp2, anti-TPX2 and anti-tubulin. The levels of TPX2 increase at 10h after the release as cells peak into mitosis, and go down after mitosis (12-14 hours), as previously described. In contrast, the levels of Hklp2 remain constant throughout the cell cycle. (P, proliferating HeLa)

Hklp2 localizes to spindle microtubules and chromosomes in mitosis

The affinity purified polyclonal antibody was used to visualize the intracellular localization of Hklp2 by immunofluorescence using confocal microscopy (Figure 8A). hTERT-RPE1 cells were chosen for this characterization because of their excellent morphology. In interphase cells the antibody did not label any subcellular structure, displaying a diffuse signal in the cytoplasm. This was also the case in other cell types (HeLa, MDK)(Figure 8B). In mitotic cells, Hklp2 localized to the spindle MTs and the chromosomes. The staining was most evident at metaphase, once the chromosomes were aligned. Hklp2 decorated the overall length of the MTs in a punctuate pattern and the chromosomes. In anaphase, the staining became more diffuse but remained on the elongating spindle. Once the cells reached telophase, no specific localization for Hklp2 could be observed. Hklp2 localization to the spindle MTs and the chromosomes seemed coherent with a putative role in spindle assembly.

Immunofluorescence analysis in HeLa cells showed identical results to the ones obtained with the hTERT-RPE1 cells (Figure 9A).

To further confirm the localization of Hklp2, a construct encoding for the full-length Hklp2 tagged with a FLAG sequence was generated. The protein was exogenously expressed in HeLa cells, and its localization visualized using a specific polyclonal antibody against the FLAG sequence (Figure 9B). FLAG-Hklp2 remained diffuse in the cytoplasm during interphase and localized to the spindle MTs and chromosomes in mitosis, confirming the previous results obtained with the specific antiHklp2 antibody.

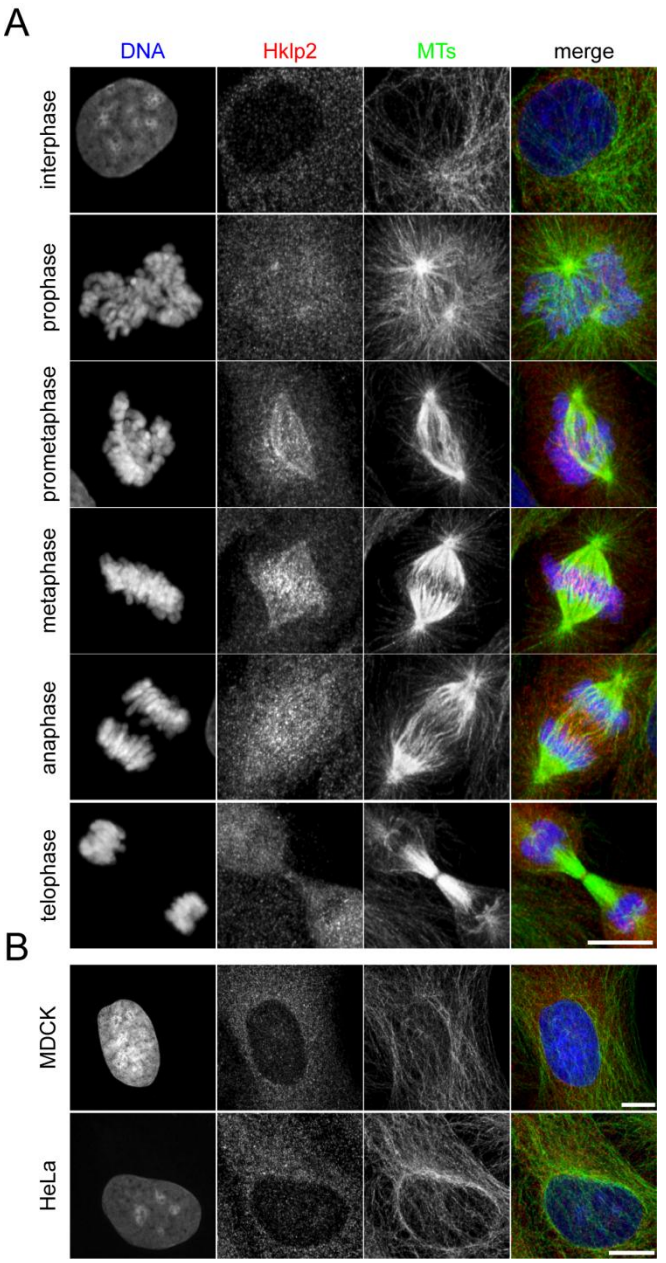


Figure 8: Hklp2 subcellular localization
A- hTERT-RPE1 cells fixed and immunostained with the anti-Hklp2 (red) and anti-tubulin (green) antibodies. Hklp2 is diffuse in the cytoplasm during interphase and associates with the spindle MTs and the chromosomes in mitosis.
B- MDCK cell and HeLa cell in interphase fixed and immunostained as in A. In both cell lines, Hklp2 does not localize to any subcellular structure in interphase.
All images are maximum projections of confocal sections acquired every 0.3 µm. DNA stained with DAPI (blue). (bars, 10 µm)

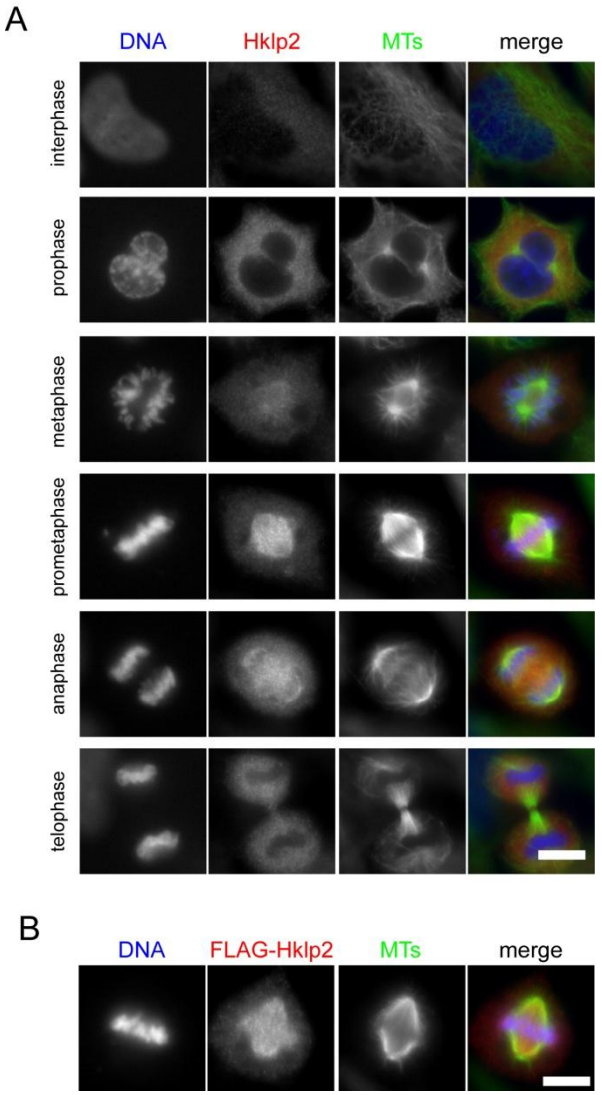


Figure 9: Hklp2 and FLAG-Hklp2 subcellular localization in HeLa cells
A- Proliferating HeLa cells were processed for immunofluorescence using anti-tubulin (green) and anti-Hklp2 (red). Pictures show the localization of Hklp2 in all stages of the cell cycle and this localization is identical to the one observed in hTERT-RPE1 cells. Hklp2 is diffuse in the cytoplasm during interphase and localizes to the spindle MTs and the chromosomes in mitosis.
B- HeLa cells transfected with FLAG-Hklp2 and immunostained with an anti-FLAG (red) and anti-tubulin (green) antibodies. The exogenously expressed protein has a similar localization to the endogenous Hklp2.
In all the cases, DNA was stained with DAPI (blue) (bars, 10 µm)

Hklp2 localization on microtubules relies on TPX2

The cell-cycle dependent localization to the MTs of Hklp2 suggested that additional factors may be required. TPX2 is a nuclear protein first identified as a factor necessary for the targeting of Xenopus kinesin Xklp2 to the MTs (Wittmann et al., 1998). Using RNA interference, TPX2 expression was silenced in HeLa cells to barely detectable levels (Figure 10A). The silenced cells were arrested in a prometaphase-like state with condensed chromatin and MTs emanating from the centrosomes but in insufficient numbers to organize a spindle (Gruss et al., 2002). In these cells, no Hklp2 was observed either on the MTs or on the chromosomes. To verify that the absence of Hklp2 localization to the MTs was due specifically to the absence of TPX2 and not to the reduced number of MTs, control and TPX2-RNAi cells were treated with taxol to stabilize MTs (Figure 10B). In these conditions, more MTs were observed in mitotic cells. However, Hklp2 was still not localized. These data confirmed that TPX2 is essential for the targeting of Hklp2 to the MTs and chromosomes.

As TPX2 is known to interact with and activate the mitotic kinase Aurora A (Eyers et al., 2003; Kufer et al., 2002; Tsai et al., 2003), we also examined if Aurora A is required for Hklp2 mitotic localization. Aurora A was silenced by siRNA transfection in HeLa cells (Figure 10C). In these cell, spindle MTs were much disorganized but Hklp2 still decorated them in a punctuate manner. Therefore Aurora A does not seem to be involved in the localization of Hklp2 to the MTs in mitosis.

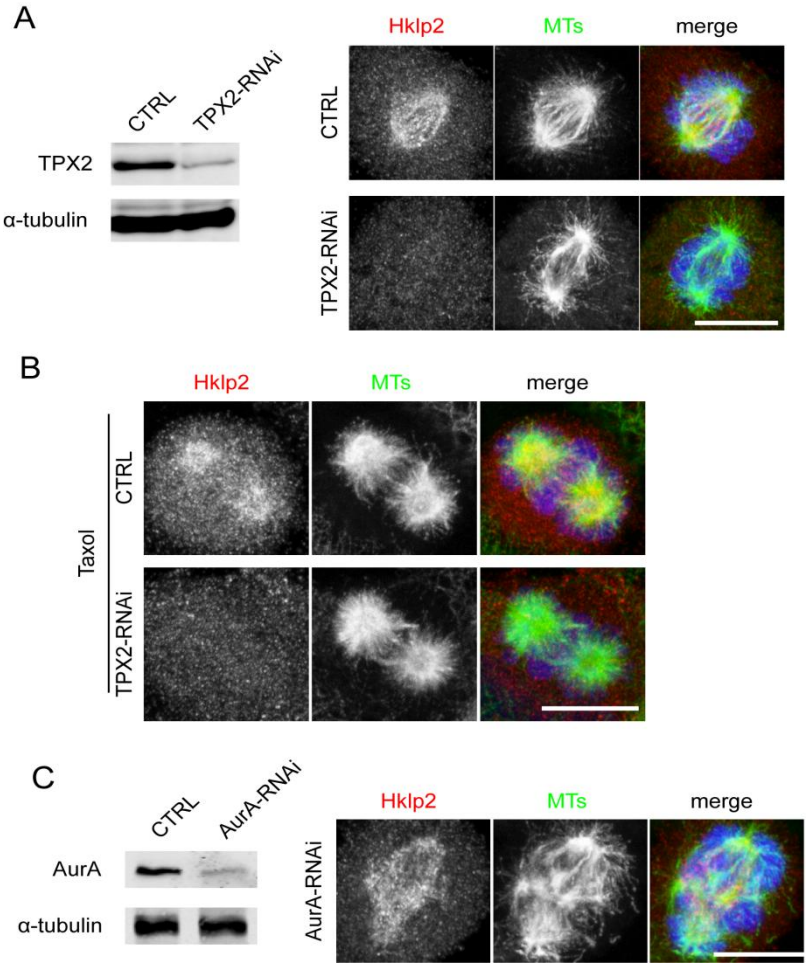
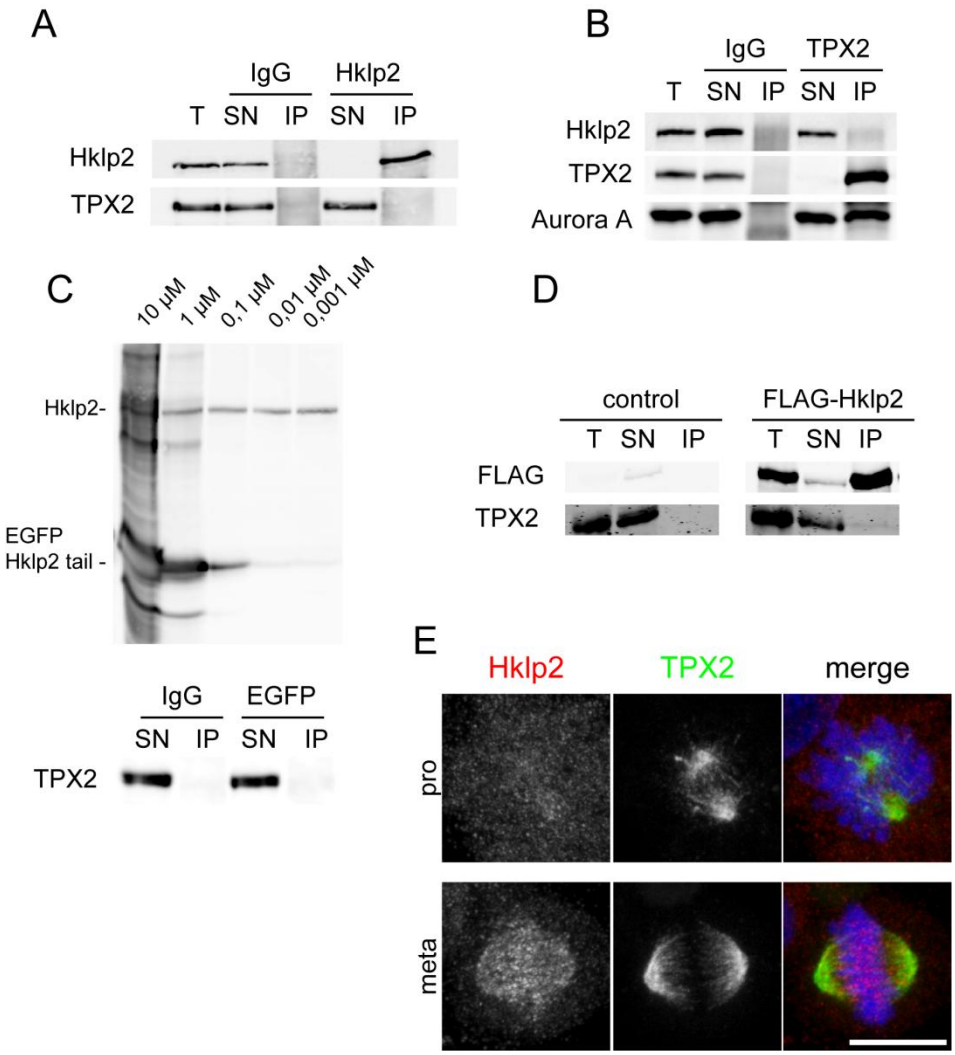


Figure 10: Hklp2 localization depends on TPX2 but not Aurora A
A- Left: Western blot showing that TPX2 levels are reduced by around 90%, 48 hours after TPX2-specific siRNAs transfection. Anti-TPX2 antibody used for detection. The level of tubulin sserved as a loading control. Right: HeLa cells silenced for TPX2 were processed for immunofluorescence with the anti-Hklp2 (red) and anti-tubulin (green) antibodies. Hklp2 fails to localize to the MTs in the absence of TPX2.
B- Control and TPX2-silenced HeLa cells were treated with microtubule stabilizer taxol and immunostained (Hklp2 (red), tubulin (green)). Hklp2 does not have any specific localization when TPX2 is absent even if the microtubules are stabilized.
C- Left panel shows a Western blot of HeLa cells transfected with control or Aurora A-specific siRNAs showing the extent of silencing. Right panel shows HeLa cells silenced for Aurora A (Hklp2 in red, MTs in green) with Hklp2 still localizing to the disorganized MTs.
In all cases DNA was stained using DAPI (blue). All images are maximum projections of confocal sections acquired every 0.3 μm. (bars, 10 μm)

Since TPX2 identification as a targeting protein for Xklp2 (Wittmann et al., 1998), numerous attempts were made in the lab to determine whether the two proteins interact directly. None of the approaches (immunoprecipitation or pull-downs from Xenopus egg extract) gave any positive results. To examine this issue in human cells, Hklp2 was immunoprecipitated from a total lysate of cells synchronized in mitosis, using the C-terminal antibody (Figure 11A). Hklp2 was efficiently immunoprecipitated but no co-precipitation of TPX2 was detected in any condition of lysis and washings tested. As a complementary approach TPX2 was immunoprecipitated in the same conditions with a polyclonal antibody generated in the lab (Figure 11B)(Gruss et al., 2002). Aurora A was present in the precipitate but Hklp2 was not.

Figure 11: Hklp2 interaction with TPX2 may be indirect
A- Western blot showing the Hklp2 and TPX2 content of an IP from mitotic HeLa cell lysate using Hklp2 antibody or unspecific IgG for control (T, total lysate; SN, supernatant; IP, immunoprecipitated material). Hklp2 is nicely immunoprecipitated from the cell lysate but TPX2 is not coprecipitating.
B- Western blot showing the Hklp2, TPX2 and Aurora A content of an IP from mitotic HeLa cell lysate using TPX2 antibody or unspecific IgG for control. TPX2 is nicely immunoprecipitated and Aurora A is present as a coprecipitant but not Hklp2.
C- Top panel shows a Western blot with increasing amount of recombinant EGFP-Hklp2 tail added to a total HeLa cell lysate (immunobloting with anti-Hklp2 antibody). The amount of 1μM of EGFP-Hklp2 tail was used as a saturating amount to be added to a mitotic HeLa cell lysate and pulled down after incubation. Bottom panel shows the Western blot analysis of the immunoprecipitate and the absence of TPX2.
D- Western blot analysis showing the FLAG-Hklp2 and TPX2 content of an immunoprecipitation from mitotic HeLa cell transfected with FLAG-Hklp2 or empty vector. Immunoprecipitation was performed with FLAG antibody coated beads. FLAG-Hklp2 is nicely immunoprecipitated but without TPX2.
E- Confocal images of a prophase and a metaphase HeLa cells processed for immunofluorescence with the anti-Hklp2 (red) and anti-TPX2 (green) antibodies and the DNA was stained with DAPI (blue). TPX2 localizes to the MTs prior to Hklp2 and becomes enriched towards the spindle poles in metaphase whereas Hklp2 shows its typical localization to the more central part of the spindle and the chromosomes. Images are maximum projections of confocal sections acquired every 0.3 μm. (bar, 10 μm)



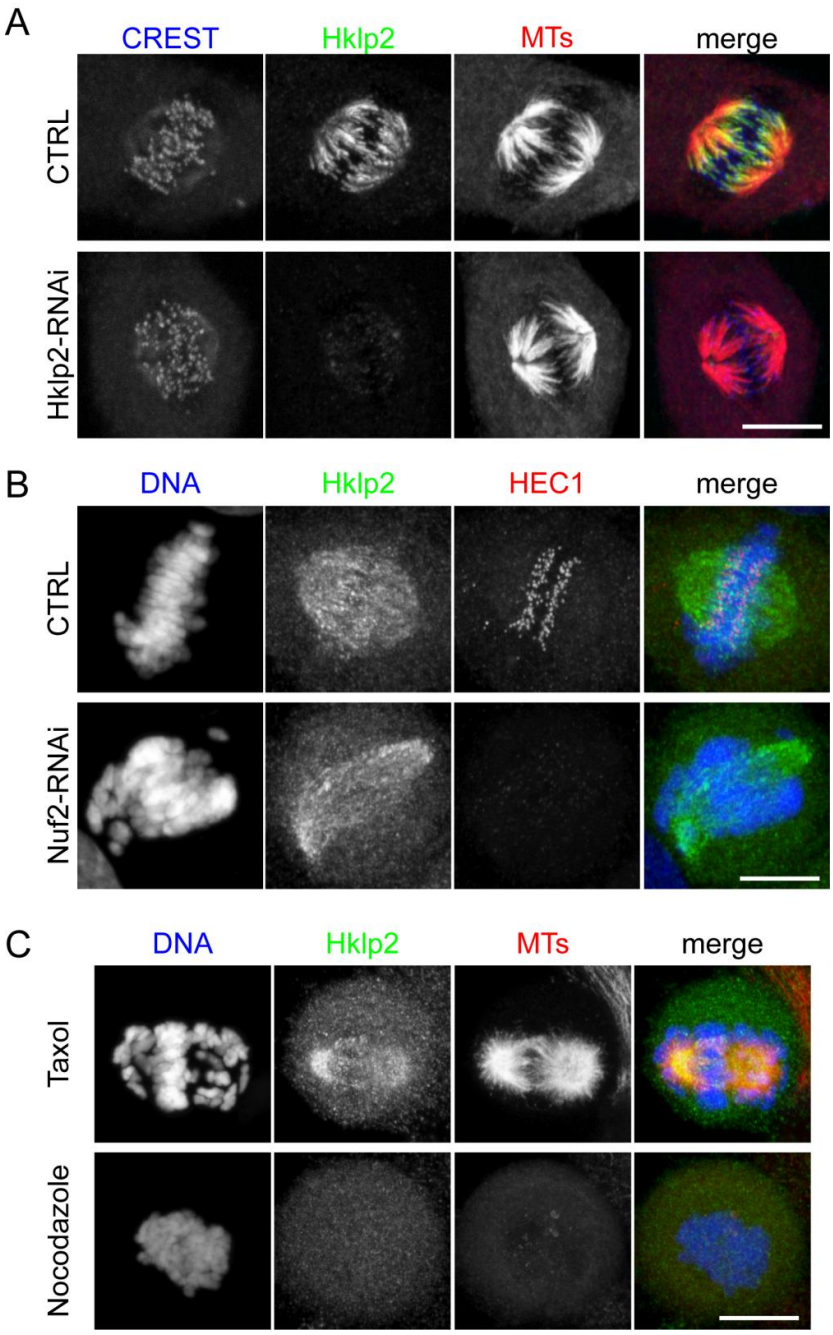
Previous data obtained in the Xenopus system showed that the C-terminal part of Xklp2 is involved in the TPX2-dependent targeting. As the polyclonal antibody that we raised against Hklp2 targets the tail and could disrupt the interaction between Hklp2 and TPX2, we tried two different approaches to overcome this potential problem. The first one was to add a saturating amount of purified EGFP-Hklp2 tail fusion protein to a cell lysate and, after incubation, to pull it down with anti-EGFP antibodies (Figure 11C). The protein content

was analysed by Western blot. TPX2 was not present. The other approach was to express exogenously the FLAG-Hklp2 fusion that localizes identically to the endogenous protein in cells synchronized in mitosis and immunoprecipitate the FLAG fusion using beads bound with a specific anti-FLAG antibody (Figure 11D). Again, TPX2 was not recovered among the coprecipitants.

As none of the attempts to detect an interaction of Hklp2 with TPX2 was successful, the mechanism underlying the TPX2-dependent targeting of Hklp2 to the mitotic MTs may not involve the formation of a stable complex but a more intricate mechanism. This idea is also supported by the clear differences in the localization patterns of the two proteins (Figure 11E). TPX2 localizes to MTs earlier than Hklp2, and becomes enriched towards the spindle poles in metaphase, whereas Hklp2 is comparatively more enriched in the central part of the spindle and the chromosomes.

Altogether the data indicate that Hklp2 localization to the spindle MTs depends on TPX2, most probably through an indirect mechanism that does not involve Aurora A.

Figure 12: Hklp2 localizes to kinetochore and non-kinetochore microtubules
A- Control and Hklp2-silenced HeLa cells treated with cold to depolymerize the more dynamic spindle MTs. The cells were stained with the anti-Hklp2 (green), and anti-tubulin (red) antibodies. Kinetochores were detected with the CREST antiserum (blue). Hklp2 localizes very strongly to the the K-fibers, but is not required for their stability.
B- Control and Nuf2-silenced HeLa cells stained with the anti-Hklp2 (green), and anti-HEC-1 (red) antibodies. Hklp2 associates to non-kinetochore MTs(Nuf2-RNAi). The efficiency of Nuf2 silencing is shown by monitoring the Ndc80 complex protein Hec-1.
C- HeLa cells treated with taxol or nocodazole and immunostained for Hklp2 (green) and tubulin (red). Impaired MT dynamics with taxol does not abolish Hklp2 localization to the mitotic MTs but the chromsome localization is lost. When MTs are completely depolymerized using nocodazole, Hklp2 does not display any specific staining.
In all cases the DNA was stained with DAPI (blue). All images correspond to maximum projections of confocal sections acquired every 0.3 μm. (bars, 10 μm)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C
DNA
Hklp2
MTs
merge
Taxol
Nocodazole

Hklp2 associates with all spindle microtubules and is strongly recruited to the kinetochore fibers

TPX2 drives MT assembly at the kinetochores, and the formation of mature kinetochore fibers (K-fibers) (Tulu et al., 2006). To examine whether Hklp2 associates preferentially to some classes of spindle MTs, HeLa cells were incubated on ice to induce the selective depolymerization of the more dynamic MTs while preserving the K-fibers (Figure 12A). Immunofluorescence analysis showed that Hklp2 was very strongly recruited to these MT bundles. The silencing of Hklp2 and subsequent cold treatment showed that Hklp2 is not required for their formation. We then examined Hklp2 localization in HeLa cells that are defective for K-fiber formation. Protein hNuf2 is part of a complex acting as a molecular linker between kinetochores and MTs (DeLuca et al., 2002). When hNuf2 is silenced by RNAi, K-fibers cannot assemble. Immunofluorescence analysis in these cells showed that Hklp2 still localized to the remaining spindle MTs (Figure 12B).

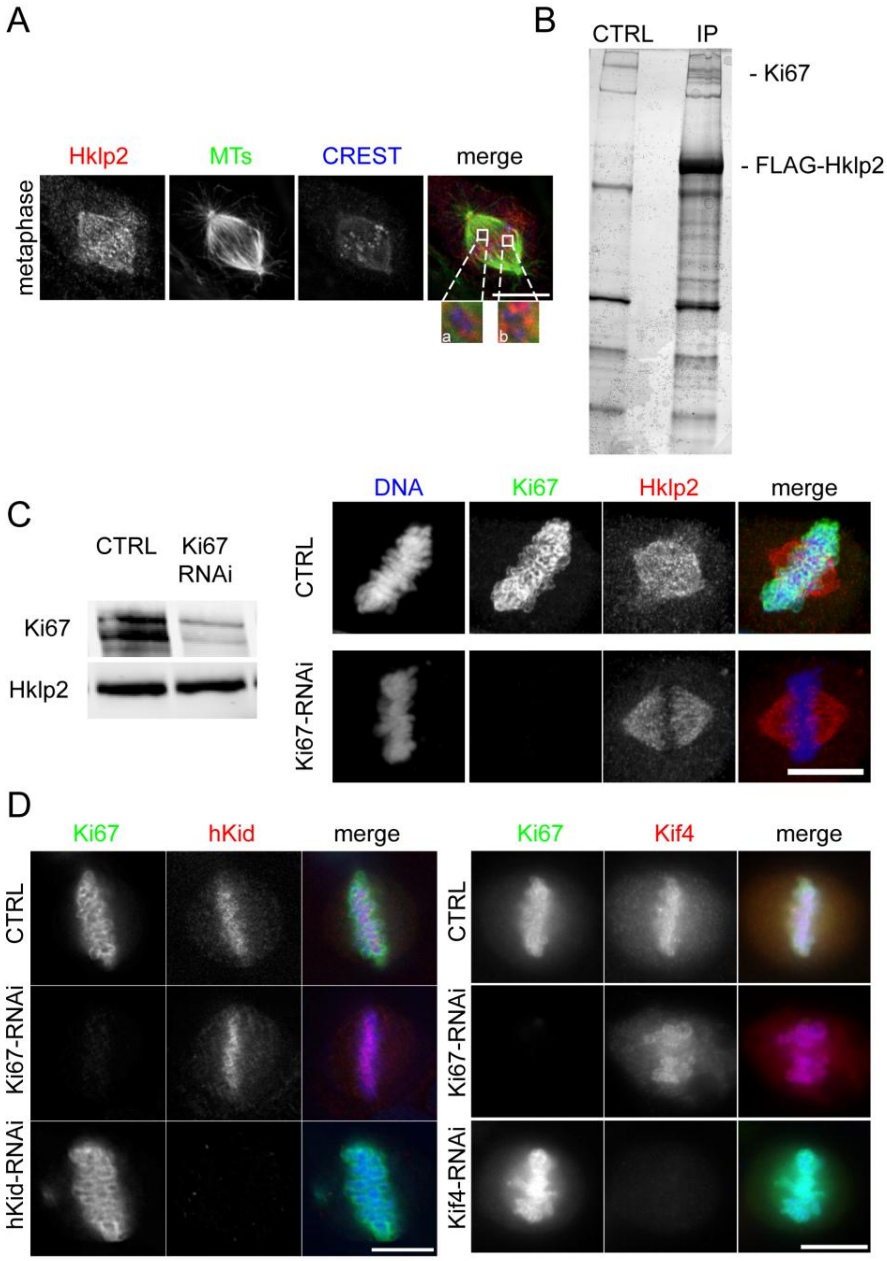
Microtubule dynamics can be altered in cells treated with nocodazole, a drug that bind the β-tubulin subunit, leading to the full depolymerization of microtubules (Jordan et al., 1992; Luduena and Roach, 1991). In the absence of MTs, Hklp2 remained diffuse in the cytoplasm of the mitotic cells (Figure 12C). On the contrary, taxol can bind β-tubulin subunits and promote the formation of overstable MTs with much reduced dynamics. In cells treated with taxol, Hklp2 was associated to the MTs but not to the chromosomes.

Therefore, Hklp2 associates with both kinetochore and non-kinetochore MTs, although it seems to have a higher affinity for the K-fibers. Interestingly, both types of MTs and their dynamic properties seem to be required for the localization of Hklp2 to the chromosomes.

Hklp2 localization on chromosomes relies on Ki67

The localization of Hklp2 close to the chromosomes in metaphase was further examined. The kinetochores were marked using a specific autoimmune human antibody, CREST (Figure 13A) and the anti-Hklp2 antibody. No specific overlapping with the Hklp2 punctuate pattern close to the chromosomes was observed. A previous report showed, by means of yeast-two-hybrid and pull-downs, that Hklp2 interacts with Ki67 (Sueishi et al., 2000), a chromosome-associated proliferation marker whose function during mitosis is currently unknown (Brown and Gatter, 2002). Immunoprecipitation experiments from FLAG-Hklp2 expressing HeLa cells confirmed the interaction between Hklp2 and Ki67 (Figure 13B). To examine whether Hklp2 localization to the chromosomes in metaphase requires Ki67, HeLa cells were transfected with siRNAs to silence Ki67. Immunofluorescence analysis of Hklp2 localization in these cells showed that the chromosomal localization of Hklp2 was completely abolished in absence of Ki67 (Figure 13C). The localization of other kinesins known for their localization to the chromosomes was checked to see if the loss of Ki67 could affect them (Figure 13D). Both chromokinesins Kif4 and Kid did not show any difference in localization between control cells and cells silenced for Ki67. These data showed that Ki67 is required specifically for the localization of Hklp2 to the mitotic chromosomes.

Figure 13: Hklp2 localizes to the chromosomes in a Ki67-dependent way
A- Confocal section through the spindle of an hTERT-RPE1 cell processed for immunofluorescence with the anti-Hklp2 (red), the anti-α tubulin (green) antibodies, and a CREST antiserum to mark the kinetochores (blue). Hklp2 associates with the spindle MTs and the chromatin. a and b are magnifications from two selected areas to show that Hklp2 does not localize to the kinetochores.
B- Silver stained gel of proteins pulled down with anti-FLAG covered beads from lysates of mitotic HeLa cells transfected with the empty vector (CTRL) or pFLAG-Hklp2 (IP). Mass spec analysis revealed the presence of Ki67 in the IP fraction.
C- Western blot showing the extent of the silencing of Ki67 in HeLa cells after siRNAs transfection is shown on the left. On the right, immunofluorescence on control and Ki67-silenced cells shows that Hklp2 fails to localize to the chromosomes in the absence of Ki67. In the overlay Ki67 is in green, Hklp2 is in red and DNA in blue. Images correspond to maximum projections of confocal sections acquired every 0.3 μm.
D- Left: HeLa cells transfected with control, anti-Ki67 or anti-hKid siRNAs processed for immunofluorescence with anti-Ki67 (green) and anti-hKid (red) antibodies. Ki67 and hKid and Ki67 localizes to the chromosomes independently.
Right: Hela cells transfected with control, anti-Ki67 or anti-Kif4 siRNAs processed for immunofluorescence with anti-Ki67 (green) and anti-Kif4 (red) antibodies. Ki67 and Kif4 localizes to the chromosomes independently.(bars, 10 μm)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- FLAG-Hklp2
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CTRL
Ki67 RNAi
Ki67
Hklp2
DNA
Ki67
Hklp2
merge
CTRL
Ki67-RNAi
D
Ki67
hKid
merge
CTRL
Ki67-RNAi
hKid-RNAi
Ki67
Kif4
merge
CTRL
Ki67-RNAi
Kif4-RNAi

Hklp2 is involved in spindle bipolarity

The different results obtained concerning Hklp2 localization and its potential interactors were coherent with the possibility of a role for Hklp2 in spindle bipolarity. To get an insight into this role, an RNA interference approach was adopted. Western blot analysis showed that specific siRNAs could reduce the Hklp2 protein level by around 80%, 72 h after transfection in HeLa cells (Figure 14A). Depletion efficiency could not be increased by repeated siRNA transfections. Immunofluorescence on HeLa cells silenced by transfection of siRNAs showed barely detectable signal of Hklp2 on the spindle and the chromosomes in mitosis. No major defects in spindle organization and chromosome alignment could be observed in these cells but we found that metaphase spindles were on average 11% shorter than controls (Figure 14B). Although relatively mild, this phenotype was statistically significant ($p < 0.001$, see also Table 1 for spindle length in Appendix) and suggested that Hklp2 could have a role in spindle pole separation during mitosis.

As the Hklp2-silenced cells were still able to assemble functional spindle, we decided to sensitize them by adding Eg5 inhibitor monastrol (Figure 14C). Therefore, cells were transfected with control and Hklp2-specific siRNAs and with 25, 50 and 100 µM monastrol for 5 hours. The cells were then fixed and processed for immunofluorescence analysis. In both control and Hklp2-silenced cells, the inhibition of Eg5 activity by monastrol promoted the formation of monopolar spindles, as expected, in a concentration dependent manner. However, at all monastrol concentrations tested, Hklp2 silencing enhanced the effect of monastrol and more spindles were unable to separate their poles. This enhancement was maximal at 50 µM monastrol with only 5% of bipolar spindles in Hklp2-silenced cells instead of 38% in control cells (average from three independent experiments; $p < 0.001$; see also Table 2 for mono/bipolar

proportions). These results suggested that indeed Hklp2 has a role in spindle bipolarity.

As previously described, the effect of monastrol is reversible. The compound can be washed out and the poles can separate to assemble a bipolar spindle due to the recovery of the activity of Eg5 (Figure 14D). These conditions allow the monitoring of the establishment of spindle bipolarity. Thus, we examined whether Hklp2 participated in the establishment of spindle bipolarity by quantifying the efficiency of spindle pole separation of monopolar structures in control and Hklp2-silenced cells, 10 minutes after monastrol wash-out. If in control cells, 26% of the monopolars had re-established bipolarity, only 9% were able to do so in Hklp2-silenced cells (average from three independent experiments; $p < 0.001$).

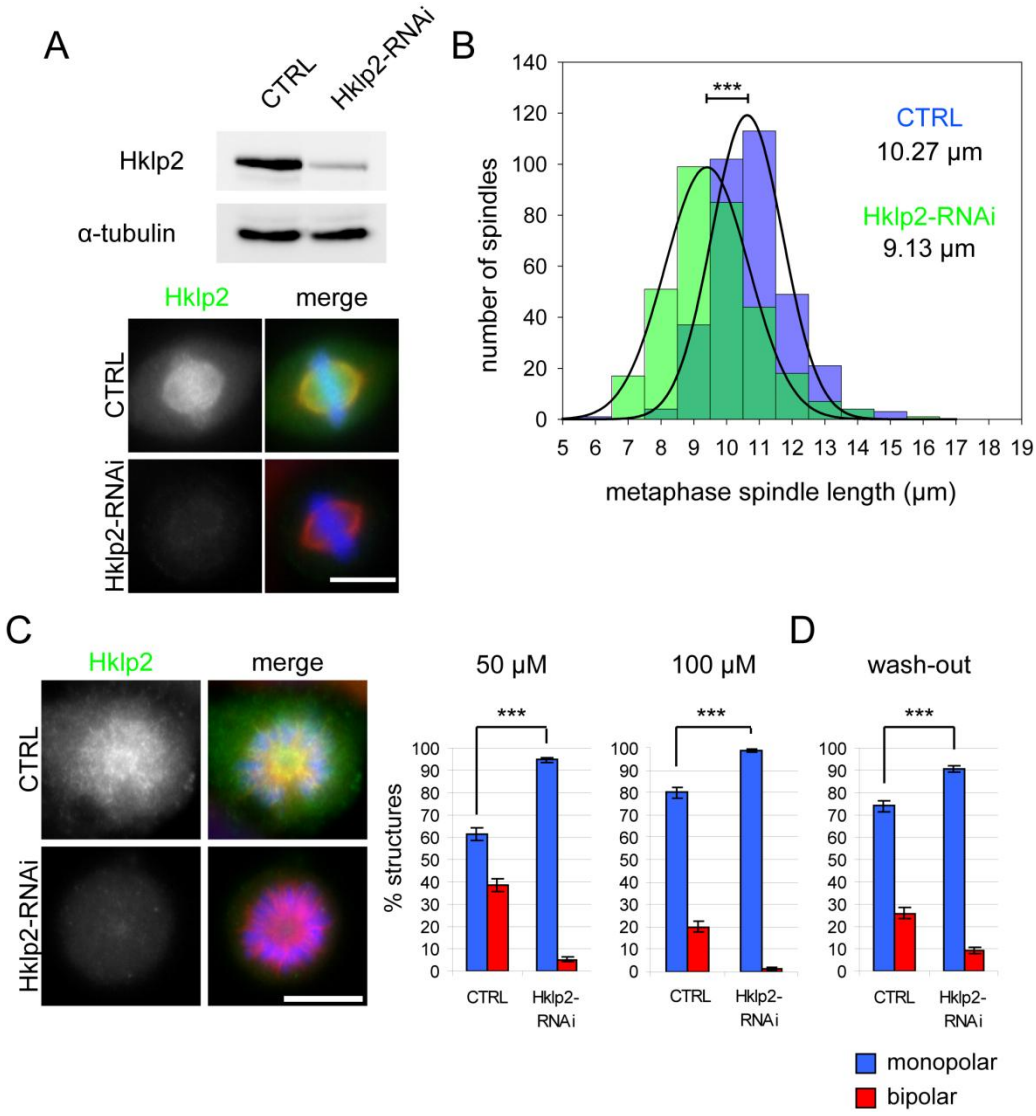
These results suggested that Hklp2 cooperates with Eg5 to promote spindle bipolarity.

Hklp2 is partly responsible for the maintenance of spindle bipolarity in metaphase

The results indicated so far that Hklp2 could have a completely redundant function with Eg5. Alternatively it could have a more distinct function that might be difficult to separate due to the dominant role of Eg5 in the establishment of spindle bipolarity. We previously showed that, in HeLa cells, Eg5 activity is not necessary for the maintenance of spindle bipolarity in metaphase (see Figure 6). Therefore, we examined if Hklp2 was required for metaphase bipolar spindle stability and responsible for the maintenance of bipolarity in absence of Eg5 activity (Figure 15A). Hklp2-silenced cells that were incubated with MG132 also arrested in metaphase with virtually all the spindles in a bipolar organization like in control. However, the addition of

monastrol to the culture media resulted in a dramatic effect on spindle bipolarity with 61% of the spindles collapsing to a monopolar state compared to only 6% in control cells (average from three independent experiments; p value < 0.001). Live cell analysis showed that the bipolar spindles blocked in metaphase were experiencing a sudden collapse upon addition of monastrol without passing through an intermediate length stage (Figure 15B). The results show that both Eg5 and Hklp2 contribute to the stability of the bipolar spindle. When Eg5 does not function, Hklp2 becomes essential and vice-versa.

Figure 14: Hklp2 silencing influences spindle pole separation
A- Western blot of control and Hklp2-specific siRNAs transfected HeLa cells showing a reduction of Hklp2 levels to around 80% in the silenced cells (upper panel). Immunofluorescence on control and Hklp2-specific siRNAs transfected HeLa cells shows that Hklp2 is barely detectable in metaphase in the silenced cells (lower panel). In the overlay, Hklp2 is in green, MTs are in red and DNA in blue.
B- Histogram of metaphase spindle lengths in control (blue) and Hklp2-silenced HeLa cells (green) obtained from > 100 spindles in each of three independent experiments. The best fit Gaussian distribution is shown as a superimposed line. The average of the spindle lengths is shown for each condition. Spindles assembled in Hklp2-silenced cells are on average 11% shorter than controls. (***, $p < 0.001$).
C- Immunofluorescence of HeLa cells transfected with control or Hklp2-specific siRNAs and incubated with 50 µM or 100 µM monastrol (Hklp2, green; MTs, red; DNA, blue). The frequency of bipolar and monopolar spindles found in each condition is shown on the right.
D- Quantification of the proportion of monopolars and reforming bipolar spindles at 10 min. after 100 µM monastrol wash-out.
Data for C and D were obtained from > 100 spindles for each of three independent experiments. Error bars correspond to the standard error done on proportions (***, $p < 0.001$).
(bars, 10 µm)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Hklp2
α-tubulin
Hklp2
merge
CTRL
Hklp2-RNAi
B
***
CTRL
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Hklp2-RNAi
9.13 µm
number of spindles
metaphase spindle length (µm)
C
Hklp2
merge
CTRL
Hklp2-RNAi
D
50 µM
100 µM
wash-out
% structures
CTRL
Hklp2-RNAi
monopolar
bipolar

Hklp2 and Eg5 probably differ in their mechanism of action

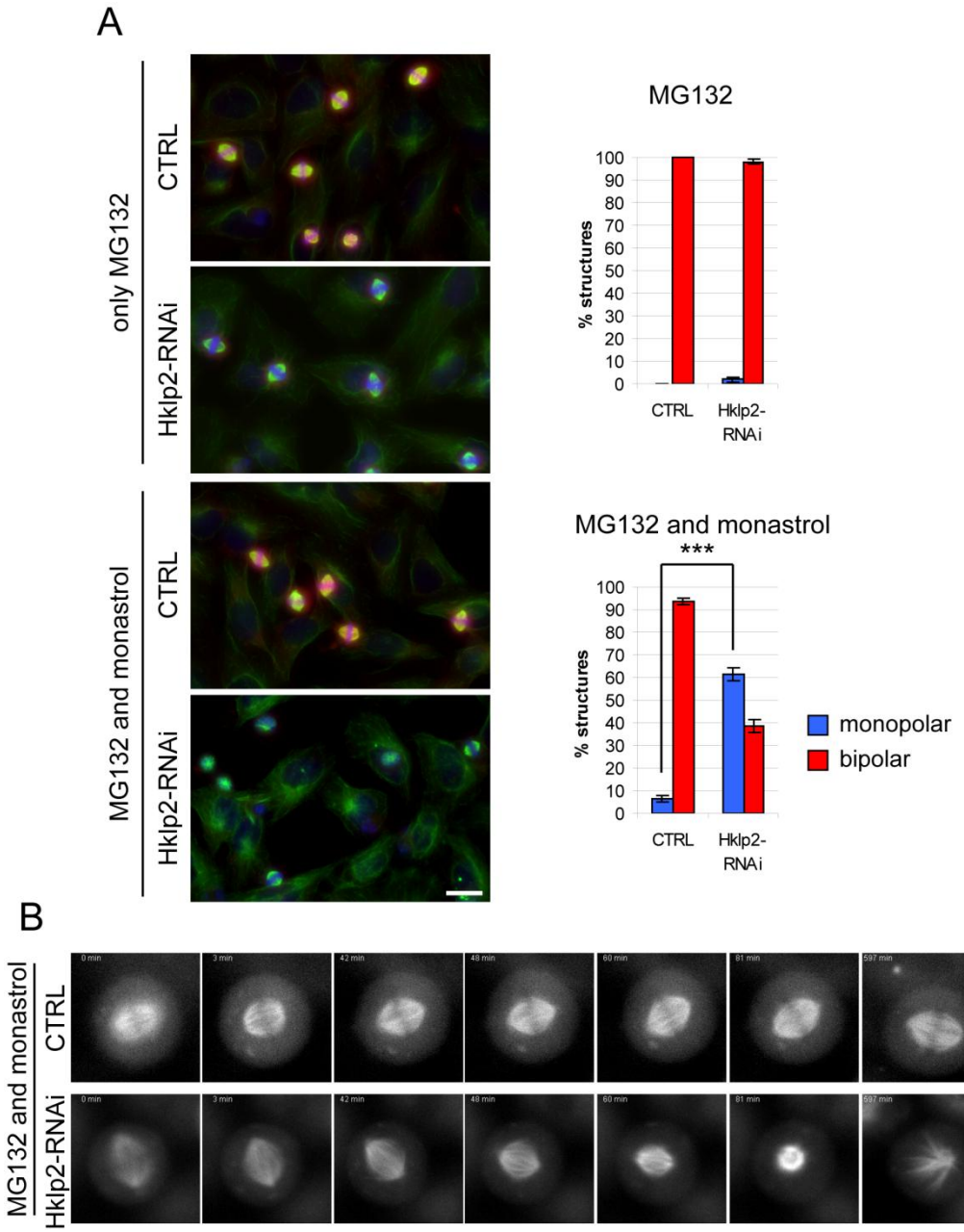
We decided to investigate the mechanism of action of Hklp2 to know if it could act on spindle bipolarity establishment and/or maintenance in a fashion different from Eg5. Hence the respective localizations of Hklp2 and Eg5 during mitosis was closely examined (Figure 16A). Double immunofluorescence analysis showed that Eg5 accumulates around the centrosomes at the onset of mitosis and is therefore positioned at the right place to drive centrosome separation and the initial establishment of spindle bipolarity. In contrast, Hklp2 is recruited to the MTs at a later stage, well after nuclear envelope breakdown, at a time when the spindle is already forming. Consequently, it is unlikely that it participates in the initial steps of spindle pole separation, before nuclear envelope break-down, suggesting that its role could be performed later. Also, at metaphase, the localizations of Eg5 and Hklp2 are also clearly distinct: Eg5 localizes to the spindle MTs with a clear enrichment towards the spindle poles, whereas Hklp2 is comparatively more prominently associated to the central part of the spindle and the chromosomes. Similarly, in cold-treated cells, Eg5 is seen close to the poles on the remaining MT bundles of the kinetochore fibers, whereas Hklp2 is more enriched towards the chromosomes (Figure 16B). These observations suggest that their main site of action is different.

Figure 15: Hklp2 is required for the maintenance of spindle bipolarity

A- Left: Control and Hklp2-silenced HeLa cells incubated with MG132 for 4 hours or 2 hours with MG132 followed by an additional 2 hours with 100 µM monastrol added. Immunofluorescence images of Hela cells treated with these conditions with Hklp2 (red), MTs (green) and DNA (blue).

Right: Quantification of the bipolar and monopolar spindles. Hklp2 silencing leads to the destabilization of the bipolar metaphase spindles. The data were obtained from > 100 spindles from each of three independent experiments. Error bars correspond to the standard error done on proportions. (***, $p < 0.001$).(bar, 20 µm)

B- Still images from a movie depicting control and Hklp2-silenced HeLa cell stably expressing RED-tubulin. The cells were treated as in A. Recording started upon addition of monastrol (time 0). Collapse of the poles in Hklp2-silenced cells started about 40 minutes after monastrol addition. The spindle goes from bipolar to monopolar without staying in an intermediated length.


A
only MG132
CTRL
Hklp2-RNAi
MG132 and monastrol
CTRL
Hklp2-RNAi
MG132
% structures
100
90
80
70
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50
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30
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10
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CTRL
Hklp2-RNAi
MG132 and monastrol
***
% structures
100
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40
30
20
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CTRL
Hklp2-RNAi
monopolar
bipolar
B
MG132 and monastrol
CTRL
Hklp2-RNAi
0 min
3 min
42 min
48 min
60 min
81 min
597 min

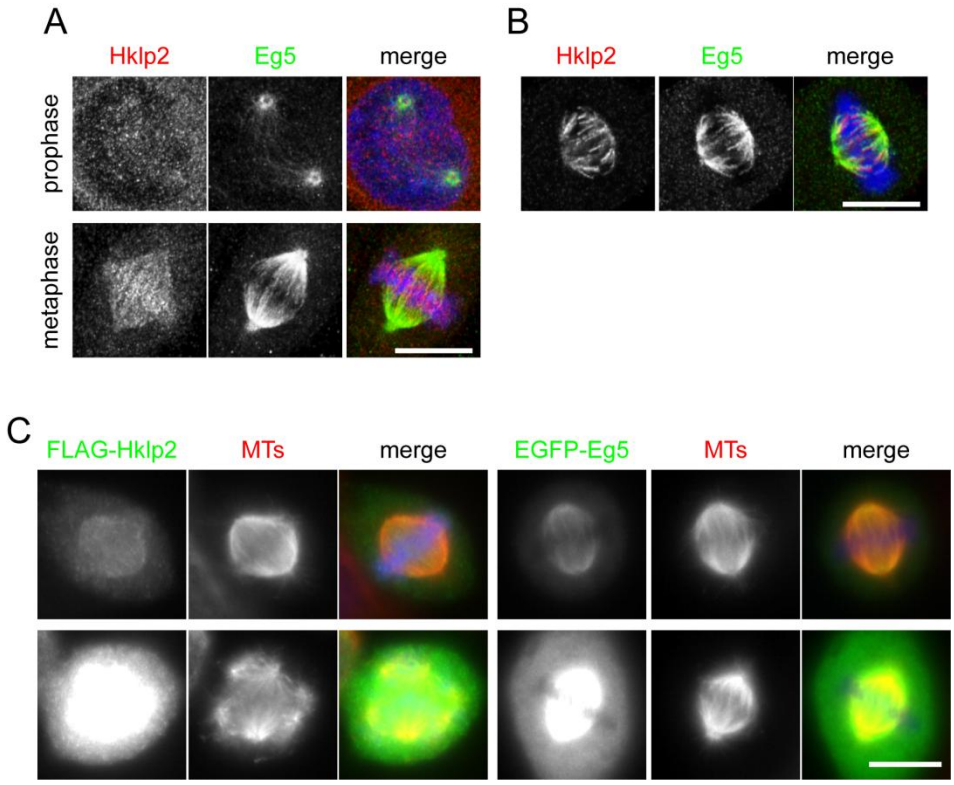


Figure 16: Hklp2 and Eg5 may have different mechanisms of action
A- Immunofluorescence images of HeLa cells in prophase and metaphase showing the respective localizations of Hklp2 (red) and Eg5 (green). Eg5 is present at the centrosomes before nuclear envelope break-down while Hklp2 is still diffuse in the cytoplasm. At metaphase Eg5 is concentrated on the MTs near the poles while Hklp2 is more proximal to the central part of the spindle and on the chromosomes.
B- Images of HeLa cells treated with cold to depolymerize the more dynamic spindle MTs and processed by immunofluorescence with the anti-Hklp2 (red), and anti-Eg5 (green) antibodies. Eg5 and Hklp2 localize to the stable MT bundles, presumably the K-fibers, but Hklp2 is more present close to the chromosomes while Eg5 is strongly enriched at the poles.
Images in A and B correspond to maximum projections of confocal sections acquired every 0.3 µm.
C- HeLa cells overexpressing FLAG-Hklp2 or EGFP-Eg5 were immunostained with anti-FLAG (green), anti-tubulin (red) antibodies. Cells expressing low amounts of any of the two kinesins form a bipolar spindle. High expression of EGFP-Eg5 does not interfere with bipolar spindle assembly whereas the high expression of FLAG-Hklp2 impairs the formation of the bipolar spindle and promotes highly aberrant MT assemblies.
In all cases DNA (blue) was marked with DAPI. (bars, 10 µm)

The overexpression of Eg5 or Hklp2 in HeLa cells has dramatically different consequences on bipolar spindle assembly (Figure 16C). Indeed, when overexpressed to really high levels, massive amounts of EGFP-Eg5 localized predominantly close to the poles on the spindle MTs and, remarkably, did not interfere with bipolar spindle formation. The spindle conserved a perfectly normal shape. On the contrary, overexpression of high levels of FLAG-Hklp2 induced very strong mitotic abnormalities including multipolar spindles and completely disorganized MT structures.

Altogether, these results strongly suggest that Eg5 and Hklp2 both contribute to the stability of the bipolar spindle through different mechanisms that may involve distinct spatial and temporal modes of action.

Different pools of Hklp2 seemingly play opposite roles in spindle pole separation

One of the main characteristic of Hklp2 and difference with Eg5 is its Ki-67 dependent localization to the chromosomes. We decided to investigate this particular feature of Hklp2 to determine how it contributes to Hklp2 function in spindle assembly and stability.

Ki67 was silenced in HeLa cells by siRNA transfection (Figure 17A). Immunofluorescence analysis showed that the spindles assembled normally in these cells but were on average 12% longer than in controls (average from three independent experiments; $p < 0.001$), suggesting that pushing forces are more efficient when Hklp2 does not interact with the chromosomes (Figure 17B). We next examined the consequences of inhibiting Eg5 with monastrol together with the silencing of Ki67 (Figure 17C). Immunofluorescence analysis showed a certain proportion of monopolar structures with Hklp2 absent from the

chromosomal area and more concentrated at the center. However, some spindles were able to maintain a bipolar configuration although the activity of Eg5 was abolished. Quantification of the structures showed that, in the absence of Ki67, a higher proportion of bipolar spindles formed compared to control (44% instead of 16% in control cells, average of three independent experiments; $p < 0.001$). Furthermore, monitoring the recovery of spindle bipolarity of the monopolar structures after monastrol wash-out showed that Ki67-silenced cells were more efficient in separating the spindle poles than control cells (Figure 17D)(73% of bipolars in Ki67-silenced cells compared to only 28% in control cells; average of three independent experiments; $p < 0.001$). We also noticed that, in the Ki67-silenced cells, Hklp2 was strongly enriched on the interpolar MTs of the re-forming spindles.

Figure 17: Hklp2 chromosomal localization through Ki67 is involved in bipolar spindle dynamics

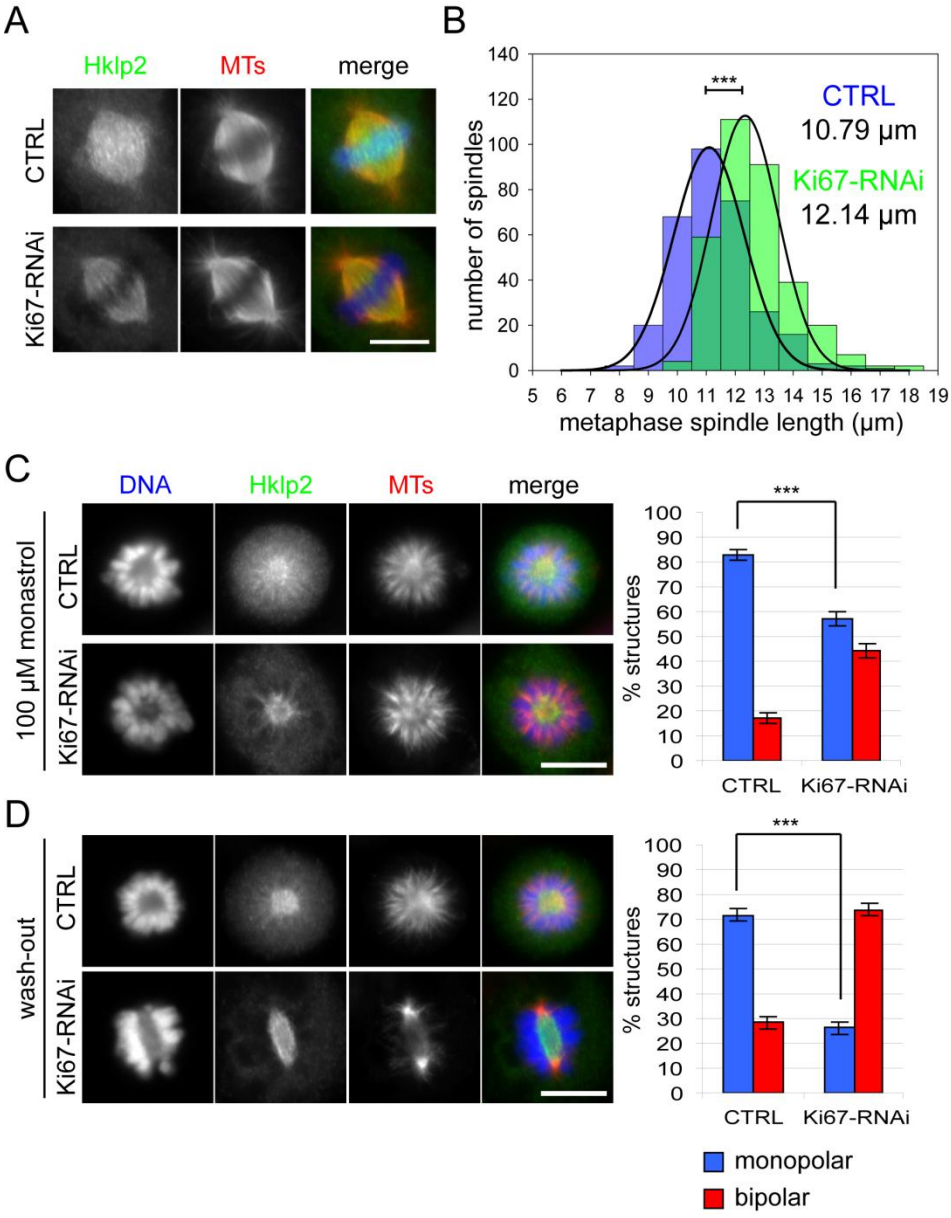
A- Immunofluorescence shows that Hklp2 fails to localize to the chromosomes in the absence of Ki67. In the overlay MTs are in red, Hklp2 is in green and DNA in blue.

B- Histogram of the metaphase spindle lengths in control (blue bars) and Ki67-silenced HeLa cells (green bars) obtained from > 100 spindles for each of three independent experiments. The best fit Gaussian distribution is shown as a superimposed line. The average of the spindle lengths is shown for each condition. Spindles assembled in Ki67-silenced cells are on average 12% longer than controls. (***, $p < 0.001$)

C- Control and Ki67-silenced cells incubated with 100 µM monastrol. In the overlay MTs are in red, Hklp2 is in green and DNA in blue. Note that in the absence of Ki67, Hklp2 is not any more associated with the chromosomes and appears to concentrate on the MTs at the center of the monoaster. The quantification is shown on the right.

D- Immunofluoresecence on HeLa cells transfected with control or specific Ki67 siRNAs treated as in C and further incubated in monastrol-free medium for 10 min. Note that in control cells, Hklp2 is strongly recruited to the MTs close to the center of the monoaster. The corresponding quantification shown on the right.The data used for C and D were obtained from > 100 spindles for each of three independent experiments. Error bars correspond to the standard error done on proportions (***, $p < 0.001$).

(bars, 10 µm)


A
Hklp2
MTs
merge
CTRL
Ki67-RNAi
B
number of spindles
metaphase spindle length (µm)
***
CTRL
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Ki67-RNAi
12.14 µm
C
DNA
Hklp2
MTs
merge
100 µM monastrol
CTRL
Ki67-RNAi
% structures
***
CTRL
Ki67-RNAi
D
wash-out
CTRL
Ki67-RNAi
% structures
***
CTRL
Ki67-RNAi
monopolar
bipolar

These results show that Ki67 plays a role in the regulation or establishment of spindle bipolarity and that it most likely exerts this role through the regulation of the steady-state distribution of Hklp2 between the MTs and the chromosomes.

The absence of Hklp2 on the chromosomes is responsible for the effects on spindle bipolarity in Ki67-silenced cells

In order to prove that the effects of Ki67-silencing on spindle bipolarity were directly due to the modified localization of Hklp2 and not other factors, we simultaneously silenced Hklp2 and Ki67 by co-transfecting the specific siRNAs (Figure 18A). Spindle length was measured in these cells and was on average 11% shorter than in controls (average from three independent experiments; $p < 0.001$), which is comparable to the data obtained in Hklp2-silenced cells. Silencing of both Ki67 and Hklp2 and incubating the cells with 100 μM monastrol did not increase the proportion of bipolar spindles compare to control, in contrast to what was observed in Ki67-RNAi conditions (Figure 18B). Rather the contrary, co-silenced cells had less bipolar than control cells (12% instead of 18%; average of three independent experiments; $p < 0.05$). Consistently, monastrol wash-out did not show a higher efficiency of spindle pole separation as the co-silenced cells only showed 22% of bipolars instead of 31% in control cells (Figure 18C)(average from 3 independent experiments; $p < 0.01$).

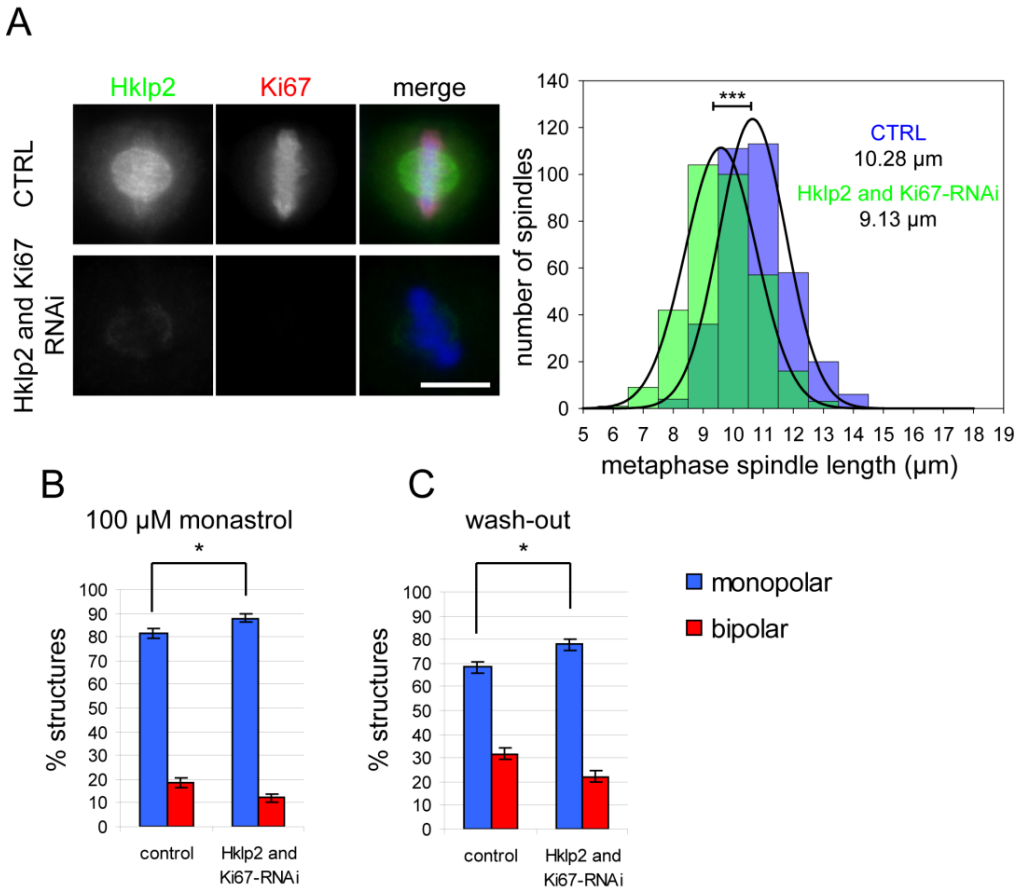


Figure 18: Hklp2 absence at the chromosomes is responsible for the spindle phenotypes observed in Ki67-silenced cells

A- Immunofluorescence images of metaphase HeLa cells co-transfected with control or both Ki67 and Hklp2 specific siRNAs (Hklp2, green; Ki67, red; DNA, blue). (bar, 10 µm)

Histogram of metaphase spindle lengths in control (blue bars) and co-silenced HeLa cells (green bars) obtained from > 100 spindles for each of three independent experiments. The best fit Gaussian distribution is shown as a superimposed line. The averages of the spindle length are shown for each condition. Spindles assembled in co-silenced cells are on average 11% shorter than controls (***, $p < 0.001$).

B- Frequency of monopolar and bipolar spindles in control and Ki67/Hklp2 co-silenced HeLa cells incubated with 100 µM monastrol. The data correspond to > 100 spindles for each of three independent experiments. Error bars correspond to the standard error done on proportions (*, $p < 0.05$).

C- Quantification of the monopolar and reforming bipolar spindles in HeLa cells treated as in B and further incubated in monastrol-free medium for 10 min. The data correspond to > 100 spindles for each of three independent experiments. Error bars correspond to the standard error done on proportions (*, $p < 0.01$).

Overall, these results show that the effect on spindle bipolarity observed in Ki67-silenced are dependent on the presence of Hklp2 and therefore the consequence of its altered steady-state localization. They suggest verystrongly that the lack of Hklp2 localization to the chromosomes and possibly its enrichment to the MTs reinforces spindle bipolarity.

Role of the different domains of Hklp2 in its localization and function

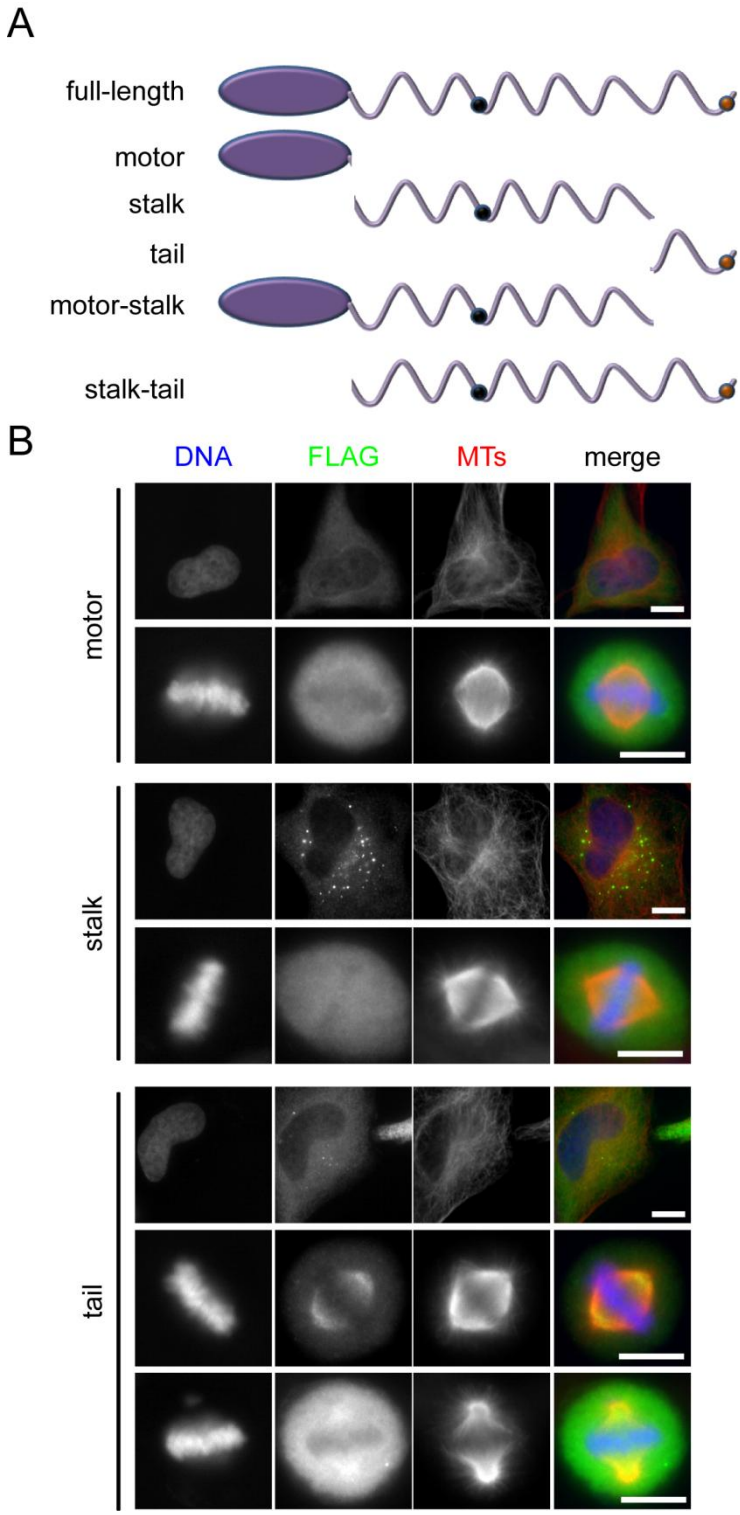
To get further insight into the mechanism of action of Hklp2, different constructs were prepared to express different regions of the protein with a N-terminal FLAG tag after transfection in cells (Figure 19A).

The first 392 amino acids are coding for the globular motor domain (Sueishi 2000). From amino acid 392 to 1138, this region called stalk consist of a long coiled-coil domain and contains a putative phosphorylation site by cdk1. The last region is called tail and consists of the amino acids from 1139 to 1388, predicted to be a coiled-coil (in contrast with other kinesins for which this domain is globular) with a Leucine zipper at the end. The leucine zipper at the C-terminal is also present in the Xenopus homologue of Hklp2 and was shown to be necessary for the dimerization of the kinesin as well as its TPX2-dependent targeting to the microtubules (Wittmann et al., 1998).

The different fragments were exogenously expressed in HeLa cells and the assembled spindles were analysed by immunofluorescence. The motor domain alone or the stalk region alone did not localize anywhere in the cell (Figure 19B). The tail region remained diffuse in the cytoplasm in interphase but localized to the spindle poles in mitosis. When overexpressed to high levels, only a limited amount of the protein localized to the poles, the rest remaining diffuse in the cytoplasm. The shape of the poles looked modified, appearing

more tight but the spindles were still bipolar. These spindles also showed moderate misalignment of chromosomes. Thus, the tail of Hklp2 has the ability to localize, during mitosis, to the MTs close to their minus-ends whether through a direct or indirect binding. Similarly, the region encompassing both stalk and tail, which consists of the whole coiled-coil region, was recruited to the poles but in a more efficient way than the tail alone. When high level of overexpression was attained, the stalk-tail almost entirely localized to the poles. Furthermore, this fragment localized to microtubules in interphase. This suggests that the last region of the coiled-coil (tail) has the capacity to bind directly or indirectly microtubules and that including the rest of the coiled-coil (stalk) increases this capacity. Overexpression of the stalk-tail generated aberrant fragmented spindle poles but did not have any impact on pole separation. The motor-stalk fragment was not observed on any subcellular structure during interphase. In mitosis, it localized to the chromosomes, thus mimicking the localization of a particular pool of endogenous Hklp2. This fragment had a dominant negative effect on spindle assembly interfering with pole separation. Cells expressing it and synchronized in metaphase using MG132 had 30% of mitotic structures in monopolar configuration. This result suggests that the pool of Hklp2 localized to the chromosomes oppose the pushing forces that separate the poles, in line with the conclusion drawn from the Ki67 silencing experiments.

Figure 19(next two pages): Localization of Hkl2p domains
A- Cartoon depicting different fragments of Hklp2 corresponding to the different regions of the kinesin cloned in a FLAG vector.
B- HeLa cells were transfected with constructs coding for Hklp2 motor, stalk, tail, motor-stalk and stalk-tail. The cells were then processed for immunofluorescence with anti-FLAG (green), anti-tubulin (red) and DNA was marked with DAPI (blue). The motor and the stalk do not localize to any subcellular structure while the tail is targeted to the spindle poles.
The fragment stalk-tail is targeted to the microtubules in interphase and to the spindle MTs in mitosis where it interferes with the pole integrity. The motor-stalk fragment is targeted to the chromosomes in mitosis and can prevent correct separation of the spindle poles. Quantification of the phenotypes generated by the motor-stalk shown on the right. (bars, 10 μm)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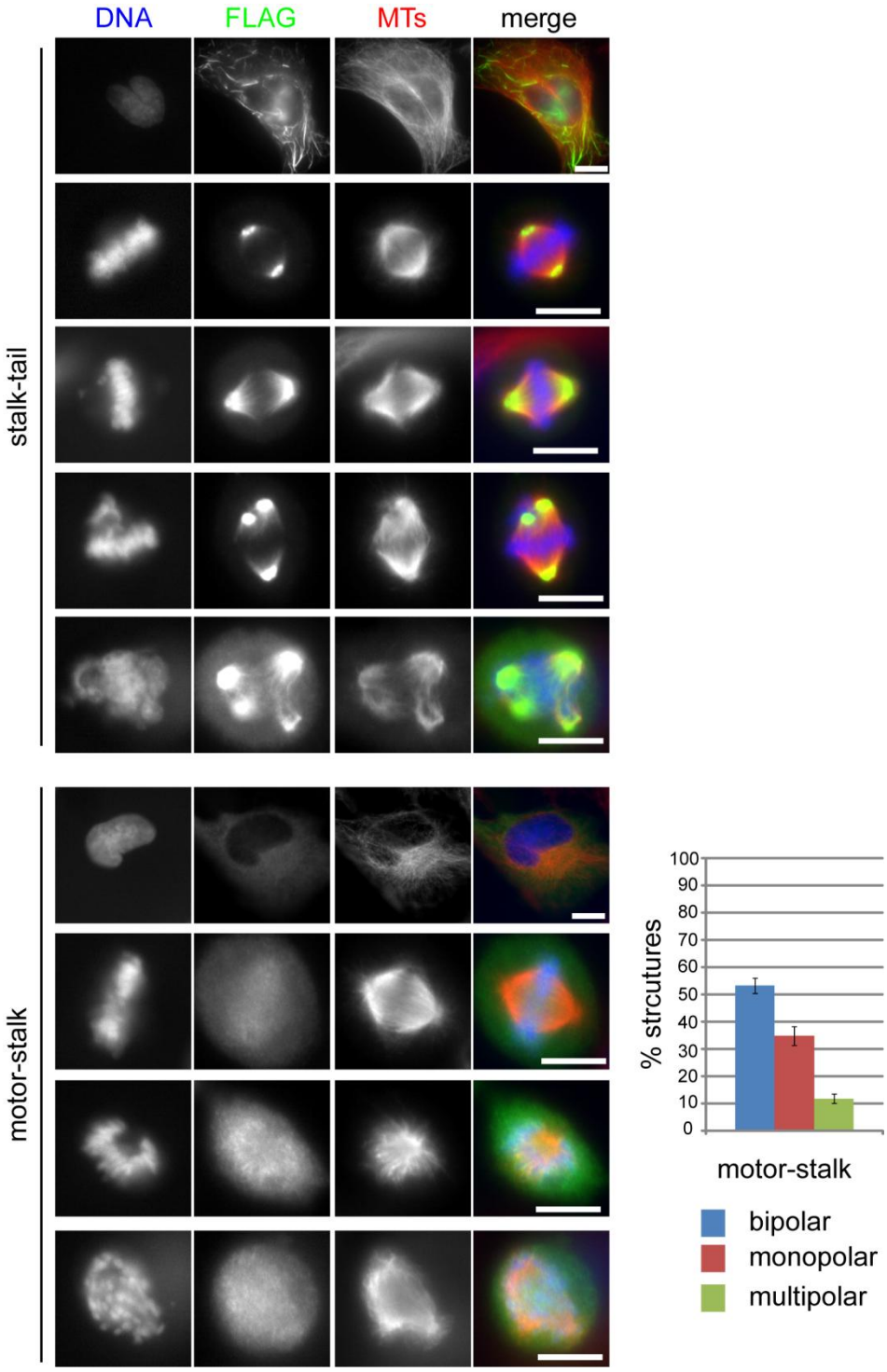
stalk
tail
motor-stalk
stalk-tail
B
DNA
FLAG
MTs
merge
motor
stalk
tail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FLAG
MTs
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90
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50
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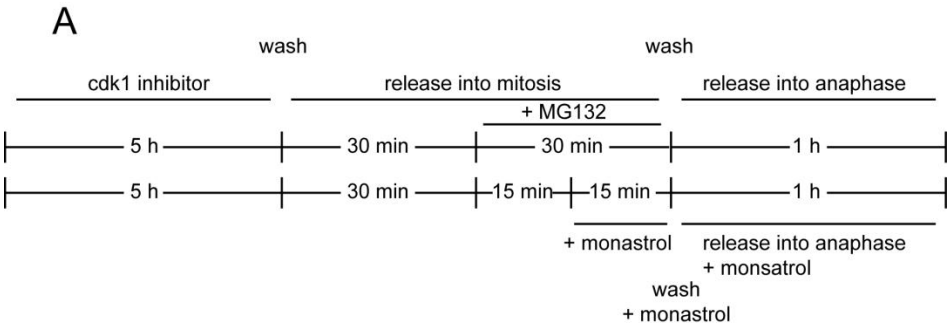
30
20
10
0
motor-stalk
bipolar
monopolar
multipolar

Pushing forces are required to maintain bipolarity at metaphase-anaphase transition

Our results showed that the bipolarity of a metaphase spindle does not depend on Eg5 or Hklp2 individually but is abolished if the two kinesins are impaired at the same time (see Figure 15). The dramatic change of shape and dynamics that happens to the spindle when it extends to separate the chromatids in anaphase, raised the question of the possible involvement of these kinesins at the crucial metaphase-anaphase transition. To explore this issue, cells were first blocked in metaphase using MG132 and then released into anaphase by washing-out the compound (Figure 20A). After 1 hour of release, 52% of the mitotic cells were in anaphase or telophase. To determine whether Eg5 plays a role in this process, cells were blocked in MG132, and subsequently incubated in monastrol in the presence of MG132. MG132 was then washed-out while keeping monastrol in the medium. In these conditions, most cells did not go into anaphase (only 8% of ana/telophase).

Figure 20: Eg5 is essential for spindle bipolarity at the metaphase-anaphase transition.
A- The different steps of the experiment are detailed at the top. Immunofluorescence on the cells treated as explained and fixed one hour after release in anaphase with or without monastrol was performed using anti-tubulin (green), anti-Hklp2 (red) and. On the right is shown the proportion of spindles in monopolar, bipolar and in ana/telophase. The absence of Eg5 activity once the cells have to go into anaphase not only prevents anaphase but collapses the poles to a monopolar structure. The data correspond to > 100 spindles for each of three independent experiments. (bar, 25 μm)
B- Immunofluorescence images of HeLa cells in metaphase and later stages of mitosis. Antibody used are anti-Hklp2 (red), anti-Eg5 (green). Part of Hklp2 localizes to spindle midzone early in anaphase before diffusing while Eg5 stays first close to the poles before going to the midzone. (bar, 10 μm)
In all cases the DNA was marked with DAPI (blue)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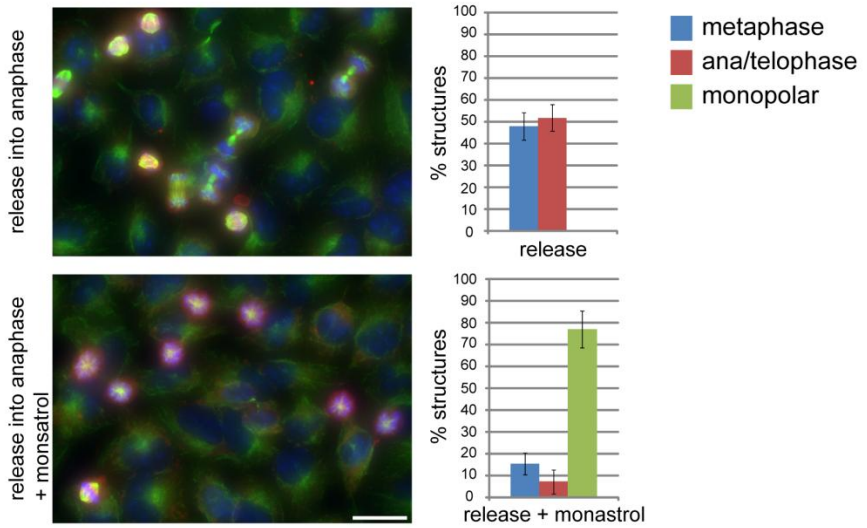
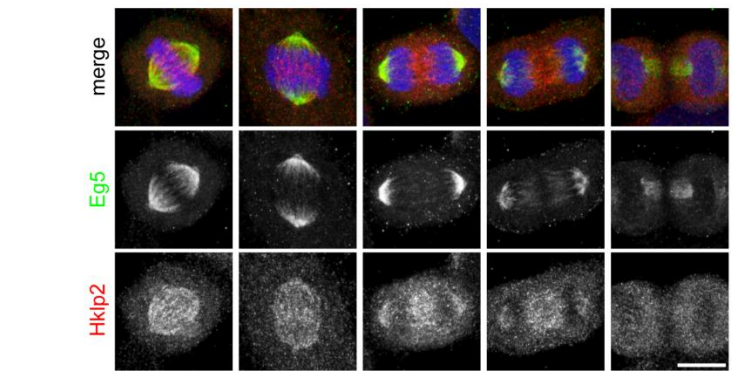
wash
wash
cdk1 inhibitor
release into mitosis
release into anaphase
+ MG132
5 h
30 min
30 min
1 h
5 h
30 min
15 min
15 min
1 h
+ monastrol
release into anaphase
+ monsatrol
wash
+ monastrol


release into anaphase
release into anaphase
+ monsatrol
% structures
release
release + monastrol
metaphase
ana/telophase
monopolar


B
merge
Eg5
Hklp2

Moreover many bipolar spindles collapsed into a monopolar structures (78% of the spindles). These results indicate that although the activity of Eg5 is not essential to maintain spindle bipolarity in metaphase, it becomes absolutely necessary for the transition from metaphase to anaphase. The comparative localization of Eg5 and Hklp2 during the metaphase-anaphase transition and later stages, showed that Eg5 stays close to the poles at the beginning of anaphase and later is also observed on the bundles of the spindle mid-zone before detaching from the microtubules to regain a diffuse pattern in late telophase (Figure 20B). In contrast, Hklp2 signal gets more diffuse at the beginning of anaphase although the protein seems to be enriched in the central region of the spindle as the chromatids are separated and move toward the poles. Experiments involving metaphase block and release will be performed in the future, in Hklp2 and Ki67-silenced cells, to examine the hypothesis of a possible role of Hklp2 in anaphase.

DISCUSSION

Kinesins in spindle assembly

The mitotic spindle is a superstructure characterized by a constant instability of its main component, the microtubules, compensated by the action of other proteins to reach a steady-state (Karsenti and Vernos, 2001). These proteins control the dynamic properties of the MTs and their spatial arrangement to build a bipolar spindle. Bipolarity is a crucial feature of the spindle as it has to segregate the chromosomes into two different sets for the two nascent daughter cells. On one hand, the inability to separate the centrosomes blocks the cells in mitosis and further causes apoptosis (Tao et al., 2005). On the other hand, and maybe even more important, supernumerary spindle poles can cause incorrect segregation, possibly leading to aneuploidy, an event that can potentially cause the death of the nascent cells or contribute to the development of a tumor (Nigg, 2002). To achieve establishment and maintenance of bipolarity, the forces generated by friction, elasticity, MT dynamics and molecular motors have to reach a balance (Dumont and Mitchison, 2009). The forces generated in the spindle by the motors (dynein and kinesins) are the ones most studied (Heald and Walczak, 1999; Sharp et al., 2000).

The aim of this thesis was to study the role of molecular motors in the establishment and maintenance of spindle bipolarity. Although the work from several labs supported a major role for the plus-end homotetrameric motor Eg5, some questions were remaining about the molecular mechanism underlying spindle bipolarity. To start, we examined some intriguing facts about Eg5 role in spindle assembly. The main focus of this work was then the characterization of a new factor involved in bipolarity, Hklp2.

Eg5 role in bipolarity and questions

In most models depicted in the literature (Ferenz et al., 2009; Gadde and Heald, 2004; Goshima et al., 2005; Kollu et al., 2009; Sharp et al., 2000), Eg5 is represented at the center of the spindle where it binds antiparallel MTs and pushes them apart. We showed here that Eg5 concentrates around the centrosomes when prophase starts while the nuclear envelope is still intact. At metaphase, Eg5 localizes to the MTs and concentrates near the poles, a location where in principle there are few antiparallel MTs, although this issue is still in debate (Mastronarde et al., 1993). So it is not clear to what kind of orientation of MTs Eg5 is bound at metaphase and consequently what kind of forces it can produce. In vitro, Eg5 can bind to both configurations of MTs, parallel and antiparallel, with a preference for antiparallel (Kapitein et al., 2005; van den Wildenberg et al., 2008).

Moreover, our results show that increasing amounts of an exogenously expressed EGFP-Eg5 on the spindle MTs does not interfere with bipolar spindle assembly. This does not fit easily with the balance of forces model. If the spindle end up in a monopolar configuration when impairing Eg5 (an outward force) and bipolarity can be restored by impairing dynein (an inward force) at the same time, then how can an excess of Eg5 have no effect? It could be explained by the displacement of another factor important for bipolarity due to the overloading of the MTs. In this case the spindle would gain outward pushing forces from supplementary Eg5 while losing at the same time another outward pushing factor that would have to be determined. Different proteins localized close to the poles could be candidates: Kif2a, a depolymerizing kinesin present at the poles, NEDD1, a structural protein localized to the centrosomes. These proteins somehow contributes to bipolarity as their absence causes monopolarity (Ganem and Compton, 2004; Haren et al., 2006). We

currently have not checked if Hklp2 is displaced from the spindle MTs when Eg5 is overexpressed. Another hypothesis could be that after a certain distance has been achieved between the poles, Eg5 activity is ineffective due to a difference in MT architecture and the lack of antiparallel MTs as suggested recently by Kollu et al (Kollu et al., 2009). Consequently, Eg5 would not generate anymore pushing forces in metaphase. In this is true, overexpression of Eg5 could speed up the initial separation of the centrosomes but would not increase the length of the spindle. In fact, the experiments performed with MG132 block and addition of monastrol showed that the inhibition of Eg5 in metaphase does not compromise spindle bipolarity. This strongly suggests that, at metaphase, Eg5 does not generate outward pushing forces anymore. It also suggests that the establishment and the subsequent maintenance of spindle bipolarity are two steps that may require different activities.

The complexity of the mechanism involved in spindle bipolarity establishment and maintenance calls for additional investigations on Eg5 and the identification of other factors involved in order to build a comprehensive model for spindle assembly.

Hklp2 as a bipolarity factor

Large and medium scale RNAi approaches have successfully identified a number of motors involved in centrosome separation, bipolar spindle assembly, chromosome movements and cytokinesis (Goshima and Vale, 2003; Goshima et al., 2007; Kittler et al., 2007; Neumann et al., ; Zhu et al., 2005). However, none of these approaches had so far revealed a function for Hklp2 during mitosis in human cells.

We decided to characterize Hklp2 and its function based on results obtained by the lab on its Xenopus homologue that indicated a possible involvement in

bipolarity establishment. Moreover, data obtained in other organisms (sea urchin, C. elegans) provided additional evidences. Recently, a screen performed on all human motors confirmed Hklp2 (or Kif15) as the major factor contributing with Eg5 to bipolarity maintenance and additional results allowed to propose a model of action (Tanenbaum et al., 2009). These results came out at the same time as ours, and although most of the conclusions are in agreement with ours, there are some differences that will be discussed here.

Under standard culture conditions, Hklp2 RNAi results in a small reduction of spindle size, a mild but statistically significant phenotype that would be missed in the context of a large screen. It has to be noted that this small reduction is comparable to the one observed when the minus-end directed motor HSET is silenced (Cai et al., 2009), reinforcing the idea that the spindle size is quite stable with no intermediates but a switch between two stable states: a monopolar and a bipolar (Goshima et al., 2005; Mitchison et al., 2005).

Results obtained from experiments involving monastrol treatment and subsequent wash-out suggest that Hklp2 is helping Eg5 to separate the poles and establish spindle bipolarity, maybe providing supplementary organization of the MTs that would increase the efficiency of the outward pushing force of Eg5. Recent results using HeLa cells showed that half the cells starts the prophase with separated centrosomes and assembles the spindle from this already bipolar situation while the other half starts with unseparated centrosomes and spindle bipolarity is established later through a different mechanism (Toso et al., 2009). Hklp2 activity could be particularly relevant in the later case.

Experiments performed on cells blocked in metaphase with MG132 show that the spindle bipolarity is more stable during this phase and does not require the activities of both Eg5 and Hklp2 but if the two activities are absent, the poles

collapse and the spindle switches to the monopolar state. This result confirms the crucial role of Hklp2 in maintaining bipolarity during metaphase.

In the situation of a loss of function of both Eg5 (outward force) and dynein (inward force), remaining forces are able to establish spindle bipolarity and maintain it (Ferenz et al., 2009; Tanenbaum et al., 2008). Our results indicate that Hklp2 could be one of these forces and future experiments with Hklp2 silencing should allow us to shed some light on that matter.

How could Hklp2 funtion?

To establish a model for Hklp2 mechanism of action, numerous data on its localization, the type of MTs it binds, the proteins it interacts with and its regulation in time and space are needed. Hklp2 has a very different pattern of localization from Eg5. Hklp2 is not present on the MTs before nuclear-envelope breakdown and, because of that, cannot be involved in the first steps of centrosome separation. Interestingly, later in mitosis, it is Hklp2 that is localized to the part of the spindle where most antiparallel MTs are, at the middle, and not Eg5. Hklp2 is likely to be a dimer by homology to what has been demonstrated for Xklp2 (Boleti et al., 1996). Hklp2 could bind two antiparallel MTs if its C-terminal had a direct or indirect interaction with them. As the motor domain has a plus-end directed activity, it could sort the MTs and in consequence help to separate the poles. It could also act as a cross-linker by binding two parallel MTs and, giving some cohesion to the whole array, helping Eg5 to generate pushing forces. A putative MT cross-linking activity from Hklp2 could act as a positive regulator of Eg5, favoring its interaction with two MTs and therefore activating its motor activity (Kapitein et al., 2008).

In the research article recently published by Tanenbaum et al, experiments show that the overexpression of the full length Hklp2 in human cells allows the

assembly of phenotypically normal and functionally capable bipolar spindles when Eg5 is impaired (Tanenbaum et al., 2009). This result could suggest that Hklp2 is functionally redundant to Eg5 and have the same mechanism of action. However, we found that overexpression of Hklp2 in cells induces the assembly of abnormal mitotic structures in contrast to the lack of effect of Eg5 overexpression. Therefore it is likely that Hklp2 may have some redundancy with Eg5 but it certainly has a specific role and its own mechanism of action.

About Hklp2 localization

The exogenous expression of fragments of Hklp2 and the observation of the localization and its consequence on spindle assembly has given some clues about its mode of action. Hklp2, like Eg5 (Blangy et al., 1995), is present in the cytoplasm during interphase with no particular localization to any subcellular structure. What is a 160 kDa protein doing in the cytoplasm? Expressed individually, none of the individual domains localizes to the MTs. The fact that the motor domain does not visibly localize to the MTs comes probably from poor dimerization, a low affinity for MTs and the presence of hydrolysable ATP. The fragment stalk-tail can localize to the MTs in interphase and mitosis. We also showed that the tail alone can bind to MTs only in mitosis while the stalk does not in any situation. This means that there is an intrinsic capacity to bind MTs in the tail, whether directly or indirectly, and that this ability is enhanced when the stalk is also present. It could be also that a longer coiled-coil (stalk-tail instead of tail) is more stable. Since full-length protein does not bind the MTs in interphase, it suggests that the protein undergoes a conformation change (like kinesin-1 (Hackney, 1995)) or is inhibited by another protein.

The expression of a motor-tail fusion could potentially localize only to the MTs but not on the chromosomes and give additional information on the role of Hklp2 at this location in the spindle.

Influence of TPX2 and Ki67 on localization and function

Full-length Hklp2 localizes to the spindle MTs after nuclear-envelope break-down. We showed that this was dependent on TPX2, a factor that is constricted to the nucleus in interphase. So it is only when TPX2 is available that the full length Hklp2 localizes to the MTs (Figure 21). Does this change in Hklp2 ability to bind MTs involve a direct interaction with TPX2 or a more complex mechanism? Our immunofluorescence analysis showed that TPX2 and Hklp2 do not completely colocalize in the spindle in any stage of mitosis. Furthermore, all the immunoprecipitation approaches tried in human cells failed to show an interaction between the two proteins. It is also noteworthy that previous attempts performed in the lab in the Xenopus system to study an interaction between the two homologues turned to be unsuccessful (data not shown). Considering all these different results, it seems very unlikely that the two proteins form a 1:1 stable complex (with TPX2 binding a MT and the motor domain of Hklp2 bound to another one) as proposed by Tanenbaum et al (Tanenbaum et al., 2009). It is important to note that the coimmunoprecipitation results shown by Tanenbaum et al were obtained by overexpressing both EGFP-Hklp2 and TPX2 in cells.

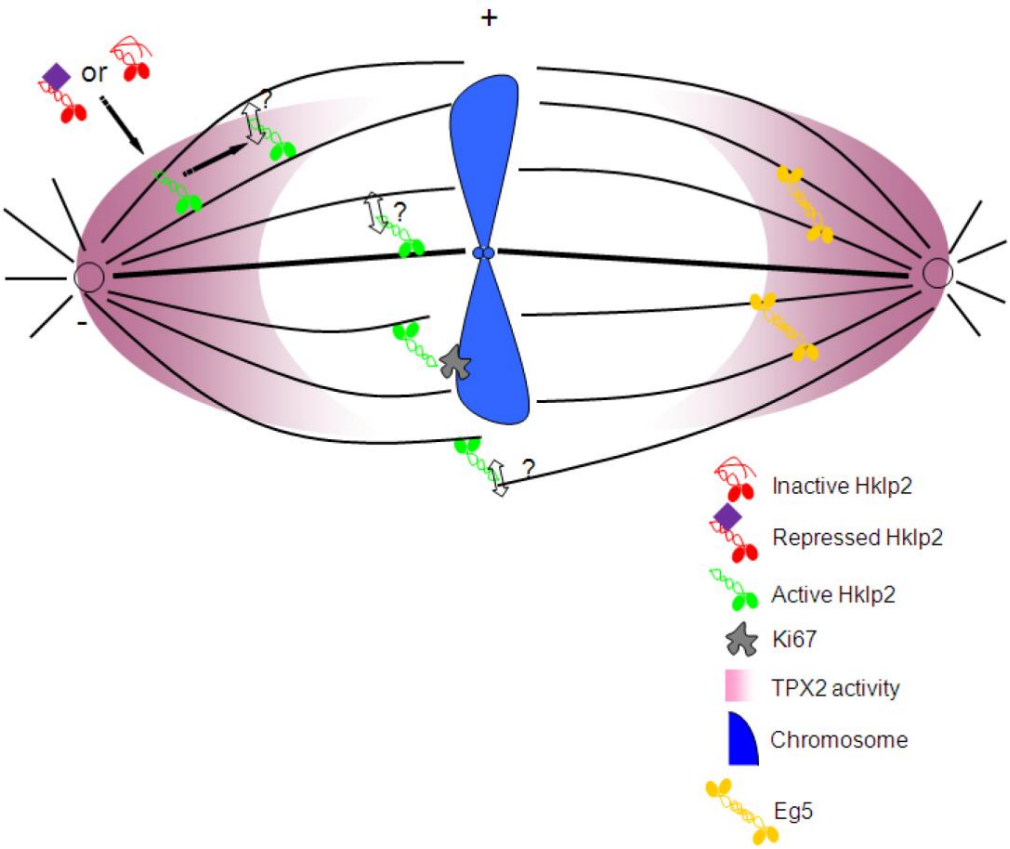


Figure 22: Model for Hklp2 role in spindle bipolarity
Hklp2 is maintained in an inactive form in the cytoplasm (red) during interphase. The mechanism (active repression from a protein (purple) or conformational inactivity) has yet to be determined. After nuclear envelope break-down, TPX2 (pink) targets Hklp2, directly or indirectly, to the MTs where the kinesin is free to walk towards the plus-ends of MTs. It probably cross-links MTs together with an as yet unidentified factor. When Hklp2 reaches the chromosomes (blue) it associates with Ki67 (gray) providing a dynamic link between the MT and the surface of the chromosome. It could also cross-link two antiparallel MT near the equator of the spindle. Eg5 (orange) is localized to the MTs, close to the poles.

In addition, EGFP-Hklp2 does not seem to function properly as it accumulates at the spindle poles and is not present near the chromosomes. Expression of an EGFP-Hklp2 fusion in our lab gave similar localization and suggests that the proximity of the EGFP tag to the motor prevents its correct functioning. Taking everything into account, the data indicate that TPX2 is responsible directly or indirectly for the targeting of Hklp2 to the MTs and its tendency to accumulate at the poles through the tail, but the mechanism is currently unclear.

Endogenous Hklp2 localizes to spindle MTs and also to the chromosomes in mitosis. We showed that Hklp2 chromosomal localization is dependent on protein Ki67, a widely used cell proliferation marker whose function is currently unknown (Brown and Gatter, 2002). The motor-stalk fragment itself has the property to bind only the chromosomes. This localization is most probably due to its interaction with Ki67, although we did not confirm this idea by expressing the motor-stalk fragment in cells silenced for Ki67. Sueishi et al. (Sueishi et al., 2000), proposed that the site of interaction between Hklp2 and Ki67 is located in the stalk. However, we found that the stalk alone cannot localize to the chromosomes. The motor domain appears to be necessary, maybe for actively bringing the protein close to the chromatin by walking towards the plus-ends of MTs.

What can be concluded from these data is that the protein could bind MTs in interphase but it is kept inactive due to a regulation through the motor head. In mitosis it is targeted to the MTs by TPX2 and dragged to the poles by its tail, the motor domain walks towards the chromosomes where it can interact with Ki67.

The phenotypes generated by the overexpression of the tail and the stalk-tail may be unspecific due to the overloading of the poles. Nonetheless, it differs from the results obtained in Xenopus egg extract where the addition of the

Xklp2-tail abolished the separation of the poles. This underlines that the two homologues may have different roles or mechanisms of action. This could also be the consequence of a difference in the requirements for bipolar stability in human and Xenopus illustrated by the fact that the inhibition of Eg5 causes a collapse of the poles in metaphase Xenopus spindles (Kapoor et al., 2000) but not in human cells. Interestingly, Hklp2 fragments localizing to the poles and interfering with its integrity can generate problems in chromosome alignment. This result is similar to what is observed when structural component of the poles NuMA is silenced (Haren et al., 2009). It underlines the interplay that exists between both ends of MTs through forces or changes of dynamics.

The monopolar phenotype generated by the presence of the motor-stalk fragment at the chromosomes confirms the somehow surprising data that was obtained in the Ki67-RNAi. These results were in a sense counterintuitive as, in principle, Hklp2 attached to the chromosomes from its coiled-coil could generate pushing forces by walking towards the MT plus-end (like hKid (Levesque et al., 2003)). In fact, the results suggest the contrary; the presence of a pool of Hklp2 at the chromosomes generates a force countering the separating activities, as observed in Ki67-silenced cells. Consistently, increasing the pool of Hklp2 present at the chromosomes by the overexpression of the motor-stalk fragment impairs the separation of the spindle poles. How is the motor functioning at the chromosomes? It is unclear whether when the stalk interacts with the chromosomes, the motor domain is attached to the MTs. One possibility is that the kinesin attaches to the plus-end of a MT and makes a pause, creating a link between the chromosome and the MT that could counteract outward pushing forces. The attachment of a motor to MTs can provoke a brake effect if it counteracts other motors moving faster. The kinesin 5 of C. elegans (homologue of Eg5) is not responsible for the pole separation in this system but rather to oppose the forces that separate the poles due to protein

friction on MTs (Saunders et al., 2007). Also, mathematical modeling demonstrated that pausing at the end of MTs can have an impact on MT organization (Nedelec, 2002; Surrey et al., 2001). Another possibility is that it locally changes MT dynamics. Other kinesins have already been linked to the control of MT dynamics like kinesins-13 (Manning et al., 2007) or, more interestingly, Kif4 (Castoldi and Vernos, 2006; Mazumdar et al., 2004) which localizes to the chromosomes. MT motility assays on reconstituted chromatin (Bieling et al.), using the stalk-tail fragment should give an answer to these questions and help to propose a model of the activity of Hklp2 at the chromosome-microtubule interface.

Furthermore, it is interesting to note that Hklp2 has two different activities depending on localization and maybe the protein interactors present there. In the Xenopus system, it has been shown that Eg5 steady-state localization at metaphase integrates a constant poleward movement dependent on dynein, indicating a direct interplay between these two motors (Uteng et al., 2008). This movement does not concern a small pool of Eg5 that localizes precisely at the middle of the spindle and that does not move. The variation of dynamics with its position is indicative of position-dependent functions of Eg5.

Altogether, these results emphasize the complexity of understanding the global role of motors as their activities and kinetics may depend on their position in the spindle.

Types of microtubules

The immunofluorescence data show that Hklp2 binds to the spindle MTs in mitosis but is absent from the astral MTs. The selective depolymerization of particular classes of MTs reveals that Hklp2 binds to non-kintochore MTs and the kinetochore bundles of MTs. The difference lies in the efficient recruitment

of the kinesin to the kinetochore fibers remaining after cold treatment. However Hklp2 is not required for their formation or stability. It could be that the protein has a preference for bundles of MTs (which its mouse homologue has been shown to have (Buster et al., 2003)) but this is not visible in untreated cells. This recruitment could also be due to the particular dynamic conditions in which these bundles are. Even if they resist the cold-treatment for some time, the bundles of MTs attached to the kinetochores are going through depolymerization. The fact that there could be recruitment in a particular dynamic state should be investigated further and shall give hints on the precise action of Hklp2 on the MTs.

Role of motors in anaphase

Our experiments using MG132 block together with monastrol treatment and Hklp2 silencing demonstrates that the spindle at metaphase is relatively stable in the bipolar configuration and that the individual activity of Eg5 or Hklp2 is not necessary to maintain it. The localization of Hklp2 and Eg5 in anaphase and later stages of mitosis suggests that they could play a role in the changes of shapes that the spindle undergoes. Using a combination of MG132 block together with monastrol and release into anaphase, we could show that, surprisingly, cells that try to go into anaphase without Eg5 activity not only cannot separate the chromatids and increase spindle length but are unable to keep the spindle poles separated and the spindles collapse into monopolars. Live experimentation will be essential for an accurate description of what happens in these conditions. Do the cells try to elongate the spindle and fail or do they go directly from bipolar metaphase to monopolar? In any case, these results indicate that at the metaphase/anaphase transition, the spindle undergoes a change in its stability that makes Eg5 necessary for the maintenance of

bipolarity. Similar experiments will be performed in the future with the silencing of Hklp2 and Ki67.

Motor proteins in cancer

Kinesins are crucial factors implicated in spindle function and cell division and, by so, are involved in cancer.

On one level, they constitute new targets for the development of a new generation of anti-cancer treatment. Most chemotherapeutic anti-cancer drugs used in clinic today interfere with the normal progression of mitosis to inhibit the proliferation of tumor cells and induce apoptosis (Lee and Schmitt, 2003). A group of drugs that has proven to be exceptionally successful is composed of so called "spindle poisons" as they bind MTs and impair the spindle function causing a cell cycle arrest in mitosis leading to cell death (Jordan and Wilson, 2004; Mollinedo and Gajate, 2003; Pasquier et al., 2006; Zhou and Giannakakou, 2005). However, these compounds act on the MTs of all cells and since interphase, resting and differentiated cells also require MT dynamics for the maintenance of cytoskeletal functions and intracellular transport process (especially in neurons), there are adverse side effects caused by these drugs (Trudeau, 1996; Zhou and Giannakakou, 2005). Also, interfering with MT dynamics causes problems in non-transformed dividing cells. For these reasons, there is an urgent need to identify drugs that interfere with proteins more specifically needed for progression of mitosis. Mitotic kinase Aurora A and polo-like kinases are good candidates, as well as kinesins and in particular Eg5. Newly developed Eg5 inhibitors are currently in clinical trials. In this study, we showed that Hklp2 was a complementary factor to Eg5 in spindle bipolarity establishment and maintenance. Identifying compounds targeting Hklp2 may provide additional therapeutic advantage, in particular to counteract the

development of resistance. A more complete description of spindle assembly and the factors involved should allow a more specific targeting for drug development.

On another level, some kinesins are important to achieve mitosis and a faithful transmission of the genetic material. Loss of function of some kinesins have been linked to aneuploidy (Mailhes et al., 2004; Weaver et al., 2003), a situation that can trigger tumorgenesis. Loss of chromokinesin KIF4 can lead to tumor formation (Mazumdar et al., 2006). The mildness of the phenotype obtained in Hklp2-silenced cells suggest that, after repeated cell divisions, the lack of or reduced activity of Hklp2 could cause some defects subtle enough to avoid the activation of the checkpoint but compromising the fidelity of chromosome segregation. A cell line stably expressing a silencing short hairpin RNA against Hklp2 is being generated in the lab to address the question of genome integrity in Hklp2 absence.

CONCLUSIONS

1. Hklp2 is a human kinesin that localizes to the cytoplasm in interphase and to the microtubules of the spindle and the chromosomes in mitosis.

2. Hklp2 binding to the microtubules is dependent on microtubule nucleation factor TPX2. The targeting is done through the C-terminus part of Hklp2 and involves an indirect mechanism. The localization to the chromosomes is dependent on an interaction between the coiled coil of Hklp2 and chromosomal protein Ki67.

2. Hklp2 binds to the interpolar microtubules with a preference for the kinetochore fibers.

3. Hklp2 contributes to the establishment and the maintenance of spindle bipolarity together with Eg5.

4. The activity of Hklp2 differs on the microtubules and the chromosomes. It contributes to the centrosome separation on the microtubules and has an opposite activity on the chromosomes.

MATERIAL AND METHODS

Cloning and protein expression

The cDNA encoding residues 1132-1388 of Hklp2 was cloned into pGEX (Amersham Pharmacia Biotech) and pHAT2-EGFP (Brunet 2004) for protein expression in E coli BL21 (DE3) and the corresponding GST-Hklp2 tail and EGFP-Hklp2 tail fusion proteins were purified using standard procedures. The cDNAs coding for full length Hklp2 (residues 1-1388), motor domain (residues 1-392), stalk domain (residues 393-1131), tail domain (residues 1132-1388), motor-stalk (residues 1-1131), stalk-tail (residues 393-1138) were cloned into pFLAG-CMV2 (Sigma) for expression in eukaryotic cells. The construct pCS2-EGFP Eg5 is a generous gift from Thomas Mayer (University of Konstanz, Germany).

Antibodies

The anti-Hklp2 antibody was generated by immunizing rabbits with GST-Hklp2 tail following a standard three weeks protocol at the antibody facility from the University of Barcelona. The sera were affinity purified on EGFP-Hklp2 tail covalently bound to a HiTrap column (GE Healthcare Life Sciences) following the company's protocol. The affinity-purified antibody was used at 1 μg/ml for immunofluorescence studies.

The following antibodies were used: a monoclonal anti-Ki67 antibody (Ab-1; Oncogene) at 1/200 dilution, a mouse monoclonal anti-α tubulin (DM1a; Sigma) at 1/1000 dilution, a CREST antibody (Antibodies Incorporated) at dilution 1/400, a mouse monoclonal anti-Hec1 (Gentex) at dilution 1/500, a mouse monoclonal anti-Eg5 antibody (BD Biosciences) at dilution 1/1000.

Alexa (Molecular Probes) or HRP (Jackson immunoresearch) conjugated secondary anti-mouse and anti-rabbit at 1/1000 dilution. The polyclonal affinity purified anti-HsTPX2 (used at 0.6 µg/ml, Gruss 2002), anti-HsAurora A (0.5 µg/ml), anti-Kif4 (0.6 µg/ml, Castoldi 2006) and anti-hKid antibodies were generated in the lab by immunizing rabbits with the corresponding recombinant proteins.

Cell Culture

HeLa cells and Hela cells stably expressing EGFP-H2B and RED-Tubulin (McAinsh 2006) and were cultured in DMEM (Cambrex) supplemented with 10% fetal bovine serum (Invitrogen) and 2 mM L-glutamine (Invitrogen). hTERT-RPE1 cells were cultured in DMEM:F12 medium (Sigma) containing 10% fetal bovine serum (Invitrogen), 0.35% sodium bicarbonate (Merck) and 2 mM L-glutamine. Cells were incubated at 37° C in a humid atmosphere with 5% CO2.

Transfection

siRNAs for Ki67 (5'-GGTCACACTGAGGAATCAA-3'), Hklp2 (5'-GGACATAAATTGCAAATAC-3'), Kid (5'-GCTCTCTAGAGATTGCTAA-3'), Kif4 (sequence from Mazumdar 2006), Nuf2 (sequence from DeLuca 2002), TPX2 (sequence from Gruss 2002), Aurora A (sequence from Kuffer 2002) or luciferase (5'-CGTACGCGGAATACTTCGA-3') as control were obtained from Dharmacon.

Transfections were performed with 100 pmol of siRNAs per well of a 6-well plate or 20 pmol per well of a 24-well plate combined with Lipofectamine 2000

(Invitrogen) according to the manufacturer's protocol. For double RNAi, 100 pmol were transfected with a ratio of 8:2 of anti-Hklp2: anti-Ki67 siRNAs.

Plasmid transfections were performed using 2 μg of DNA per well of a 6-well plate and 36 μg per 175 cm^2 in combination with Fugene 6 (Roche) according with the manufacturer's protocol.

Drug treatments

For monastrol experiments, cells were incubated in medium containing 100 or 50 μM monastrol (Sigma) for 5 hours. After this treatment, cells were either fixed or released after three washings with PBS and incubated in fresh medium for 10 min. before fixation.

For experiments with metaphase block, cells were either incubated 4 hours in 10 μM MG132 or incubated 2 hours in medium with 10 μM MG132 (Sigma) and then for 2 hours more in 10 μM MG132, 100 μM monastrol.

For experiments with release into anaphase, HeLa cells were first synchronized with cdk1 inhibitor for 5 hours then released after three washings with PBS in fresh medium for 30 minutes. Then MG132 was added to the medium to reach a 1.25 μM concentration and left for an additional 45 minutes. Then the cells were washed three times during 5 minutes with fresh medium and then left for release into anaphase in fresh medium for 1 hour. For conditions with Eg5 inhibitor monastrol, it was added, to a concentration of 100 μM, 15 minutes before the end of the incubation with MG132 and used for every subsequent steps.

For nocodazole and taxol experiments, cells were incubated for 8 hours in 25 nM taxol (Sigma) or 350 nM nocodazole (Sigma).

For depolymerization of non-kinetochore microtubules, HeLa cells were treated as described previously Lampson 2005.

Immunofluorescence

Cells grown on coverslips were fixed for 10 minutes in -20ºC methanol. Blocking and incubation with the antibodies were done in PBS; 2% BSA (Sigma); 0.1% Triton (Sigma) for 20 minutes at room temperature. The coverslips were mounted on Mowiol (0.1 M Tris-HCl (Sigma) pH 8.2; 25% Glycerol (Merck); 10% Mowiol 4-88 (Calbiochem).

For hKid immunofluorescence, cells were fixed first with 3.7% formalin in PBS for 1 min then further fixed and extracted with -20°C methanol for 5 min.

Fluorescence microscopy and live cell imaging

Fixed cells were observed using an inverted widefield fluorescent microscope from Leica (model DMI-6000) with 63× or 40× objective. For high-resolution images, TCS SPE confocal microscopy (Leica) was used with 63× objectives. Pictures were acquired with the Leica Application Suite software. For confocal microscopy, three-dimensional optical section images were taken at 0.3 μm intervals and projected to single planes. For live cell observations, samples were maintained in standard culture condition in an incubation chamber built on the Zeiss Cell Observer system and images were taken every 3 min. with Axiovision software using a 60× objective.

Nuclear cytoplasmic separation

HeLa cells were collected by centrifugation after trypsinization. The pelleted cells were washed twice with PBS and incubated in 5 times their volume of buffer A (10 mM Tris pH 7,9 ; 1,5 mM $MgCl_2$ (Merck); 10 mM KCl (Merck); 0,5 mM DTT (Sigma); protease inhibitors cocktail) on a rotating wheel at 4°C for 10 min at 4°C. The cells were pelleted and resuspended in twice their volume of ice cold buffer A, supplemented with 0,1% NP40, followed by homogenization with a pestle on ice. The breakage of cytoplasmic membranes was checked under the microscope, before centrifugation for 10 min. The supernatant, corresponding to the cytoplasmic fraction, and the pellet containing the nuclei, nuclear fraction, were collected. For the supernatant, one tenth of its volume of buffer B (0,3 M Tris pH 7,9 ; 30 mM $MgCl_2$; 1,4 M KCl, protease inhibitors cocktail) was added before centrifugation at 100.000g for 60 min. The resulting supernatant was used as the cytoplasmic fraction for Western blot analysis. The nuclear pellet was first washed twice with buffer A to remove traces of cytoplasm. Then one volume of buffer C (20 mM Tris pH 7,9 ; 25% glycerol (Merck); 0,42 NaCl ; 1,5 mM $MgCl_2$; 0,2 mM EDTA (Sigma); 0,5 mM DTT ; protease inhibitors cocktail) was added and the mixture homogenized with pestle before incubation on a rotating wheel for 30 min. The homogenate was centrifuged at 20.000g for 30 min. The resulting supernatant was used as the nuclear fraction for Western blot analysis.

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APPENDIX

	CTRL	Hklp2-RNAi	
Number of spindles	334	326	Difference (%)
Average length (µm)	10.27	9.13	-11.13%
SD	1.21	1.44	
SEM	0.07	0.08	
t test (p value)		< 0.001	
	CTRL	**Ki67-RNAi**	
Number of spindles	311	335	Difference (%)
Average length (µm)	10.79	12.14	12.44%
SD	1.33	1.31	
SEM	0.08	0.07	
t test (p value)		< 0.001	
	CTRL	**Hklp2 and Ki67-RNAi**	
Number of spindles	349	332	Difference (%)
Average length (µm)	10.28	9.13	-11.24%
SD	1.21	1.14	
SEM	0.06	0.06	
t test (p value)		< 0.001	

Table 1: Spindle length in cells silenced for Hklp2, Ki67 or both
Pole-to-pole distance was measured for > 100 spindles in each experiment. For each condition, three independent experiments giving the same result were pooled to make an average and perform statistical test.

	100 µM monastrol			wash-out		
	monopolar (%)	bipolar (%)	Z (p value)	monopolar (%)	bipolar (%)	Z (p value)
CTRL	79.87 ± 2.32	20.13 ± 2.32		73.98 ± 2.46	26.02 ± 2.46	
Hklp2-RNAi	98.72 ± 0.64	1.28 ± 0.64	< 0.001	90.75 ± 1.58	9.25 ± 1.58	< 0.001
CTRL	83.07 ± 2.12	16.93 ± 2.12		71.52 ± 2.57	28.48 ± 2.57	
Ki67-RNAi	56.39 ± 2.77	43.61 ± 2.77	< 0.001	26.28 ± 2.49	73.72 ± 2.49	< 0.001
CTRL	81.49 ± 2.21	18.51 ± 2.21		68.48 ± 2.61	31.76 ± 2.61	
Hklp2 and Ki67-RNAi	87.82 ± 1.85	12.18 ± 1.85	< 0.05	77.99 ± 2.36	22.01 ± 2.36	< 0.01

	50 µM monastrol		
	monopolar (%)	bipolar (%)	Z (p value)
CTRL	61.45 ± 2.62	38.55 ± 2.62	
Hklp2-RNAi	94.77 ± 1.24	5.23 ± 1.24	< 0.001

	MG132		
	monopolar (%)	bipolar (%)	Z (p value)
CTRL	0 ± 0	100 ± 0	
Hklp2-RNAi	1.88 ± 0.93	98.12 ± 0.93	> 0.05

	MG132 and 100 µM monastrol		
	monopolar (%)	bipolar (%)	Z (p value)
CTRL	6.49 ± 1.40	93.51 ± 1.40	
Hklp2-RNAi	61.37 ± 2.72	38.63 ± 2.72	< 0.001

Table 2: Mono/bipolar spindle frequencies in cells silenced for Hklp2, Ki67 or both
Results for each condition come from the pooling of three independent experiments giving the same result with > 100 structures quantified for each experiment. The error corresponds to the error on proportions. Z statistics performed to obtain a p value

Vanneste D, Takagi M, Imamoto N, Vernos I. The role of Hklp2 in the stabilization and maintenance of spindle bipolarity. Curr Biol. 2009; 19(20): 1712-7.

Supplemental Data

Experimental procedures

Cloning and protein expression

The cDNA encoding residues 1132-1388 of Hklp2 was cloned into pGEX (Amersham Pharmacia Biotech) and pHAT2-EGFP [1] for protein expression in E coli BL21 (DE3) and the corresponding GST-Hklp2 tail and EGFP-Hklp2 tail fusion proteins were purified using standard procedures. The full length Hklp2 cDNA sequence was cloned into pFLAG-CMV2 (Sigma) for expression in eukaryotic cells. The construct pCS2-EGFP Eg5 is a generous gift from Thomas Mayer (University of Konstanz, Germany).

Antibodies

The anti-Hklp2 antibody was generated by immunizing rabbits with GST-Hklp2 tail following a standard three weeks protocol at the antibody facility from the University of Barcelona. The sera were affinity purified on EGFP-Hklp2 tail covalently bound to a HiTrap column (GE Healthcare Life Sciences) following the company's protocol. The affinity-purified antibody was used at 1 μg/ml for immunofluorescence studies.

The following antibodies were used: a monoclonal anti-Ki67 antibody (Ab-1; Oncogene) at 1/200 dilution, a mouse anti-CENP-E antibody (1H12; Abcam) at 1/200 dilution, a mouse monoclonal anti-α tubulin (DM1a; Sigma) at 1/1000 dilution, a CREST antibody (Antibodies Incorporated) at dilution 1/400, a mouse monoclonal anti-Hec1 (Gentex) at dilution 1/500, a mouse monoclonal anti-Eg5 antibody (BD Biosciences) at dilution 1/1000 and a monoclonal anti-HsTPX2 antibody obtained in the lab. Alexa (Molecular

Probes) or HRP (Jackson immunoresearch) conjugated secondary anti-mouse and anti-rabbit at 1/1000 dilution. The polyclonal affinity purified anti-HsTPX2 (used at 0.6 μg/ml, [2]), anti-HsAurora A (0.5 μg/ml), anti-Kif4 (0.6 μg/ml, [3]) and anti-hKid antibodies were generated in the lab by immunizing rabbits with the corresponding recombinant proteins.

Cell Culture

HeLa cells, Hela cells stably expressing EGFP-H2B and RED-Tubulin [4] and HeLa cells stably expressing GFP-H2B fusion protein [5] were cultured in DMEM (Cambrex) supplemented with 10% fetal bovine serum (Invitrogen) and 2 mM L-glutamine (Invitrogen). hTERT-RPE1 cells were cultured in DMEM:F12 medium (Sigma) containing 10% fetal bovine serum (Invitrogen), 0.35% sodium bicarbonate (Merck) and 2 mM L-glutamine. Cells were incubated at 37° C in a humid atmosphere with 5% CO2.

Transfection

siRNAs for Ki67 (5'-GGTCACACTGAGGAATCAA-3') was obtained from Qiagen and siRNAs for Hklp2 (5'-GGACATAAATTGCAAATAC-3'), Kid (5'-GCTCTCTAGAGATTGCTAA-3'), Kif4 (sequence from [6]), Nuf2 (sequence from [7]), TPX2 (sequence from [2]), Aurora A (sequence from [8]) or luciferase (5'-CGTACGCGGAATACTTCGA-3') as control were obtained from Dharmacon.

Transfections were performed with 100 pmol of siRNAs per well of a 6-well plate or 20 pmol per well of a 24-well plate combined with Lipofectamine 2000 (Invitrogen)

according to the manufacturer's protocol. For double RNAi, 100 pmol were transfected with a ratio of 8:2 of anti-Hklp2: anti-Ki67 siRNAs.

For live cell imaging experiments with Ki67 RNAi, transfection of siRNAs was performed during the release period between the two thymidine incubations.

Plasmid transfections were performed using 2 μg of DNA per well of a 6-well plate and 36 μg per 175 cm^2 in combination with Fugene 6 (Roche) according with the manufacturer's protocol.

Drug treatments

For monastrol experiments, cells were incubated in medium containing 100 or 50 μM monastrol (Sigma) for 5 hours. After this treatment, cells were either fixed or released after three washings with PBS and incubated in fresh medium for 10 min. before fixation.

For experiments with metaphase block, cells were either incubated 4 hours in 10 μM MG132 or incubated 2 hours in medium with 10 μM MG132 (Sigma) and then for 2 hours more in 10 μM MG132, 100 μM monastrol.

For nocodazole and taxol experiments, cells were incubated for 8 hours in 25 nM taxol (Sigma) or 350 nM nocodazole (Sigma).

For the live observation of cells silenced for Ki67, HeLa cells were synchronized for 16 h with 2 mM thymidine, and then released by three washings of PBS and incubation during 8 hours in fresh medium. The cells were blocked a second time with a 16 hour incubation in 2 mM thymidine before being released for imaging.

For depolymerization of non-kinetochore microtubules, HeLa cells were treated as described previously [9].

Immunofluorescence

Cells grown on coverslips were fixed for 10 minutes in -20ºC methanol. Blocking and incubation with the antibodies were done in PBS; 2% BSA (Sigma); 0.1% Triton (Sigma) for 20 minutes at room temperature. The coverslips were mounted on Mowiol (0.1 M Tris-HCl (Sigma) pH 8.2; 25% Glycerol (Merck); 10% Mowiol 4-88 (Calbiochem).

Cells shown in Supplemental Figure 4 A were fixed with 3.7% formalin in PBS prewarmed to 37°C. For hKid immunofluorescence, cells were fixed first with formalin in PBS for 1 min then further fixed and extracted with -20°C methanol for 5 min. For human CENP-E immunofluorescence, cells were extracted with detergent before fixation essentially as described [10]. In all these cases, the cells were processed for immunofluorescence as described previously [11].

Fluorescence microscopy and live cell imaging

Fixed cells were observed using an inverted widefield fluorescent microscope from Leica (model DMI-6000) with 63× objective. For high-resolution images, TCS SP2 or SPE confocal microscopy (Leica), LSM510 confocal microscopy (Zeiss) or a microscope model IX-71; (Olympus) were used with 60x objectives. Pictures were acquired with the Leica Application Suite software or DeltaVision SoftWorx (Applied Precision). Three-dimensional optical section images were taken at 0.2 μm intervals, processed by an iterative deconvolution method (only for those taken with the last system), and projected to single planes. For live cell observations (Suppl. Movie 1 and 2), images were taken every 3 min. with Zeiss Cell observer and Axiovision software using a 60x objective. For

Suppl. Movie 3 and 4, images were collected every 10 min. from a focal section on a microscope (model IX-71; Olympus) controlled by DeltaVision SoftWorx (Applied Precision) using a 40x objective.

Nuclear Cytoplasmic separation

HeLa cells were collected by centrifugation after trypsinization. The pelleted cells were washed twice with PBS and incubated in 5 times their volume of buffer A (10 mM Tris pH 7,9 ; 1,5 mM $MgCl_2$ (Merck); 10 mM KCl (Merck); 0,5 mM DTT (Sigma); protease inhibitors cocktail) on a rotating wheel at 4°C for 10 min at 4°C. The cells were pelleted and resuspended in twice their volume of ice cold buffer A, supplemented with 0,1% NP40, followed by homogenization with a pestle on ice. The breakage of cytoplasmic membranes was checked under the microscope, before centrifugation for 10 min. The supernatant, corresponding to the cytoplasmic fraction, and the pellet containing the nuclei, nuclear fraction, were collected. For the supernatant, one tenth of its volume of buffer B (0,3 M Tris pH 7,9 ; 30 mM $MgCl_2$; 1,4 M KCl, protease inhibitors cocktail) was added before centrifugation at 100.000g for 60 min. The resulting supernatant was used as the cytoplasmic fraction for Western blot analysis. The nuclear pellet was first washed twice with buffer A to remove traces of cytoplasm. Then one volume of buffer C (20 mM Tris pH 7,9 ; 25% glycerol (Merck); 0,42 NaCl ; 1,5 mM $MgCl_2$; 0,2 mM EDTA (Sigma); 0,5 mM DTT ; protease inhibitors cocktail) was added and the mixture homogenized with pestle before incubation on a rotating wheel for 30 min. The homogenate was centrifuged at 20.000g for 30 min. The resulting supernatant was used as the nuclear fraction for Western blot analysis.

Supplemental Figures legends

Supplemental Figure 1: Hklp2 is a cytoplasmic protein whose level is not cell cycle regulated

A- Left: Brightfield pictures of proliferating Hela cells treated with hypotonic buffer before and after homogenization with a pestle, showing that the cytoplasm was broken while preserving the nucleus. Right: Western blot of cytoplasmic and nuclear fractions prepared from proliferating HeLa cells probed with the anti-Hklp2, anti-TPX2 and anti-α-tubulin antibodies. Hklp2 is clearly enriched in the cytoplasmic fraction as α-tubulin whereas TPX2 is enriched in the nuclear fraction as expected.

B- Confocal images of interphase hTERT-RPE1 cell processed for immunofluorescence with the anti-Hklp2 (red) and the anti-α tubulin (green) antibodies. The DNA was detected with DAPI (blue). Hklp2 does not show any specific localization in interphase. The images correspond to maximum projections of confocal sections acquired every 0.3 μm.

C- Confocal section through the spindle of an hTERT-RPE1 cell processed for immunofluorescence with the anti-Hklp2 (red), the anti-α tubulin (green) antibodies, and a CREST antiserum to mark the kinetochores (blue). Hklp2 associates with the spindle MTs and the chromatin. a and b are magnifications from two selected areas to show that Hklp2 does not colocalize to the kinetochores.

(bars, 10 μm)

D- Western blot analysis of lysates of HeLa cells taken at the indicated time points after the release from a double thymidine block and probed with antibodies to detect Hklp2, TPX2 and α-tubulin. The levels of TPX2 increase at 10h after the release as cells peak into mitosis, and go down after mitosis (12-14 hours), as previously described. In contrast, the protein levels of Hklp2 remain constant throughout the cell cycle.

(P, proliferating HeLa cells)

Supplemental Figure 2: Hklp2 localization depends on TPX2 but not Aurora A

A- Western blot of HeLa cells transfected with control or TPX2 siRNAs showing the extent of silencing.

B- Control and TPX2-silenced HeLa cells were treated with microtubule stabilizer taxol and immunostained (Hklp2 (red), αtubulin (green), DNA (blue)). Hklp2 does not have any specific localization when TPX2 is absent even if the microtubules are stabilized.

C- Left panel shows a Western blot of HeLa cells transfected with control or Aurora A-specific siRNAs showing the extent of silencing. Right panel shows HeLa cells silenced for Aurora A (Hklp2 in red, MTs in green and DNA in blue) with Hklp2 still localizing to the disorganized MTs.

All images shows maximum projection of confocal sections acquired every 0.3 μm. (bars, 10 μm)

Supplemental Figure 3: Hklp2 localizes to kinetochore and non-kinetochore microtubules

A- Confocal images of control and Hklp2-silenced HeLa cells treated with cold to depolymerize the more dynamic spindle MTs and processed by immunofluorescence with the anti-Hklp2 (green), and anti-tubulin (red) antibodies. Kinetochores were detected with the CREST antiserum (blue). Hklp2 localizes very strongly to the stable MT bundles, presumably the K-fibers, but its presence is not required for their stability.

B- Confocal images of control and Nuf2-silenced HeLa cells processed for immunofluorescence with the anti-Hklp2 (green), and anti-HEC-1 (red) antibodies, showing that Hklp2 is still associated to the MTs in the absence of K-fibers (Nuf2-RNAi). The efficiency of Nuf2 silencing is shown by monitoring the Ndc80 complex protein Hec-1.

C- Confocal images of HeLa cells treated with taxol or nocodazole and immunostained for Hklp2 (green) and tubulin (red). Impaired MT dynamics with taxol does not abolish Hklp2 localization to the mitotic MTs but the chromsome localization is lost. When MTs are completely depolymerized using nocodazole, Hklp2 does not display any specific staining.

D- Confocal images of HeLa cells treated with cold to depolymerize the more dynamic spindle MTs and processed by immunofluorescence with the anti-Hklp2 (green), and anti-Eg5 (red) antibodies. Eg5 and Hklp2 localize to the stable MT bundles, presumably the K-fibers, but Hklp2 is more present close to the chromosomes while Eg5 is strongly enriched at the poles.

In all cases the DNA was stained with DAPI (blue). All images correspond to maximum projections of confocal sections acquired every 0.3 μm.

(bars, 10 μm)

Supplemental figure 4: Characterization of Ki67-silenced HeLa cells

A- Western blot showing the extent of the silencing of Ki67 in HeLa cells after siRNAs transfection is shown on the left. In the middle, images of immunofluorescence performed on control and Ki67-silenced HeLa cells to visualize Ki67 (red), MTs (green) show different chromosome alignment defects in Ki67-silenced cells (Type I, normal; Type II, tightly packed congressed chromosomes; Type III, chromosome misalignment). Quantifications of the phenotypes observed are shown on the right (data obtained from 3 independent experiments; total number of counted cells; 441 for control and 384 for Ki67-silenced cells; error bars = standard error of the mean).

B- HeLa cells transfected with control, anti-Ki67 or anti-hKid siRNAs processed for immunofluorescence with anti-Ki67 (green) and anti-hKid (red) antibodies. Ki67 and hKid and Ki67 localizes to the chromosomes independently.

C- Hela cells transfected with control, anti-Ki67 or anti-Kif4 siRNAs processed for immunofluorescence with anti-Ki67 (green) and anti-Kif4 (red) antibodies. Ki67 and Kif4 localizes to the chromosomes independently.

D- Hela cells transfected with control or anti-Ki67 siRNAs processed for immunofluorescence with anti-Ki67 (red) and anti-CENP-E (green) antibodies. Examples of cells with completely congressed or uncongressed chromosomes are shown. The localization of CENP-E is not affected by the silencing of Ki67.

In all the cases, DNA was detected with DAPI (blue). (bars, 10 μm)

Supplemental Figure 5 : Hklp2 is required for the spindle phenotypes observed in Ki67-silenced cells

A- Immunofluorescence images of metaphase HeLa cells co-transfected with control or both Ki67 and Hklp2 specific siRNAs (Hklp2, green; Ki67, red; DNA, blue). (bar, 10 μm)

Histogram of metaphase spindle lengths in control (blue bars) and co-silenced HeLa cells (green bars) obtained from > 100 spindles for each of three independent experiments. The best fit Gaussian distribution is shown as a superimposed line. The averages of the spindle length are shown for each condition. Spindles assembled in co-silenced cells are on average 11% shorter than controls (***, $p < 0.001$).

B- Frequency of monopolar and bipolar spindles in control and Ki67/Hklp2 co-silenced HeLa cells incubated with 100 μM monastrol. The data correspond to > 100 spindles for each of three independent experiments. Error bars correspond to the error done on proportions (*, $p < 0.05$).

C- Quantification of the monopolar and reforming bipolar spindles in HeLa cells treated as in B and further incubated in monastrol-free medium for 10 min. The data correspond to > 100 spindles for each of three independent experiments. Error bars correspond to the error done on proportions (*, $p < 0.01$).

Supplemental Movie 1 and 2: Time-lapse of live control and Hklp2-silenced HeLa cells arrested in metaphase with MG132 and further incubated with monastrol. HeLa cells expressing RED-α tubulin and GFP-H2B were transfected with control (movie 1) or specific Hklp2 siRNAs (movie 2) and treated with MG132 and monastrol.

Only the tubulin signal is shown. Images were taken every 3 minutes. The bipolar spindle of the control cell has a stable bipolar configuration throughout the 600 min. period after addition of monastrol whereas the HKlp2 silenced cells is unable to maintain the bipolar spindle that collapses into a monopolar structure.

Supplemental Movie 3 and 4: Time-lapse of live cell division in control and Ki67-silenced HeLa cells

HeLa cells expressing histone H2B-GFP were synchronized by a double thymidine block and transfected with control (movie 3) or specific Ki67 siRNAs (movie 4). Images were taken every 10 min. Chromosome alignment is delayed in cells lacking Ki67.

Table 1: Spindle length in cells silenced for Hklp2, Ki67 or both

Pole-to-pole distance was measured for > 100 spindles in each experiment. For each condition, three independent experiments giving the same result were pooled to make an average and perform statistical test.

Table 2: Mono/bipolar spindle frequencies in cells silenced for Hklp2, Ki67 or both

Results for each condition come from the pooling of three independent experiments giving the same result with > 100 structures quantified for each experiment. The error corresponds to the error on proportions. Z statistics performed to obtain a p value.

Table 1 : Spindle length in cells silenced for Hklp2, Ki67 or both

	CTRL	Hklp2-RNAi	
Number of spindles	334	326	Difference (%)
Average length (µm)	10.27	9.13	-11.13%
SD	1.21	1.44	
SEM	0.07	0.08	
t test (p value)		< 0.001	
	CTRL	**Ki67-RNAi**	
Number of spindles	311	335	Difference (%)
Average length (µm)	10.79	12.14	12.44%
SD	1.33	1.31	
SEM	0.08	0.07	
t test (p value)		< 0.001	
	CTRL	**Hklp2 and Ki67-RNAi**	
Number of spindles	349	332	Difference (%)
Average length (µm)	10.28	9.13	-11.24%
SD	1.21	1.14	
SEM	0.06	0.06	
t test (p value)		< 0.001	

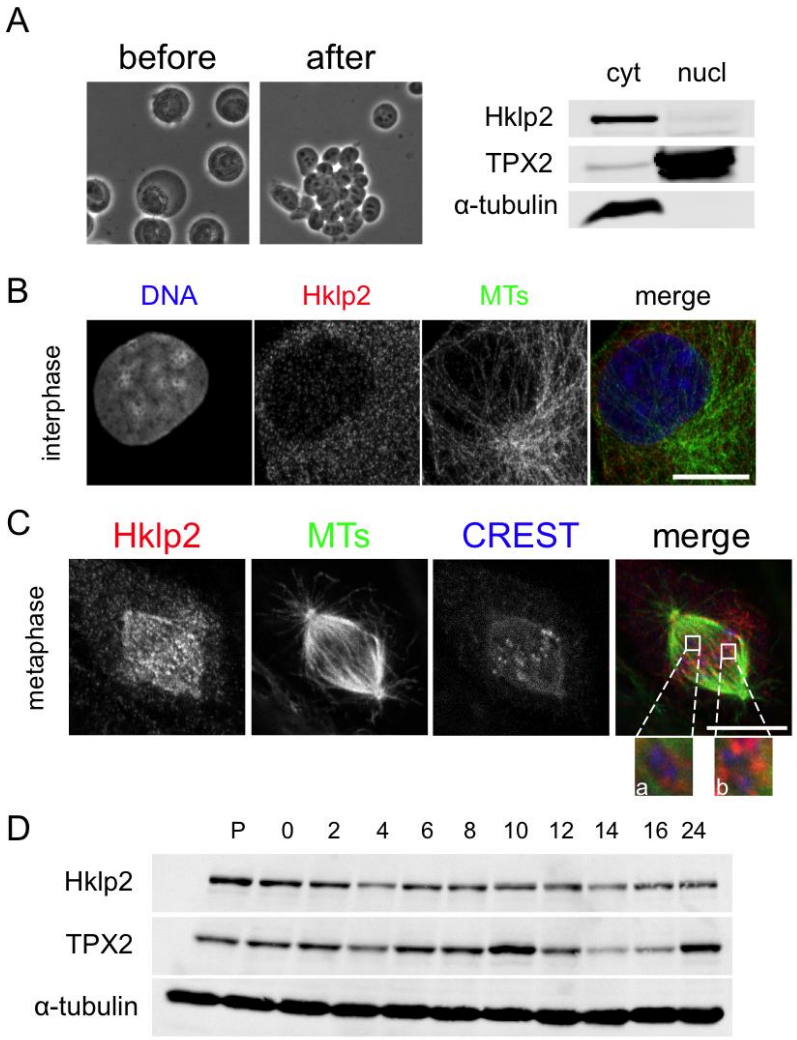
Table 2: Mono/bipolar spindle frequencies in cells silenced for Hklp2, Ki67 or both

	100 µM monastrol			wash-out		
	monopolar (%)	bipolar (%)	Z (p value)	monopolar (%)	bipolar (%)	Z (p value)
CTRL	79.87 ± 2.32	20.13 ± 2.32		73.98 ± 2.46	26.02 ± 2.46	
Hklp2-RNAi	98.72 ± 0.64	1.28 ± 0.64	< 0.001	90.75 ± 1.58	9.25 ± 1.58	< 0.001
CTRL	83.07 ± 2.12	16.93 ± 2.12		71.52 ± 2.57	28.48 ± 2.57	
Ki67-RNAi	56.39 ± 2.77	43.61 ± 2.77	< 0.001	26.28 ± 2.49	73.72 ± 2.49	< 0.001
CTRL	81.49 ± 2.21	18.51 ± 2.21		68.48 ± 2.61	31.76 ± 2.61	
Hklp2 and Ki67-RNAi	87.82 ± 1.85	12.18 ± 1.85	< 0.05	77.99 ± 2.36	22.01 ± 2.36	< 0.01

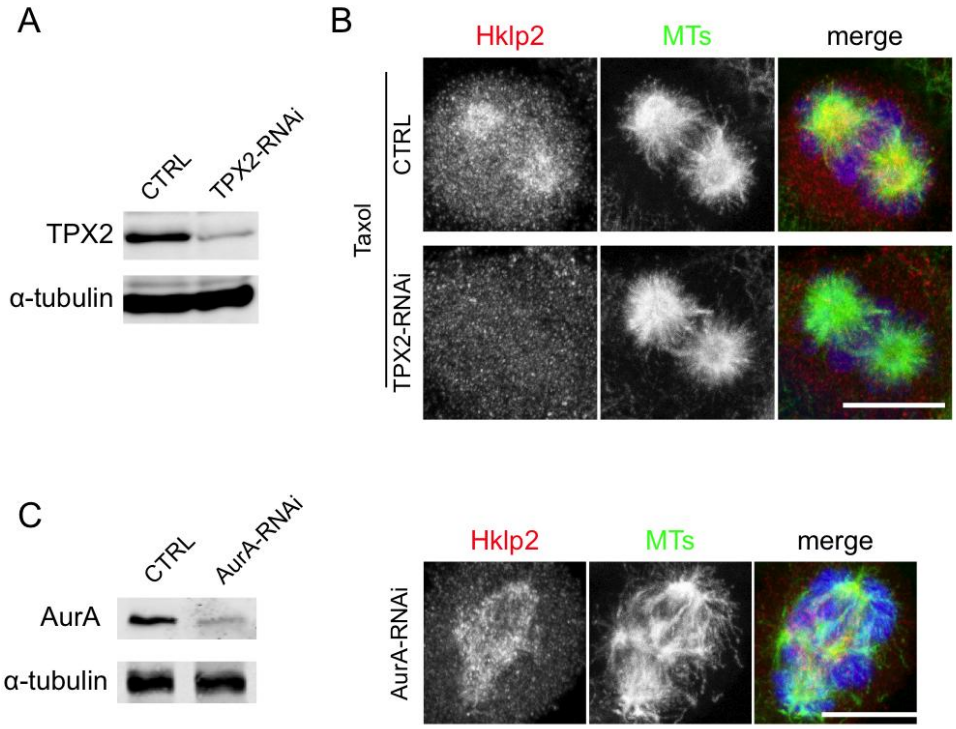
	50 µM monastrol		
	monopolar (%)	bipolar (%)	Z (p value)
CTRL	61.45 ± 2.62	38.55 ± 2.62	
Hklp2-RNAi	94.77 ± 1.24	5.23 ± 1.24	< 0.001

	MG132		
	monopolar (%)	bipolar (%)	Z (p value)
CTRL	0 ± 0	100 ± 0	
Hklp2-RNAi	1.88 ± 0.93	98.12 ± 0.93	> 0.05

	MG132 and 100 µM monastrol		
	monopolar (%)	bipolar (%)	Z (p value)
CTRL	6.49 ± 1.40	93.51 ± 1.40	
Hklp2-RNAi	61.37 ± 2.72	38.63 ± 2.72	< 0.001



Supplemental Figure 1



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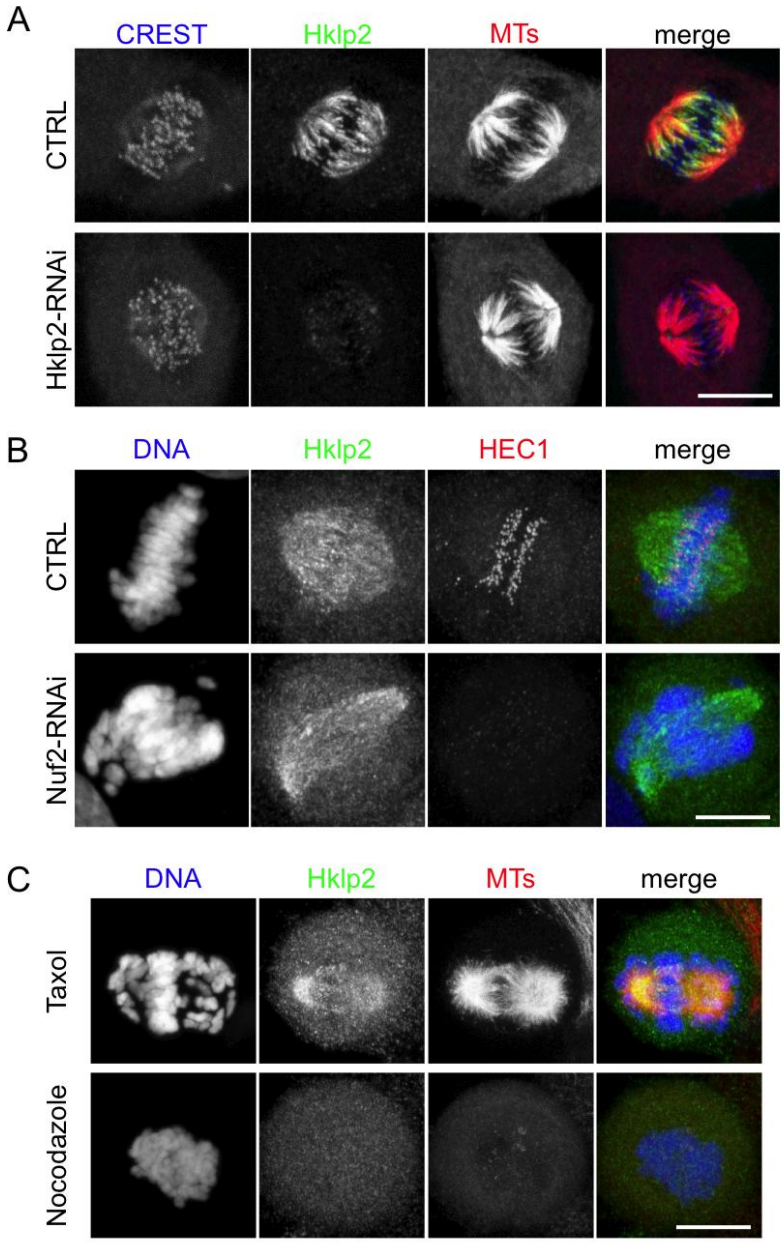

Supplemental Figure 3

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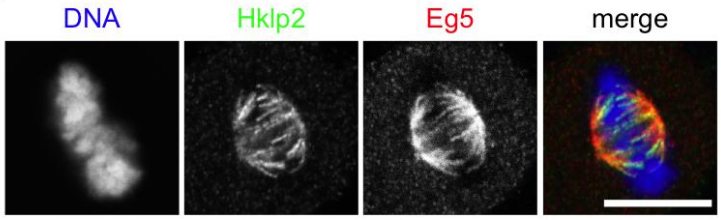

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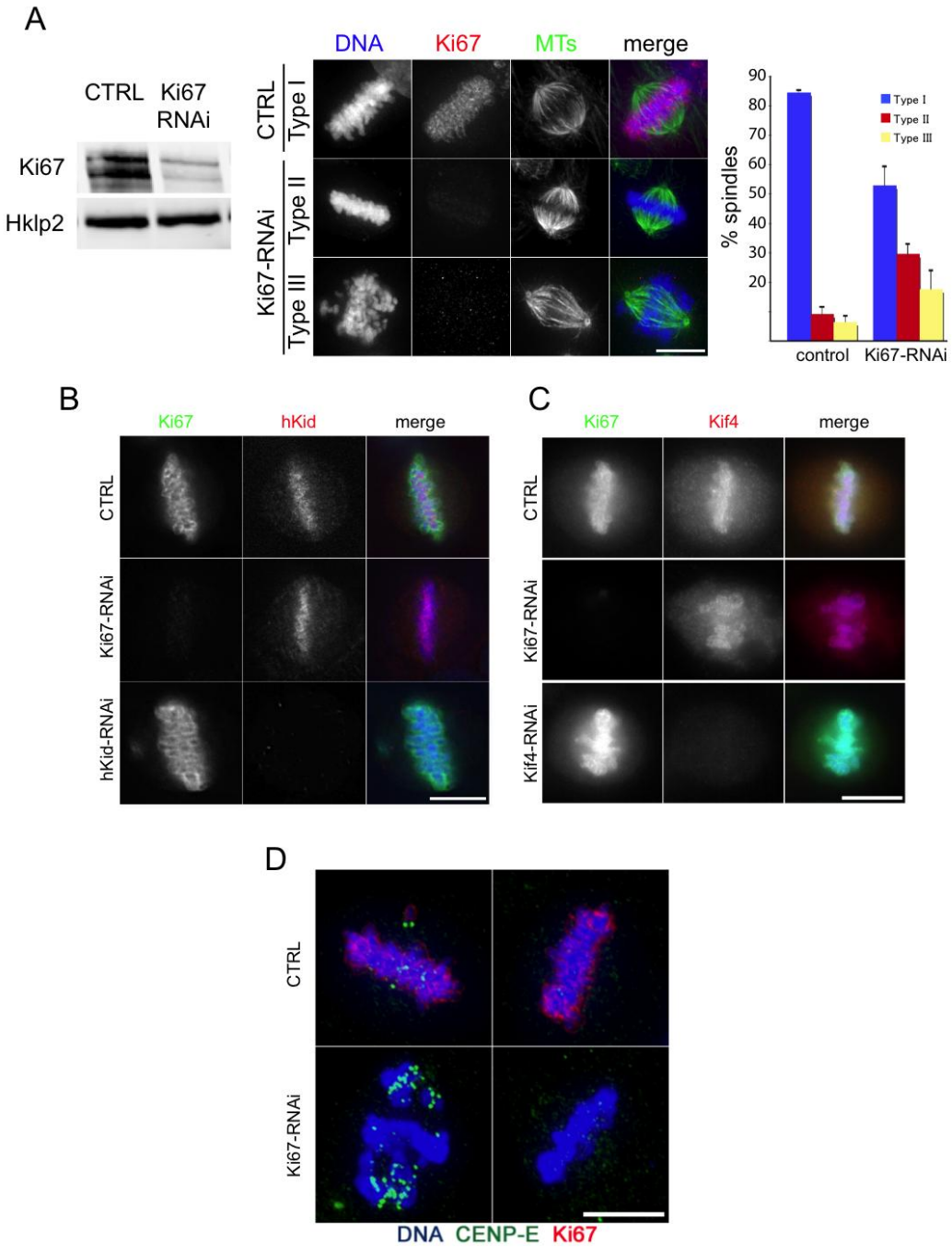

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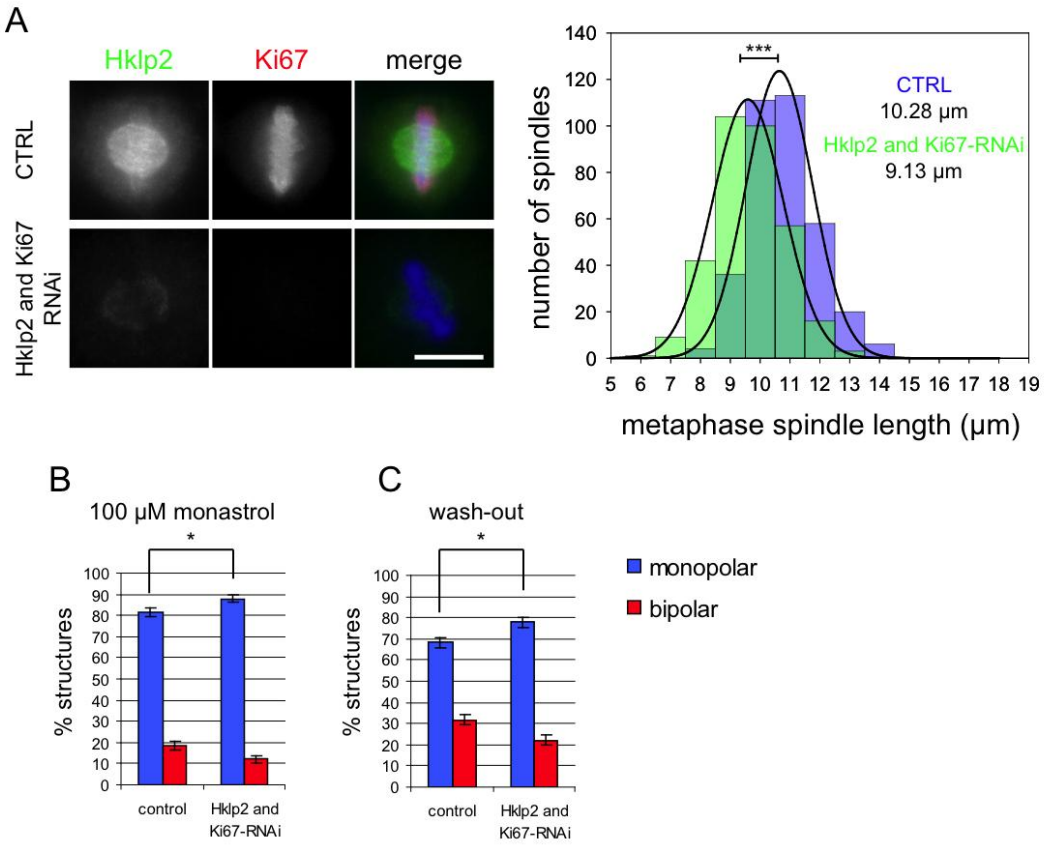

Supplemental Figure 5

Supplemental References

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