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Report

The Role of Hklp2 in the Stabilization and Maintenance of Spindle Bipolarity

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Summary

Spindle bipolarity relies on a fine balance of forces exerted by various molecular motors [1–4]. In most animal cells, spindle bipolarity requires sustained outward forces to push the spindle poles apart, an activity that is provided by Eg5, a conserved homotetrameric plus-end-directed kinesin that crosslinks and slides antiparallel microtubules apart [5]. These pushing forces are balanced by inward minus-end-directed forces. Impairing both Eg5 and dynein restores the formation of functional bipolar spindles [4], although the mechanism at play is far from clear. The current model also fails to explain why in some systems Eg5 inhibition does not promote bipolar spindle collapse [6, 7] or why increasing Eg5 levels does not interfere with bipolar spindle assembly [8]. Moreover, the *C. elegans* Eg5 ortholog is not required for bipolar spindle formation [9]. We show here that the kinesin Hklp2 participates in the assembly and stabilization of the bipolar spindle. Hklp2 localizes to the mitotic microtubules in a TPX2-dependent manner and to the chromosomes through Ki67. Our data indicate that its mechanism of action is clearly distinct from and complementary to that of Eg5, providing an additional understanding of the mechanism driving the formation and maintenance of the bipolar spindle.

Results and Discussion

We previously identified Xklp2 as a *Xenopus* kinesin-like protein [10] required for centrosome separation, although further experiments did not provide additional support for this idea [11]. Xklp2 orthologs in sea urchin [12] and *C. elegans* [13] also have roles in bipolar spindle assembly, but overall, the function of this family of motors is poorly understood. Here we characterized the human member of this family, Hklp2, also named Kif15 [14, 15]. We raised polyclonal antibodies against the C-terminal part of Hklp2 to determine its subcellular localization in HeLa and hTERT-RPE1 cells (Figures 1A and 1B; see also Figures S1A–S1C available online). Immunofluorescence analysis showed that Hklp2 does not localize to the microtubules (MTs) or any other structure during interphase (Figure S1B), although it is a cytoplasmic protein whose expression is not cell-cycle regulated (Figures S1A and S1D). Sometime after nuclear envelope breakdown, Hklp2 could be visualized on the MTs as well as at the periphery of the chromosomes (Figure 1B). At metaphase, Hklp2 decorated the spindle (but not the astral MTs) and the chromosomes in a punctate pattern without colocalizing with the kinetochores (Figure S1C). After metaphase, Hklp2 localization became more diffuse. A similar localization pattern was observed for FLAG-Hklp2 in transfected HeLa cells (Figure 1C). The lack of Hklp2 localization to interphase and early mitotic MTs suggested that its MT-binding properties are tightly regulated. We previously identified the nuclear protein and RanGTP-regulated factor TPX2, as a *t*argeting *p*rotein for *X*klp2 during mitosis [16]. Immunofluorescence analysis on TPX2-silenced HeLa cells [17] showed that Hklp2 did not localize to the mitotic MTs (even after incubation with paclitaxel; Figure S2B) or to the chromosomes (Figure 1D; Figure S2A) in the absence of TPX2. However, the TPX2 partner Aurora A was not required for Hklp2 MT localization (Figure S2C). Pull-down experiments with GST-Xklp2-T [16] and TPX2 recombinant proteins showed that these proteins do not interact directly in vitro (unpublished data), suggesting that the TPX2-dependent targeting of Hklp2 to the mitotic MTs might not involve a stable interaction but a more complex mechanism. This idea was also supported by the clear differences in their localization patterns (Figure 1E). TPX2 localized to MTs earlier than Hklp2 and became enriched toward the spindle poles in metaphase, whereas Hklp2 was comparatively more enriched in the central part of the spindle and the chromosomes. Hklp2 may therefore be in an inactive form in the cytoplasm and become activated or directly loaded onto the MTs by TPX2 or a TPX2-dependent complex after nuclear envelope breakdown. As a plus-end-directed motor (by analogy with Xklp2) [10], Hklp2 could "walk" along the MTs reaching and binding to the chromosomes (see model below).

TPX2 drives MT assembly at the kinetochore and the formation of mature kinetochore fibers [18]. To examine whether Hklp2 associates preferentially to some classes of spindle MTs, we incubated HeLa cells on ice to induce the selective depolymerization of the more dynamic MTs while preserving the K-fibers. Immunofluorescence analysis showed that Hklp2 was very strongly recruited to these MT bundles, although it was not required for their formation (Figure S3A). We then examined Hklp2 localization in Nuf2-silenced HeLa cells that are defective for K-fiber formation [19] and found that Hklp2 also localized to spindle MTs in these cells (Figure S3B). Nocodazole-induced depolymerization of all spindle MTs resulted in the complete loss of Hklp2 localization (Figure S3C). Therefore, Hklp2 associates with both kinetochore and nonkinetochore MTs, although it seems to have a higher affinity for the K-fibers. Interestingly, both types of MTs seem to be required for the localization of Hklp2 to the chromosomes.

Hklp2 Is Involved in Bipolar Spindle Assembly and Stabilization

To gain an insight into Hklp2 function, we silenced its expression by small interfering RNA (siRNA) transfection in HeLa cells. Silenced cells showed an 80% reduction of Hklp2 levels by western blot analysis and barely detectable Hklp2 signal by immunofluorescence (Figure 2A). Although mitotic cells

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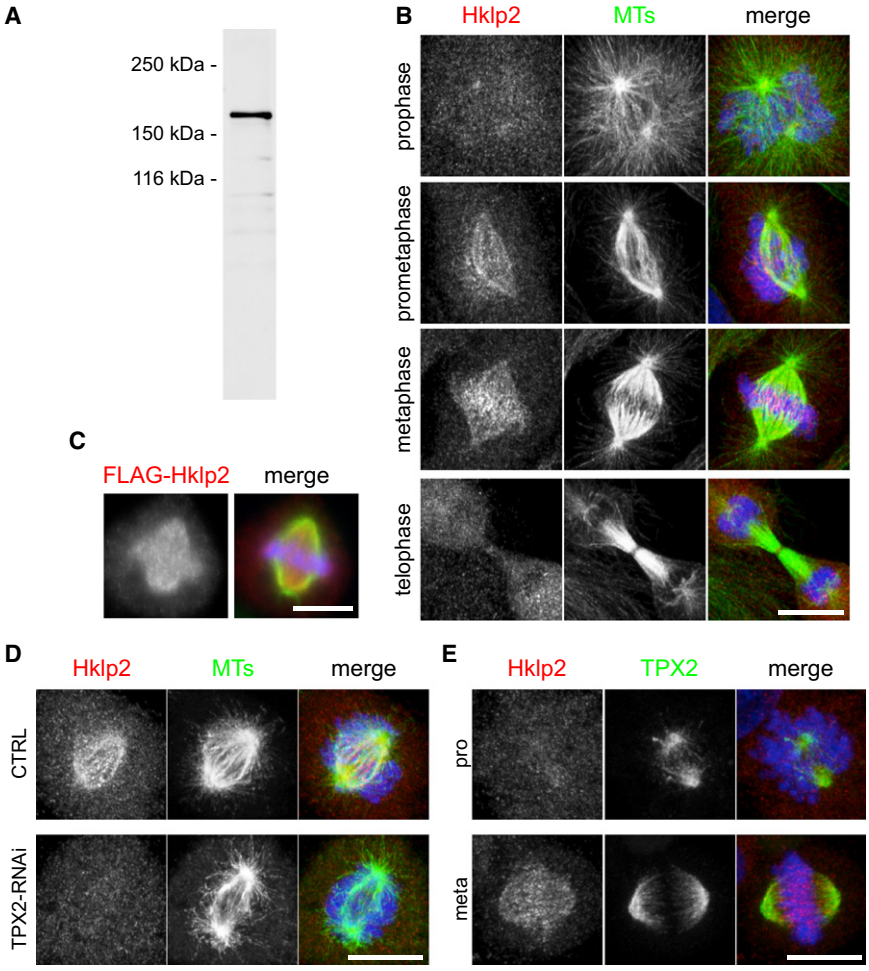

Figure 1. Hklp2 Subcellular Localization

(A) 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 corresponding to the Hklp2 predicted size.

(B) Confocal images of hTERT-RPE1 cells processed for immunofluorescence with anti-Hklp2 (red) and anti-tubulin (green) antibodies. The images are maximum projections of confocal sections acquired every 0.3 μm. Hklp2 associates with the spindle microtubules (MTs) and the chromosomes in metaphase.

(C) HeLa cells transfected with FLAG-Hklp2 and immunostained with anti-FLAG (red) and anti-tubulin (green) antibodies. The exogenously expressed Hklp2 has a localization similar to the endogenous Hklp2 protein.

(D) HeLa cells transfected with control or TPX2-specific siRNAs were processed for immunofluorescence with anti-Hklp2 (red) and anti-tubulin (green) antibodies. Hklp2 fails to localize to the MTs in the absence of TPX2. The images are maximum projections of confocal sections acquired every 0.3 μm.

(E) Immunofluorescence in prophase (pro) and metaphase (meta) HeLa cells showing the localizations of Hklp2 (red) and TPX2 (green). The images are maximum projections of confocal sections acquired every 0.3 μm. TPX2 localizes to the MTs prior to Hklp2 and becomes enriched toward the spindle poles in metaphase, whereas Hklp2 typically localizes to the more central part of the spindle and the chromosomes. In (B)–(E), DNA was stained with DAPI (blue); scale bars represent 10 μm.

had no apparent defects, metaphase spindles were on average 11% shorter than controls ($p < 0.001$) (Figure 2B; Table S1), suggesting that Hklp2 could play a role in spindle pole separation during mitosis. To further investigate this idea, we sensitized control and Hklp2-silenced cells by interfering with Eg5 activity via a range of monastrol concentrations. Immunofluorescence analysis showed that monastrol promoted the formation of monopolar spindles in both control and Hklp2-silenced cells in a concentration-dependent manner (Figure 2C). However, the proportion of monopolar spindles was significantly increased in Hklp2-silenced cells by up to 30% (95% instead of 61% in control cells at 50 μM monastrol, average from three independent experiments; $p < 0.001$) (Figure 2C; Table S2). We next examined whether Hklp2 could participate in the separation of the spindle poles after monastrol washout. We found that when 26% of the control cells had already recovered spindle bipolarity, only 9% of the Hklp2-silenced cells were in a similar state (average from three independent experiments; $p < 0.001$) (Figure 2D), indicating that the separation of the spindle poles was less efficient in the absence of Hklp2. These results further supported a role for Hklp2 in bipolar spindle formation. However, it was not clear whether Hklp2 had a completely redundant function with Eg5 or whether it had a more distinct function that might be difficult to separate because of the dominant role of Eg5 in the establishment of spindle bipolarity. We first examined more closely the respective localizations of Eg5 and Hklp2 during mitosis. Double immunofluorescence analysis showed that Eg5 accumulates around the centrosomes at the onset of mitosis and is therefore positioned at the right location 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, when the spindle is already forming (Figure 3A). It is therefore unlikely that it participates in the initial steps of spindle pole separation, suggesting that its role could take place later. Interestingly, the localization at metaphase of Eg5 and Hklp2 is also clearly distinct: Eg5 localizes to the spindle MTs with a clear enrichment toward the spindle poles, whereas Hklp2 is comparatively more prominently associated with the central part of the spindle and the chromosomes (Figure 3A). Similarly, in cold-treated cells, Eg5 is seen on the remaining MT bundles close to the poles, whereas Hklp2 is more enriched toward the chromosomes (Figure S3D). These observations suggest that their main site of action is different.

Although the stability of the bipolar spindle is believed to rely on a balance of forces exerted by plus- and minus-end motors, in particular Eg5 and dynein [3, 4], there are some contradictory data on the role of Eg5 in bipolar spindle maintenance. Inhibiting Eg5 does not induce bipolar spindle collapse in monkey and marsupial cell lines [6, 7]; however, it does so in *Xenopus* egg extracts [3]. To determine whether Eg5 is required for the maintenance of spindle bipolarity in HeLa cells, we used the proteasome inhibitor MG132 to promote a metaphase arrest with a fully assembled bipolar spindle (Figures 3B and 3C). Addition of monastrol to these cells did

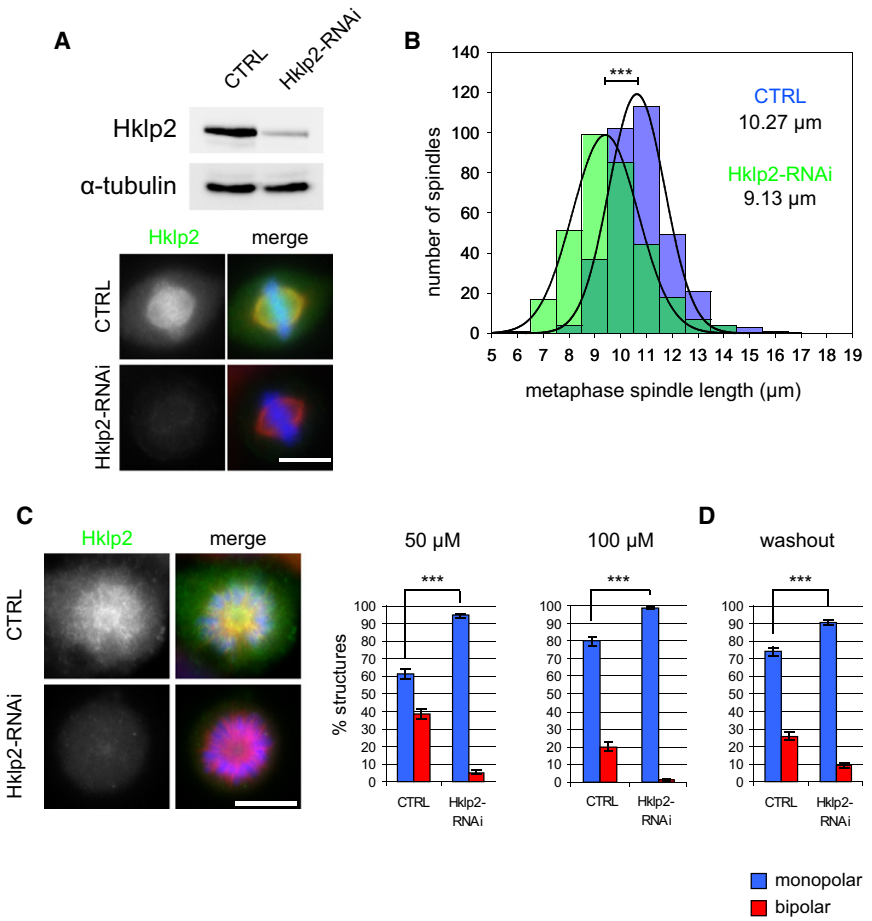

Figure 2. Hklp2 Silencing Influences Spindle Pole Separation

(A) Top: western blot of control and Hklp2-specific siRNA-transfected HeLa cells showing a reduction of Hklp2 levels to around 80% in the silenced cells. Bottom: immunofluorescence in control and Hklp2-specific siRNA-transfected HeLa cells showing that Hklp2 is barely detectable in metaphase in the silenced cells. In the merged overlay, Hklp2 is green, MTs are red, and DNA is blue.

(B) Histogram of metaphase spindle lengths in control (blue bars) and Hklp2-silenced (green bars) HeLa cells 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) Left: immunofluorescence in HeLa cells transfected with control or Hklp2-specific siRNAs and incubated with 50 μM or 100 μM monastrol (Hklp2, green; MTs, red; DNA, blue). Right: frequency of bipolar and monopolar spindles found in each condition. Scale bars in (A) and (C) represent 10 μm.

(D) Quantification of the proportion of monopolar spindles and reforming bipolar spindles at 10 min after 100 μM monastrol washout. The data for (C) and (D) were obtained from >100 spindles in each of three independent experiments. Error bars correspond to the standard error of the proportion (***$p < 0.001$).

not promote bipolar spindle collapse (Figures 3B and 3C; Movie S1), strongly suggesting that Eg5 activity is not essential for the maintenance of spindle bipolarity in HeLa cells. We then tested whether Hklp2 could play a role in this process. Hklp2-silenced cells incubated with MG132 also arrested in metaphase with control-like bipolar spindles (Figures 3B and 3C). However, adding monastrol to the medium resulted in a dramatic effect on spindle bipolarity with a 10-fold increase of metaphase spindle collapse without passing through an intermediate-length stage as observed in live cells (61% of monopolar spindles compared to 6% in control cells, average from three independent experiments; $p < 0.001$) (Figures 3B and 3C; Movie S2). Therefore, both Eg5 and Hklp2 contribute to the stability of the bipolar spindle. When Eg5 does not function, Hklp2 becomes essential, and vice versa.

But are the mechanisms of action of these two kinesin motors similar? Their different spatial and temporal localization patterns indicate that they are likely to be very different. In addition, Eg5 is a homotetramer, whereas Hklp2 is a dimer that requires TPX2 (a nuclear protein) to localize to the MTs in mitosis. Moreover, overexpression of Eg5 or Hklp2 in HeLa cells has dramatically different consequences on bipolar spindle assembly. Indeed, the overexpression of GFP-Eg5, even at extremely high levels, remarkably did not interfere with bipolar spindle formation, whereas Hklp2 overexpression induced strong mitotic abnormalities including multipolar spindles and completely disorganized MT structures (Figure 3D).

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

Hklp2 Chromosomal Localization Regulates Its Activity during Mitosis

One characteristic of Hklp2 is its chromosomal localization. A previous report showed that Hklp2 interacts with Ki67 [20], a chromosome-associated proliferation marker whose function during mitosis is currently unknown [21]. Pull-down experiments from FLAG-Hklp2-expressing HeLa cells confirmed this interaction (data not shown), and we also found that Hklp2 chromosomal localization was completely abolished in Ki67-silenced cells (Figure 4A).

Ki67-silenced cells formed bipolar spindles, but some chromosome alignment defects were observed in fixed (Figure S4A) and live (Movies S3 and S4) cells. Immunofluorescence analysis did not reveal any change in the localization of chromosome-associated kinesins (Kid, Kif4, and CENP-E; Figures S4B–S4D) and other spindle-associated factors (TPX2 and Aurora A; data not shown) in mitotic Ki67-silenced cells. We therefore used Ki67-silenced cells to examine the functional consequences of impairing Hklp2 association with the chromosomes. Measuring metaphase spindle length in Ki67-silenced cells showed that they were on average 12% longer than in control cells (average from three independent experiments; $p < 0.001$) (Figure 4B). This suggested that pushing forces are more efficient when Hklp2 does not interact with the chromosomes. To test this idea, we treated the Ki67-silenced cells with monastrol and found that these cells indeed

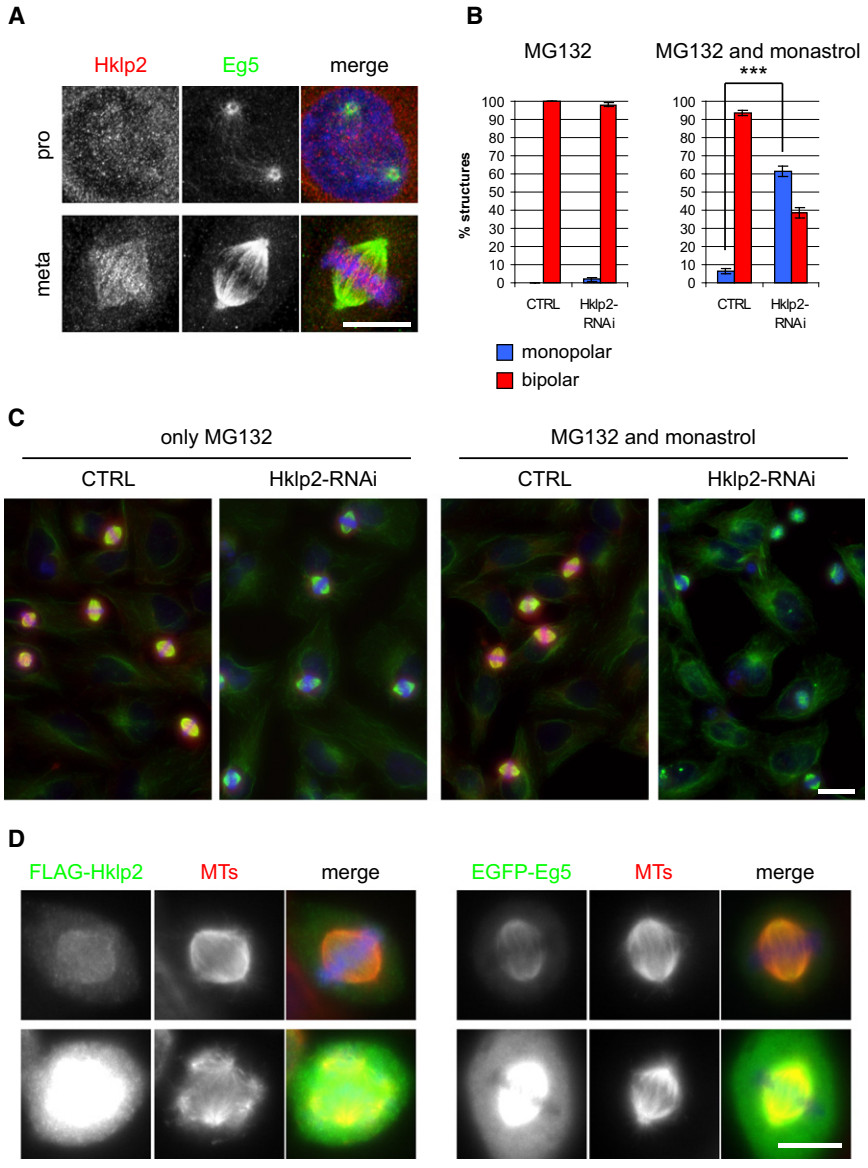

Figure 3. Hklp2 Is Required for the Maintenance of Spindle Bipolarity

(A) Immunofluorescence images of HeLa cells in prophase and metaphase showing the respective localizations of Hklp2 (red) and Eg5 (green). Scale bar represents 10 μm.

(B) Quantification of bipolar and monopolar spindles in control and Hklp2-silenced HeLa cells incubated for 4 hr with MG132 or for 2 hr with MG132 followed by an additional 2 hr in the presence of 100 μM monastrol. Hklp2 silencing leads to the destabilization of the bipolar metaphase spindles. Data were obtained from >100 spindles in each of three independent experiments. Error bars correspond to the standard error of the proportion (***$p < 0.001$).

(C) Immunofluorescence images of HeLa cells treated as in (B) (Hklp2, red; MTs, green; DNA, blue). Scale bar represents 20 μm.

(D) HeLa cells overexpressing FLAG-Hklp2 or EGFP-Eg5 were immunostained with anti-FLAG (green) and anti-tubulin (red) antibodies. DNA (blue) was marked with DAPI. Cells expressing low amounts of the proteins form a bipolar spindle. High expression of EGFP-Eg5 does not interfere with bipolar spindle assembly, whereas high expression of FLAG-Hklp2 impairs the formation of the bipolar spindle and promotes highly aberrant MT assemblies. Scale bar represents 10 μm.

were less sensitive to Eg5 inhibition than control cells: they were able to form 44% of bipolar spindles, whereas control cells formed only 17% of bipolar spindles (average from three independent experiments; $p < 0.001$) (Figure 4C). Ki67-silenced cells were also more efficient in recovering the bipolar state after monastrol washout (73% of bipolar spindles versus 28% in control cells, average of three independent experiments; $p < 0.001$) (Figure 4D). To rule out the possibility that other factors apart from Hklp2 could be responsible for these phenotypes, we simultaneously silenced Ki67 and Hklp2 by transfecting HeLa cells with both specific siRNAs. All of the phenotypes observed in these cosilenced cells were similar to those observed in cells silenced only for Hklp2 (spindle length, monastrol treatment, and monastrol washout; Figure S5). These results indicate that the phenotypes observed in Ki67-silenced cells are essentially generated by the lack of Hklp2 association with the chromosomes. One possibility is that the interaction of Hklp2 with the chromosomes alters the rate of its dissociation from the MT plus ends participating in the balance of forces generated at the chromosome surface (Figure 4E). Because this involves other motors, in particular the chromokinesins, a combination of experimental and theoretical computer simulations will be required to obtain a global understanding of the contribution of these forces in spindle assembly. In any case, our results suggest that the steady-state distribution of Hklp2 between the spindle MTs and the chromosomes is important for spindle dynamics.

In summary, our data provide evidence for a role of Hklp2 in bipolar spindle assembly and stability, in agreement with the results of Tanenbaum et al. ([22], this issue of *Current Biology*), suggesting the existence of different mechanisms to provide a general cohesion and stability to this dynamic structure. Further work will be required to fully understand how, through their distinct spatial and temporal modes of action, Eg5 and Hklp2 contribute to the maintenance of genomic stability.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, five figures, two tables, and four movies and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01697-2.

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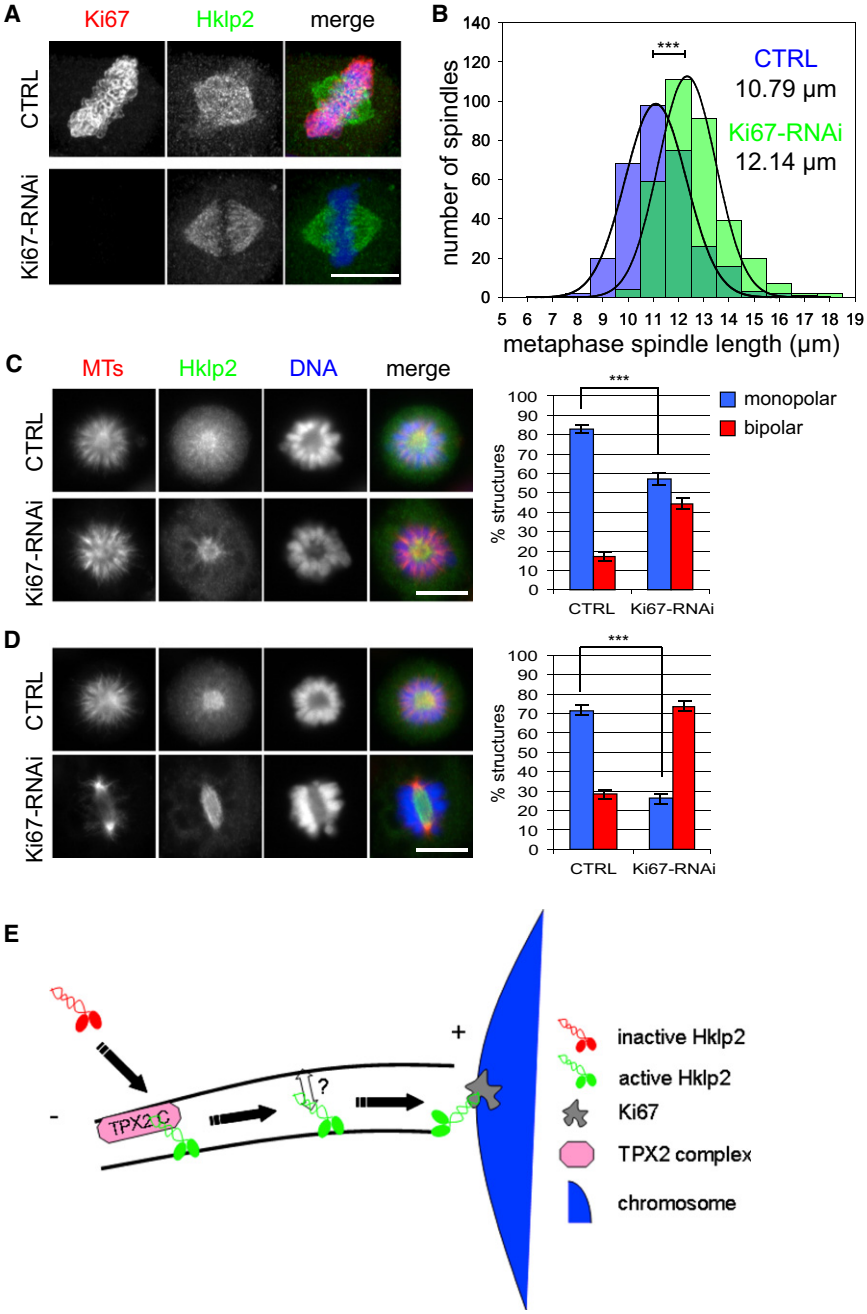

Figure 4. Hklp2 Chromosomal Localization through Ki67 Is Involved in Bipolar Spindle Dynamics

(A) Immunofluorescence in control and Ki67-silenced cells shows that Hklp2 fails to localize to the chromosomes in the absence of Ki67. In the merged overlay, Ki67 is red, Hklp2 is green, and DNA is blue.

(B) Histogram of metaphase spindle lengths in control (blue bars) and Ki67-silenced (green bars) HeLa cells 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 Ki67-silenced cells are on average 12% longer than controls. ***$p < 0.001$.

(C) Left: immunofluorescence in HeLa cells transfected with control or Ki67-specific siRNAs and incubated with 100 μM monastrol. In the merged overlay, MTs are red, Hklp2 is green, and DNA is blue. Note that in the absence of Ki67, Hklp2 is no longer associated with the chromosomes and appears to concentrate on the MTs at the center of the monaster. Right: corresponding quantification of monopolar and bipolar spindles.

(D) Left: immunofluorescence in HeLa cells transfected with control or Ki67-specific 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 monaster. Right: corresponding quantification of monopolar and bipolar spindles. The data for (C) and (D) were obtained from >100 spindles in each of three independent experiments. Error bars correspond to the standard error of the proportion (***$p < 0.001$). Scale bars in (A), (C), and (D) represent 10 μm.

(E) Hklp2 is in an inactive state in the cytoplasm (red). After nuclear envelope breakdown, it is targeted to the MTs by a TPX2-dependent pathway and, together with some as yet unidentified factor, crosslinks MTs, perhaps helping Eg5 sliding activity. By "walking" toward MT plus ends, Hklp2 reaches the chromosomes and associates with Ki67 (gray), providing a dynamic link between the MT and the surface of the chromosome.

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