Current Biology, Vol. 14, 1712–1722, October 5, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.cub.2004.09.049

Roles of Polo-like Kinase 1 in the Assembly of Functional Mitotic Spindles

Izabela Sumara,[1,4,5] Juan F. Giménez-Abián,[1,2,4] Daniel Gerlich,[3] Toru Hirota,[1] Claudine Kraft,[1] Consuelo de la Torre,[2] Jan Ellenberg,[3] and Jan-Michael Peters[1,*]

[1]Research Institute of Molecular Pathology
Dr. Bohr-Gasse 7
1030 Vienna
Austria

[2]Centro de Investigaciones Biológicas
Consejo Superior de Investigaciones Científicas
Ramiro de Maeztu, 9
28040 Madrid
Spain

[3]European Molecular Biology Laboratory
Meyerhofstrasse 1
69117 Heidelberg
Germany

Summary

Background: The stable association of chromosomes with both poles of the mitotic spindle (biorientation) depends on spindle pulling forces. These forces create tension across sister kinetochores and are thought to stabilize microtubule-kinetochore interactions and to silence the spindle checkpoint. Polo-like kinase 1 (Plk1) has been implicated in regulating centrosome maturation, mitotic entry, sister chromatid cohesion, the anaphase-promoting complex/cyclosome (APC/C), and cytokinesis, but it is unknown if Plk1 controls chromosome biorientation.

Results: We have analyzed Plk1 functions in synchronized mammalian cells by RNA interference (RNAi). Plk1-depleted cells enter mitosis after a short delay, accumulate in a preanaphase state, and subsequently often die by apoptosis. Spindles in Plk1-depleted cells lack focused poles and are not associated with centrosomes. Chromosomes attach to these spindles, but the checkpoint proteins Mad2, BubR1, and CENP-E are enriched at many kinetochores. When Plk1-depleted cells are treated with the Aurora B inhibitor Hesperadin, which silences the spindle checkpoint by stabilizing microtubule-kinetochore interactions, cells degrade APC/C substrates and exit mitosis without chromosome segregation and cytokinesis. Experiments with monopolar spindles that are induced by the kinesin inhibitor Monastrol indicate that Plk1 is required for the assembly of spindles that are able to generate poleward pulling forces.

Conclusions: Our results imply that Plk1 is not essential for mitotic entry and APC/C activation but is required for proper spindle assembly and function. In Plk1-depleted cells spindles may not be able to create enough tension across sister kinetochores to stabilize microtubule-kinetochore interactions and to silence the spindle checkpoint.

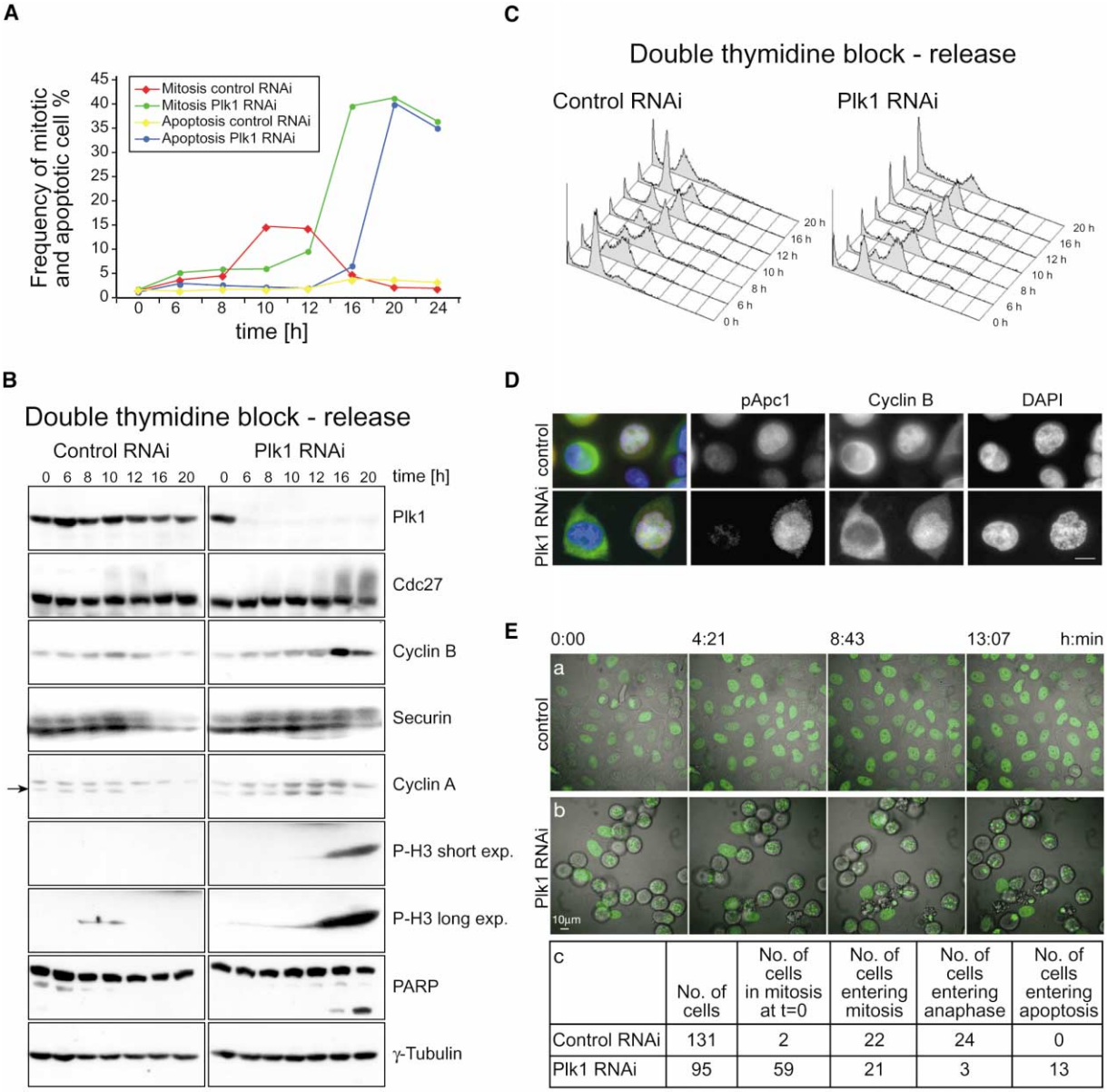
*Correspondence: peters@imp.univie.ac.at
[4]These authors contributed equally to this work.
[5]Present address: Institute of Biochemistry, Eidgenoessische Technische Hochschule, Hoenggerberg, Zuerich, Switzerland.

Introduction

Sister chromatid separation and the subsequent formation of two genetically identical daughter cells depend on the symmetrical attachment of all chromosomes to the mitotic spindle, a process called chromosome biorientation. The assembly of bipolar spindles is preceded by the movement of centrosomes toward opposite poles of the cell, where these structures function as nucleation sites for microtubule polymerization. In prometaphase, microtubules emanating from the spindle poles establish contacts with kinetochores on chromosomes through a stochastic "search and capture" mechanism [1]. The binding of microtubules to kinetochores is reversible as long as physical tension at the kinetochore is low. Once both sister kinetochores of one chromosome have been captured by microtubules from opposite spindle poles (amphitelic attachments), spindle pulling forces generate tension across sister kinetochores, and this tension is thought to stabilize the microtubule-kinetochore interactions [2, 3]. The stabilization of microtubule-kinetochore interactions allows the kinetochore to become eventually fully occupied with microtubules in the case of mammalian cells with a total of 15–30 microtubules per kinetochore (reviewed in [4]).

Sister chromatid separation in anaphase is initiated only once all chromosomes have been attached to both poles of the spindle with a full complement of microtubules [5, 6]. Before this metaphase stage has been reached, anaphase initiation is inhibited by the spindle checkpoint, a surveillance mechanism that monitors the presence of kinetochores that are not fully occupied with microtubules (reviewed by [7]). At such kinetochores, Mad2 and possibly other checkpoint proteins are activated [8]. These proteins inhibit the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase that initiates anaphase by mediating the destruction of the separase inhibitor securin and of B type cyclins, the activating subunits of cyclin-dependent kinase 1 (Cdk1). Once activated, the protease separase enables sister chromatid separation by cleaving the Scc1 subunit of cohesin, a protein complex that mediates cohesion between sister chromatids (reviewed by [9]).

Several mitosis-specific protein kinases have been implicated in bipolar spindle assembly and chromosome biorientation. For example, Cdk1 is known to phosphorylate Eg5, a kinesin that is required for the migration of centrosomes and is thus needed for the formation of bipolar spindles [10, 11]. Eg5 phosphorylation is required for its recruitment to centrosomes [10]. Cdk1 may therefore control the onset of centrosome migration by phosphorylating Eg5. Another kinase, called Aurora B, is essential for proper chromosome biorientation (reviewed by [12]). During spindle assembly, chromosomes can not only become attached to opposite spindle poles, but occa-



c	No. of cells	No. of cells in mitosis at t=0	No. of cells entering mitosis	No. of cells entering anaphase	No. of cells entering apoptosis
Control RNAi	131	2	22	24	0
Plk1 RNAi	95	59	21	3	13

Figure 1. Plk1 Depletion Delays Entry into Mitosis and Progression through Prometaphase

(A–D) HeLa cells were synchronized at the G1/S transition by double thymidine treatment. Transfection mixtures without (control RNAi) or with Plk1 siRNAs (Plk1 RNAi) were added to the cells for 4 hr, beginning at the time of second thymidine addition. Cells were collected at the indicated time points after the second thymidine release. (A) Cells were analyzed by chromosome spreading and Giemsa staining, and the frequency of mitotic and apoptotic cells was calculated. (B) Cell lysates were generated and analyzed by immunoblotting with antibodies to the proteins indicated. For histone H3 phosphorylated on Ser10 (P-H3), short and long exposures (exp.) are shown. Cyclin A is indicated by an arrow. The slower migrating band in the same blot is a crossreacting protein. (C) Cells were fixed, stained with propidium iodide, and DNA content was analyzed by flow cytometry. (D) Cyclin B1 nulcear uptake in Plk1-depleted cells. Synchronized cells transfected either with control or Plk1 siRNA were fixed and immunostained with phopho-Apc1 (red) and cyclin B1 (green), and DNA was couterstained with DAPI (blue). Late prophase cells were defined by nuclear phospho-Apc1 staining [26], and scored for nuclear cyclin B staining. Scale bar, 10 μm.

(E) Time-lapse imaging of cells transfected with control (a) or Plk1 (b) siRNA mixtures. Logarithmically proliferating HeLa cells expressing EGFP-tagged histone H2B were filmed for 13 hr, beginning 28 hr after transfection. Projections of 3D image stacks of EGFP were overlaid on transmission light images. Time is indicated in hrs and minutes (h:min). (c) Quantification of the 13 hr time-lapse experiment. No., number; t = 0, start of filming.

sionally both sister kinetochores are also captured by microtubules emanating from the same pole. Such syntelic states are corrected by an Aurora B-dependent mechanism [13, 14]. Aurora B is also required for spindle checkpoint signaling at kinetochores that are not under tension [14, 15], possibly because Aurora B's correction function leads to the formation of partly unattached kinetochores at which Mad2 can be activated [14].

A third kinase that is required for bipolar spindle assembly is Polo. Mutation of Polo in *Drosophila* leads to

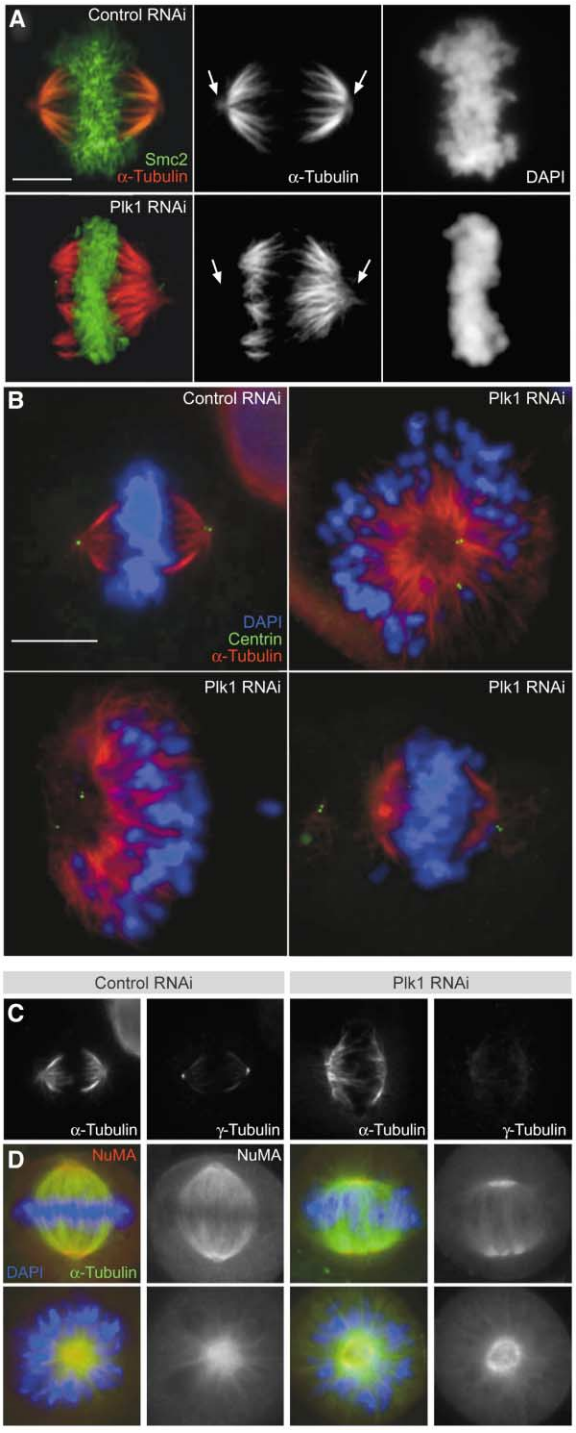


Figure 2. Spindle Defects in Plk1-Depleted Cells

HeLa cells were synchronized and transfected as in Figures 1A–1D, collected 13 hr after the release from the second thymidine block, and processed for immunofluorescence microscopy. Repesentative pictures of cells transfected with control or Plk1 siRNA are shown as indicated. Bars indicate 10 μm.

(A) Spindles in Plk1 RNAi cells lack spindle poles. Cells were stained with Smc2 antibodies (green) to probe chromosome axes, together with α-tubulin antibodies (red). Note that focused spindle poles indicated by arrows are seen in control cells, but not in Plk1 RNAi cells.

(B) Plk1 depletion causes the formation of acentrosomal spindles. Cells were costained with antibodies to centrin (green) and α-tubulin (red). DNA was conterstained with DAPI (blue). Examples of a spindle in polar view (upper right), a monopolar spindle in side-on view (lower left) and a bipolar spindle in side-on view (lower right) are shown. Additional examples of Plk1 RNAi cells are shown in the Supplemental Figure S2B.

(C) Plk1 is required for γ-tubulin recruitment to centrosomes. Cells were costained with antibodies to γ-tubulin and α-tubulin. Note that γ-tubulin levels are unchanged in Plk1 RNAi cells (Figure 1B), indicating that loss of γ-tubulin from centrosomes is due to delocalization and not to destruction of the protein.

(D) NuMA enrichment at spindle poles is not abolished by Plk1 depletion. Cells were stained with antibodies to α-tubulin (green) and to NuMA (red).

the accumulation of cells that contain monopolar spindles, spindles with broad poles, or multipolar spindles [16, 17]. Inactivation of Polo orthologs in fission yeast and human cells also results in the formation of monopolar spindles [18, 19]. These spindle abnormalities might be caused by centrosomal defects, because inactivation of Polo or its orthologs leads to failures in centrosome maturation and migration [16, 17, 19]. In *Drosophila*, Polo phosphorylates the Asp protein, which is required for the recruitment of γ-tubulin to centrosomes and for the formation of spindles with focused poles [20]. γ-tubulin is part of a complex that is required for nucleating microtubules at centrosomes [21]. In human cells, Polo-like kinase 1 (Plk1) is also required for the association of γ-tubulin with centrosomes [19], and in both species, the kinase itself is enriched at centrosomes (reviewed in [22]).

Inactivation of Polo or its orthologs also results in other phenotypes besides defects in spindle assembly, presumably reflecting the existence of numerous critical kinase substrates. In vertebrates, Plk1 has been found to be required for entry into mitosis, for the dissociation of cohesin complexes from chromosomes, for activation of the APC/C, and for cytokinesis (reviewed in [22]). Consistent with having multiple functions, Plk1 is not only enriched at centrosomes but is also found at kinetochores, the midspindle, and dispersed throughout the nucleocytoplasmic space of mitotic cells [22].

Here, we have systematically analyzed early mitotic functions of Plk1 in synchronized mammalian cells by using RNA interference (RNAi). Our data imply that Plk1 is required for the formation of spindles that are able to generate proper poleward pulling forces. In the absence of Plk1, spindles seem unable to create the tension across sister kinetochores that is needed to stabilize microtubule-kinetochore interactions. As a result, chromosomes do not become stably attached to the spindle, and cells arrest due to activation of the spindle checkpoint. Our results further indicate that normal Plk1 levels are not essential for mitotic entry and for activation of the APC/C.

Results and Discussion

Plk1 Depletion Delays Entry Into Mitosis

The *Xenopus* ortholog of Polo kinase, Plx1, is required for mitotic entry [23, 24], but mutation of Polo in *Drosophila* [16] or of Polo-like kinases in yeasts (reviewed

Table 1. Quantification of Spindle Phenotypes in Control and Plk1-Depleted Cells

Spindle Morphology			Control RNAi	Plk1 RNAi
Side-on view	Bipolar	Focused	62%	1%
		Unfocused	4.5%	36%
	Monopolar	Focused	2%	0%
		Unfocused	0%	18%
	Multipolar	Focused	2.9%	0%
		Unfocused	0%	0%
Polar view		Focused	27%	2%
		Unfocused	0.4%	43%

500 control and 500 Plk1 RNAi cells were analyzed.

in [22]) leads to accumulation of cells in mitosis. Injection of Plk1 antibodies causes human HeLa cells to accumulate in mitosis, whereas Plk1 antibodies prevent mitotic entry in nonimmortalized fibroblasts [19]. To further investigate if Plk1 is required for mitotic entry in human cells, we used siRNA transfection to inhibit Plk1 expression in HeLa cells that were synchronized in S phase by double thymidine treatment. Because Plk1 is degraded during mitotic exit [25] once the transfected cells are released from the S phase arrest, this method allows the depletion of almost all Plk1 molecules that are detectable by immunoblotting (Figure 1B) and immunofluorescence microscopy [26]. We then analyzed progression from S phase throughout the subsequent mitosis in the absence of Plk1 (Figures 1A–1E). Quantitative analysis by chromosome spreading and Giemsa staining indicated that Plk1 RNAi cells entered mitosis 2–4 hr later than control cells, and either remained in mitosis until the end of the experiment (24 hr) or entered a state with highly condensed and fragmented chromatin that resembled apoptosis. In contrast, control transfected cells entered G1 between 16 and 18 hr (Figure 1A). FACS analysis of DNA content confirmed that the bulk of Plk1-depleted cells failed to complete cell division (Figure 1C). Plk1 has been reported to be required for nuclear uptake of cyclin B-Cdk1 [27], but immunofluorescence microscopy showed that all late prophase cells, identified by staining of intact nuclei with antibodies to a phosphoepitope on the APC/C subunit Apc1, contained cyclin B, independent of whether Plk1 had been depleted (63/63) or not (46/46; Figure 1D). Immunoblot analyses revealed that cyclin B, securin, and phosphorylated forms of histone H3 and the APC/C subunit Cdc27 accumulated in Plk1 RNAi cells, further supporting the notion that many of these cells accumulated in a mitotic state (Figure 1B). Cleavage products of the enzyme poly-ADP ribose polymerase (PARP) were also seen (Figure 1B), consistent with the apoptotic appearance of many cells in the chromosome spreads (Figure 1A). Apoptosis in Plk1 RNAi cells has also been reported by Liu and Erikson [28].

Mitotic entry of vertebrate cells depends on activation of Cdk1 by the phosphatase Cdc25C and on nuclear uptake of cyclin B-Cdk1 (reviewed in [29]). Both events are thought to depend on Plx1/Plk1 [23, 24, 27]. Our data are consistent with this notion insofar as Plk1 RNAi cells show a significant delay in mitotic entry. However, the observation that most Plk1-depleted cells eventually enter mitosis and translocate cyclin B into the nucleus implies that in somatic human cells normal Plk1 levels are not absolutely essential for these processes. In contrast to Toyoshima-Morimoto et al., (2001) [27], Jackman et al. (2003) have reported that overexpression of constitutively active Plk1 does not accelerate the nuclear uptake of cyclin B [30]. The latter findings and our results together imply that Plk1 is neither required nor sufficient for translocation of cyclin B1 into the nucleus.

Plk1 Depletion Causes a Prometaphase Arrest

To analyze how Plk1 depletion affects progression through mitosis, we depleted Plk1 from logarithmically proliferating HeLa cells stably expressing an EGFP-tagged version of histone H2B [31] and filmed them by time-lapse video microscopy (Figure 1E). 28 hr after control transfection, 2% (2/131) of all cells were in mitosis, and 17% of all cells (22/131) entered mitosis during the subsequent 13 hr observation period. All mitotic cells (24/24) entered anaphase and completed cytokinesis normally (Figures 1Ea and 1Ec; Movie 1). In contrast, 62% (59/95) of Plk1 RNAi cells were in a prometaphase-like state 28 hr after transfection; i.e., they had failed to form normal metaphase plates, further supporting that Plk1 depletion causes a mitotic arrest. 22% of all cells (21/95) entered mitotis during the subsequent 13 hr (Figures 1Eb and 1Ec; Movie 2). Only 3.8% of all mitotic Plk1 RNAi cells (3/80) entered anaphase, 16% (13/80) underwent apoptosis, and the remaining cells maintained a prometaphase-like morphology. Prometaphase delay was also observed when Plk1 was depleted from cultured rat NRK cells (Supplemental Figure S1). These results indicate that Plk1 is required for chromosome congression and subsequent anaphase initiation. Plk1 may also be required for the completion of cytokinesis (Supplemental Figure S1; [32]), but in our experiments we were not able to distinguish if the cytokinetic defects were a direct effect of Plk1 depletion or an indirect consequence of chromosome segregation defects.

Plk1 Is Required for the Formation of Focused Spindle Poles that Are Associated with Centrosomes

Because abnormal spindles are observed in *Drosophila polo* mutants [16, 17] and in human cells injected with Plk1 antibodies [19], we analyzed by immunofluorescence microscopy if spindle defects could be responsible for the prometaphase delay in Plk1 RNAi cells. In 66.5% of control HeLa cells, bipolar spindles could be seen in side-on views, and most of these contained

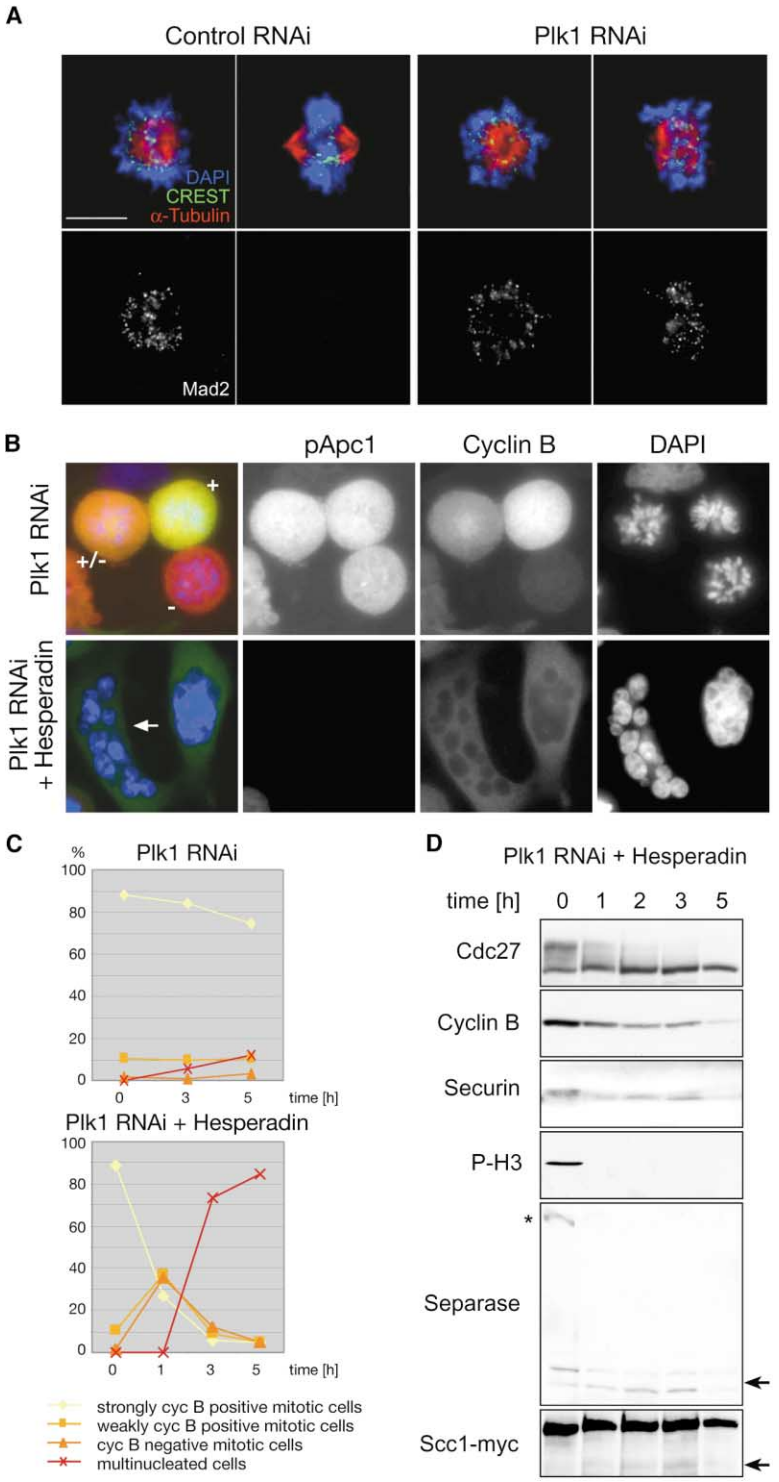


Figure 3. Plk1 Depletion Activates the Spindle Checkpoint

(A) Localization of Mad2 at kinetochores. HeLa cells transfected with either control or Plk1 siRNAi as in Figures 1A–1D were costained with antibodies to kinetochores (CREST; green in upper panel), α-tubulin (red in upper panel), and Mad2 (lower panel). DNA was counterstained with DAPI (blue in upper panel). The control cells shown are in prometaphase (left) and metaphase (right). In the Plk1 RNAi cells spindles are seen in polar (left) and side-on views (right). Size bar 10 μm.

(B–D) Hesperadin treatment causes mitotic exit in Plk1 RNAi cells. Plk1 RNAi cells were obtained as in Figures 1A–1D. 100 nM Hesperadin was added 13 hr after the second thymidine release. (B and C) At the indicated time points after Hesperadin addition, cells were fixed with paraformaldehyde and analyzed by immunofluorescence through antibodies to a mitosis-specific phosphosite on the APC/C subunit Apc1 (pApc1) and to cyclin B. DNA was stained with DAPI. (B) shows representative images of cells with high (+), weak (+/−) or no (−) cyclin B (cyc B) staining and a multinucleated cell (indicated by an arrow). (C) Statistics of the same experiment as in (B). Between 56 and 91 cells were counted for each time point. (D) Cell lysates were generated at the indicated time points after Hesperadin addition and analyzed by immunoblotting with the indicated antibodies. Uncleaved separase is indicated by a star, and separase and Scc1-myc cleavage products are indicated by arrows. The form of histone H3 that is phosphorylated on Ser10 (P-H3) disappears more rapidly after Hesperadin addition than cyclin B, securin, and the slowly migrating phosphorylated form of Cdc27 because Hesperadin inhibits Aurora B, which is required for H3-Ser10 phosphorylation [14]. The data in (B and C) and (D) are from separate experiments.

clearly focused spindle poles. In contrast, only 37% of Plk1 RNAi cells contained bipolar spindles in side-on views, and almost all of these (97%) were lacking focused poles (Figures 2A–2D, Table 1). We also observed high frequencies of monopolar spindles that could be seen in side-on views in Plk1 RNAi cells (18% compared to 2% in control cells; Figure 2B, lower left) and of spindles whose axes were oriented perpendicular to the plane of the culture dish, resulting in polar views (45% in Plk1 RNAi cells compared to 27.4% in control cells; Figure 2B, upper right). In the latter case we could not unequivocally determine the frequency of monopolar versus bipolar spindles due to the orientation of the spindle axes. However, both in side-on views of monopolar spindles and in polar views it was apparent that almost all Plk1 RNAi cells were lacking focused spindle poles.

In a few Plk1-depleted cells, staining with α-tubulin

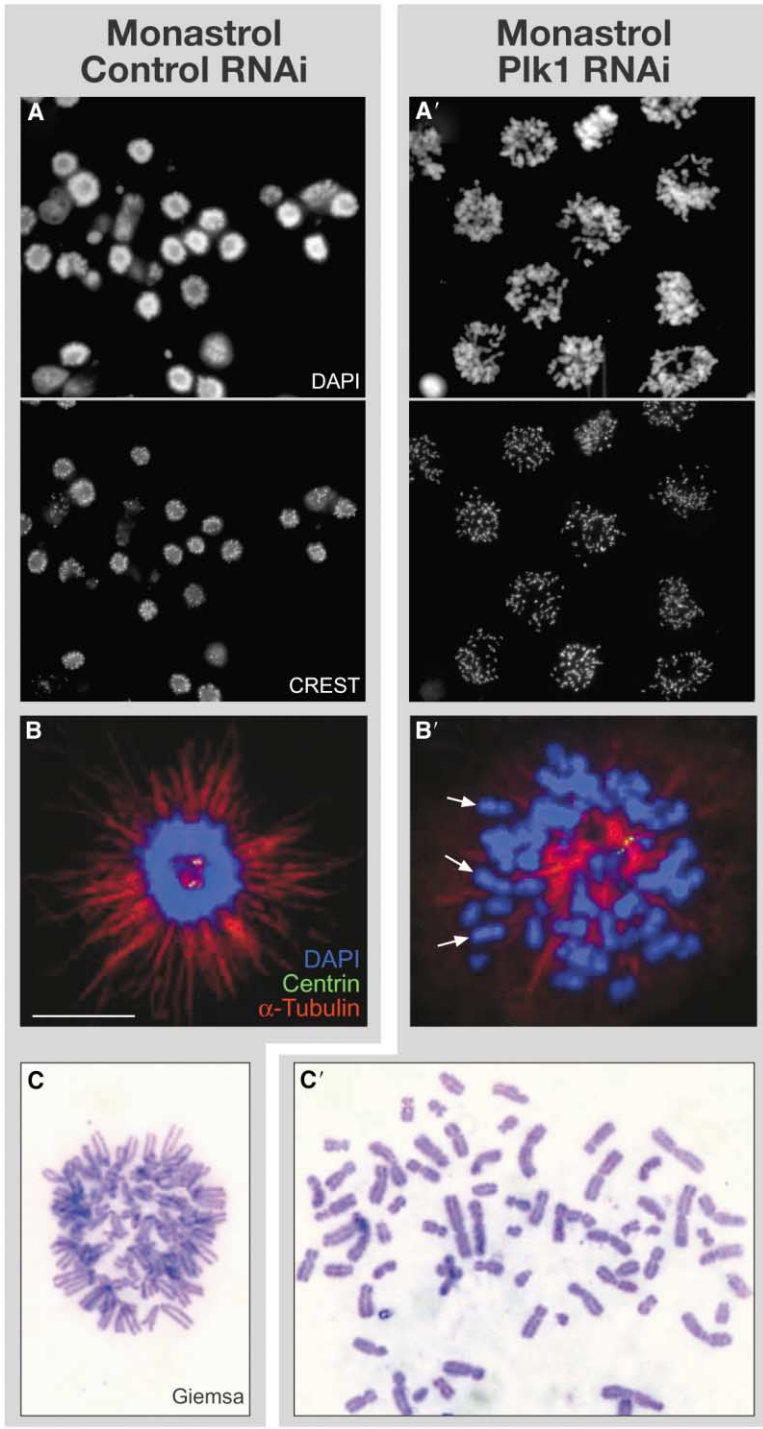


Figure 4. Chromosomes Do Not Attach Properly to Monopolar Spindles in Plk1 RNAi Cells

HeLa control (A–C) and Plk1 RNAi cells (A′–C′) were synchronized in S phase by thymidine treatment and released for 13 hr from the thymidine block in the presence of 100 μM Monastrol. (A–B′) Cells were processed for immunofluorescence microscopy as in Figure 2 and were stained with CREST antibodies (A and A′) or were costained with antibodies to centrin (green) and α-tubulin (red; B, B′). DNA was counterstained with DAPI (blue). The arrows in (B′) indicate chromosomes that show a straight appearance. Scale bar, 10 μm. (C and C′) Cells were analyzed by chromosome spreading and Giemsa staining.

antibodies revealed rosette-like structures in the cell periphery that looked like spindle poles that were detached from the mitotic spindle (Figure 2B, lower right, Supplemental Figure S2), but in most cases astral microtubules could not be seen. We therefore analyzed centrosomes in Plk1 RNAi cells by staining with antibodies to centrin and γ-tubulin, proteins that are part of centrioles and the pericentriolar material, respectively [33]. In control cells both centrin and γ-tubulin antibodies stained centrosomes that formed the center of spindle poles. Centrin antibodies often stained two double dots that represent the two pairs of centrioles. Plk1 RNAi cells also contained two centriole pairs that in most cases were separated from each other, but often these seemed to be randomly distributed in the area of the spindle or even outside of it (Figure 2B). γ-tubulin could not be detected at centrosomes in Plk1 RNAi cells (Figure 3C), confirming earlier observations [19, 34]. Disruption of dynein/dynactin-dependent transport of the NuMA protein to minus ends of microtubules causes spindle pole defects similar to those seen after Plk1 depletion [35]. However, NuMA was also enriched at spindle poles

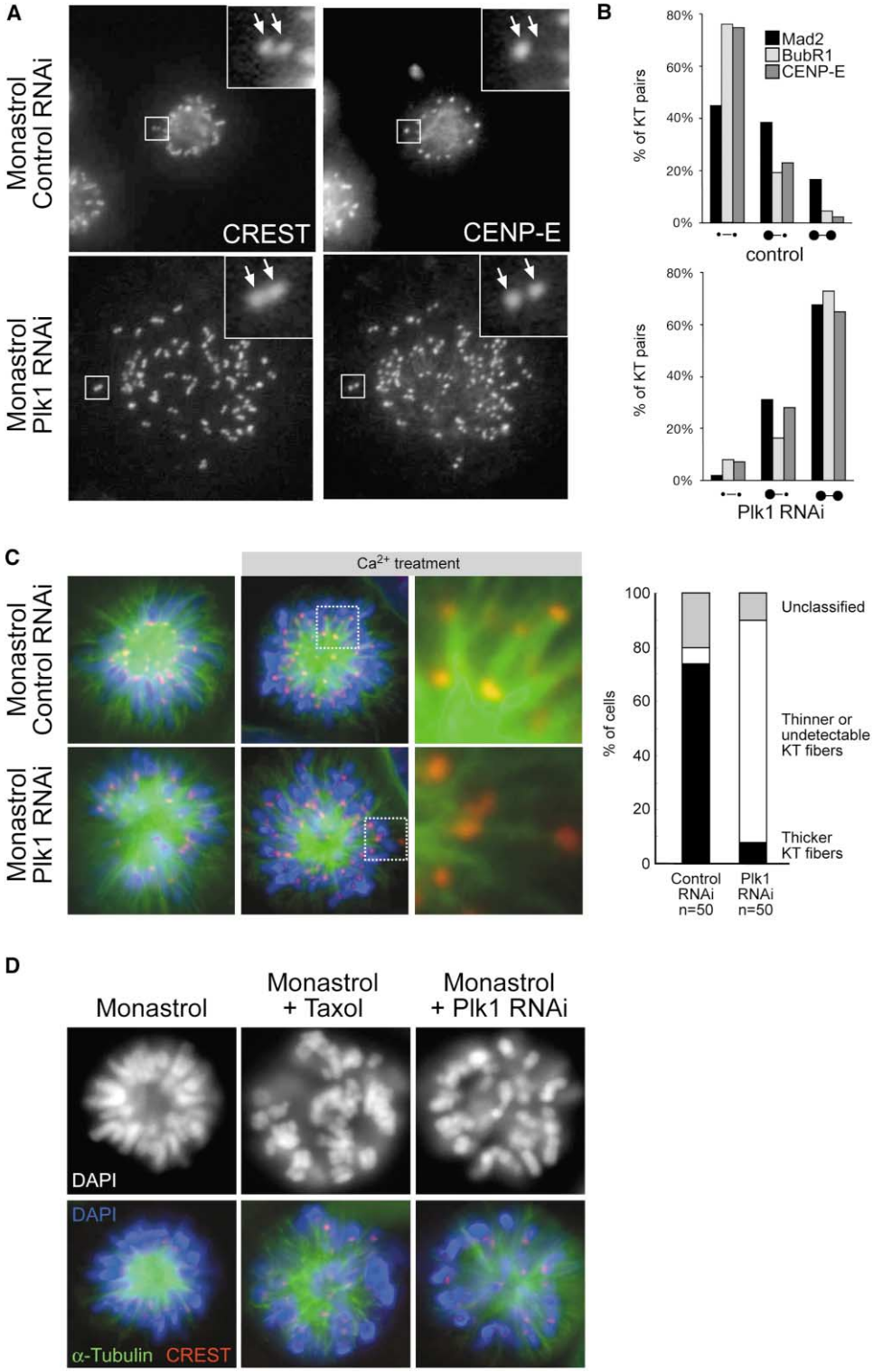


Figure 5. Impaired Kinetochore-Microtubule Attachment in Plk1-Depleted Cells

(A) Cells were treated as in Figure 4 and were costained with CREST sera and CENP-E antibodies. The boxes indicate areas that have been magnified in the insert in the upper right corner. The position of sister kinetochores is indicated by arrow pairs.

(B) Statistics of Mad2, BubR1, and CENP-E staining at kinetochores. Staining patterns were grouped into the following categories: weak or undetectable staining at both sister kinetochores, one of both sister kinetochores clearly positive, or both sister kinetochores clearly positive. Examples of the two last categories are shown for CENP-E staining in the inserts in (A). Examples of Mad2 and BubR1 staining are shown in Supplemental Figure S6. At least 10 cells and 210 kinetochore pairs were analyzed in each experiment.

(C) Plk1 is required for stable kinetochore microtubule attachment. Monastrol-treated cells in which Plk1 had either been depleted or not

in Plk1 RNAi cells (Figure 2D), suggesting that defects in NuMA localization are not responsible for the spindle pole defects in Plk1-depleted cells.

These results indicate that Plk1 is required for the stable association of centrosomes with spindle microtubules, possibly because γ-tubulin recruitment depends on Plk1. Bipolar spindles can also form in the absence of centrosomes by microtubules that are nucleated in the vicinity of chromosomes ([36]; reviewed in [37]). It is therefore possible that the acentrosomal spindles in Plk1-depleted cells were formed predominantly by microtubules nucleated at chromosomes.

Plk1 Depletion Activates the Spindle Checkpoint

Defects in spindle assembly can activate the spindle checkpoint, which inhibits the ability of the APC/C to initiate anaphase (reviewed in [9]). In *Drosophila*, some *polo* mutants cause activation of the spindle checkpoint [34], and overexpression of a Plk1 mutant activates the spindle checkpoint in human cells [38], but it is not known how Plk1 defects cause checkpoint activation. To address if the prometaphase delay caused by Plk1 depletion in our experiments is also due to activation of the spindle checkpoint, we analyzed the presence of Mad2 at kinetochores. Mad2 is only enriched at kinetochores that are either not attached to the spindle or that are not fully occupied by microtubules [8], whereas full occupation of kinetochores with microtubules leads to loss of Mad2 from the kinetochore and checkpoint silencing ([39] and references therein). In control cells we observed that Mad2 was enriched on numerous kinetochores in prometaphase, but not in metaphase (Figure 3A). However, in 98.5% (68/69) of mitotic Plk1 RNAi cells many kinetochores contained Mad2, even in cells that had a metaphase-like appearance because bipolar spindles had formed and chromosomes had congressed to the equatorial plane (Figure 3A). The failure of many Plk1 RNAi cells to enter anaphase may therefore be caused by activation of the spindle checkpoint.

It has been reported that Plk1 can phosphorylate and activate the APC/C in vitro [40, 41] and is required for destruction of the APC/C inhibitor Emi1 [42], raising the alternative possibility that loss of Plk1 causes anaphase delays by preventing APC/C activation. However, we have recently found that degradation of the APC/C substrate cyclin A is initiated without delay in Plk1 RNAi cells, indicating that Plk1 is not essential for APC/C's ability to ubiquitinate cyclin A [26]. In contrast, cyclin B and securin are not degraded in Plk1 RNAi cells (Figure 1B; [26]), suggesting that Plk1 is either required for APC/C's ability to ubiquitinate these substrates or that Plk1 depletion activates the spindle checkpoint and thereby prevents cyclin B and securin degradation. To distinguish between these possibilities we treated Plk1 RNAi cells with the small molecule Hesperadin, which silences the spindle checkpoint by inhibiting the activity of the Aurora B kinase [14]. If Plk1 depletion prevented cyclin B and securin destruction by activation of the spindle checkpoint, Hesperadin treatment should promote the degradation of these proteins and lead to mitotic exit. Hesperadin treatment caused cyclin B and securin degradation in Plk1 RNAi cells as judged by immunofluorescence microscopy and immunoblotting (Figures 3B–3D), and time-lapse video microscopy revealed that these cells exited mitosis without anaphase and cytokinesis, resulting in the formation of reconstitution nuclei (Supplemental Figure S3). Processes that are normally seen in cells initiating anaphase, such as separase and cohesin cleavage [43] and relocation of the motor protein CENP-E from kinetochores to spindle microtubules [44], could also be observed under these conditions (Figure 3D; Supplemental Figure S4). These observations imply that Plk1 depletion delays anaphase entry primarily by activating the spindle checkpoint.

Plk1 May Be Required for the Generation of Spindle Pulling Forces that Stabilize Microtubule-Kinetochore Interactions by Creating Tension

Chromosome movements could be seen in cells depleted of Plk1 (Figure 1Eb; Supplemental Figure S1; Movie 2), chromosomes had in most cases congressed to the equatorial plane in Plk1 RNAi cells containing bipolar spindles (Figure 2), and chromosomes appeared to be attached to microtubules in monopolar spindles (Figure 2B, upper right). These observations indicate that kinetochore-microtubule attachments can be formed in the absence of normal Plk1 levels. This interpretation is also consistent with our observation that Hesperadin treatment of Plk1-depleted cells causes rapid exit from mitosis, because we observed previously that Hesperadin causes rapid silencing of the spindle checkpoint only in cells in which microtubule-kinetochore interactions can be formed [14]. Mad2 was nevertheless enriched on many kinetochores in Plk1 RNAi cells (Figure 3A), indicating that kinetochores were not bound to a full complement of microtubules [8, 39]. We also observed that most Plk1 RNAi cells contained chromosomes whose sister kinetochores contained unequal amounts of CENP-E (Supplemental Figure S5). The levels of CENP-E at kinetochores decrease in metaphase when kinetochores acquire their full complement of microtubules [45]. The asymmetric CENP-E staining, there-

were preextracted in the absence or presence of 100 μM calcium ions to depolymerize microtubules that were not attached to kinetochores and processed for immunoflorescence microscopy. Cells were stained with antibodies to α-tubulin (green) and with CREST sera (red). DNA was stained with DAPI (blue). 50 mitotic cells from each sample were examined for the presence or absence of thick or thin kinetochore microtubule bundles (KT fibers). The right panels show higher magnifications of the boxed regions in the middle panels.

(D) Taxol treatment phenocopies the Plk1 depletion. Cells were synchronized by double thymidine treatment, and Monastrol was added 7 hr after the release from the second thymidine block. At 10.5 hr from the release, cells were treated either with 1 μM taxol (left panels) or an equivalent volume of DMSO (middle panels) for 30 min, followed by immunofluorescent analysis as indicated. In parallel, synchronous Plk1 RNAi cells were treated with Monastrol (right panels). Note that typical V shaped chromosomes are seen in the control, whereas straight chromosomes are seen after both taxol treatment and Plk1 RNAi. Although the overall shape of chromosomes is similar after the latter two treatments, sister chromatids are less resolved in the absence of Plk1 [52].

fore, also implies that many kinetochores in Plk1 RNAi cells were not fully occupied with microtubules.

We next investigated if kinetochore attachment defects in Plk1-depleted cells might be caused by abnormalities in spindle forces. In these experiments we analyzed chromosome behavior in cells that were treated with the Eg5 inhibitor Monastrol [11], because in these cells the presence of spindle forces can be easily inferred from the position and shape of chromosomes. Eg5 inhibition leads to the formation of monopolar spindles [10, 11] in which chromosomes are typically positioned in a rosette-like arrangement that is thought to be the result of opposing spindle forces. A polar ejection force mediated by the chromokinesin Kid moves chromosome arms away from the spindle pole, whereas pulling forces of kinetochore-associated motor proteins moves centromeres close to the pole [46]. As a result, chromosomes acquire a V shape in which the centromere faces the pole whereas the arms point away from it ([46]; Figure 4C). To analyze if these chromosome-spindle interactions depend on Plk1 we treated Plk1 RNAi cells with Monastrol and analyzed them either by immunofluorescence microscopy or by chromosome spreading followed by Giemsa staining. In all cells monopolar spindles were observed in which chromosomes were scattered throughout the spindle area (Figures 4A′–4C′). Most chromosomes were located much further away from the pole than in Monastrol-treated cells in which Plk1 had not been depleted. Remarkably, chromosomes in Plk1 RNAi cells did not show the V shape that is typically seen after Monastrol treatment but instead showed a straight appearance (Figures 4B′ and 4C′).

In Monastrol-treated cells, most kinetochores are attached to the monopolar spindle, and correspondingly Mad2 [14, 47], CENP-E, and the checkpoint kinase BubR1 (Figures 5A and 5B; Supplemental Figure S6) are enriched only at few kinetochores. In contrast, all three proteins were enriched at the majority of kinetochores when Plk1 was depleted in Monastrol-treated cells (Figures 5A and 5B; Supplemental Figure S6). About two thirds of all chromosomes showed enrichment of these proteins on both sister kinetochores, implying that the majority of chromosomes in these cells was not properly attached to the mitotic spindle, consistent with their peripheral location relative to the spindle pole and their straight appearance. When Monastrol-arrested cells were treated with calcium-containing buffers, which preferentially destabilize microtubules that are not kinetochore bound [47], thinner chromosome bound microtubule bundles were seen in Plk1-depleted cells than in control cells (Figure 5C), further supporting the notion that kinetochores are not fully occupied with microtubules in the absence of Plk1.

The lack of V shaped chromosomes in Monastrol-treated cells lacking Plk1 would be consistent with a defect in the polar ejection force. However, inhibition of this force results in movement of chromosomes toward the monopole [46], whereas in our experiments chromosomes were located further away from the pole, implying that the polar ejection force still exists in Plk1-depleted cells. It is therefore possible that Plk1 RNAi cells are defective in pulling chromosomes toward the pole, allowing the polar ejection force to move chromosomes away from the pole. To test this hypothesis we compared the effects of Plk1 depletion with the effects of the microtubule-stabilizing compound Taxol in Monastrol-treated cells. Taxol inhibits the generation of spindle pulling forces and prevents the stabilization of kinetochore attachments [8], possibly because microtubules are detached in an Aurora B-dependent manner from kinetochores that are not under tension [14]. Like Plk1 depletion, Taxol treatment resulted in a more peripheral location of chromosomes in Monastrol-treated cells, and chromosomes had a straight appearance instead of a V shape (Figure 5D). These observations are consistent with the idea that reduced microtubule occupancy of kinetochores and activation of the spindle checkpoint in Plk1-depleted cells are caused by defects in spindle pulling forces. The hypothesis that Plk1 is required for spindle force generation would also explain the observation that no anaphase chromosome movements could be seen when Plk1-depleted cells exited mitosis after Hesperadin treatment (Supplemental Figure S3), although Hesperadin treatment did not abolish anaphase-like chromosome movements in cells containing Plk1 [14].

Conclusions

Plk1 has previously been implicated in several mitotic processes, including the regulation of mitotic entry, activation of the APC/C, centrosome maturation and migration, and the assembly of bipolar mitotic spindles. Our data imply that normal Plk1 levels are needed for the correct timing of mitotic entry, but that after a short delay in G2 phase, mammalian cells can enter mitosis and translocate cyclin B into the nucleus also in the absence of detectable Plk1 levels. These observations suggest that Plk1's role in Cdk1 activation is either minor or redundant with other enzymes.

The predominant cell cycle phenotype in Plk1-depleted mammalian cells is an arrest in prometaphase. This delay does not seem to be caused directly by defects in APC/C activation but instead by maintenance of spindle checkpoint signaling. Our data suggest that Plk1 is required for the assembly of spindles that contain focused poles, that are able to generate pulling forces, and that can attach stably to chromosomes. The inability of Plk1-depleted cells to silence the spindle checkpoint might therefore be caused by defects in spindle function. After submission of this manuscript, similar observations have also been published by van Vugt et al. [48].

The anchorage of microtubule minus ends at spindle poles is required to generate proper tension across sister kinetochores [49]. It is therefore possible that Plk1's role in the formation of focused spindle poles is indirectly required for spindle force generation, which, in turn, would stabilize kinetochore attachments and would silence the spindle checkpoint once full kinetochore occupancy has been achieved. Alternatively, Plk1 could directly contribute to force generation and stabilization of kinetochore attachments by phosphorylating motor proteins or other substrates at kinetochores where the kinase has been observed both in human cells and *Drosophila* [50, 51]. The identification of Plk1's critical substrates in these processes will be required to distinguish between these possibilities.

Experimental Procedures

The following monoclonal mouse antibodies were used: Cyclin A (06-138, Upstate), Cyclin B1 (GNS1, Santa Cruz), Mad2 (PRB-452C, Babco), PARP (H-250 sc-7150, Santa Cruz), γ-tubulin (T3559, Sigma), and NuMA (Ab-2, Oncogene). Monoclonal rat antibodies against α-tubulin were from Abcam (ab9266). Rabbit polyclonal antibodies against CENP-E and BubR1 were from Tim Yen, Fox Chase Cancer Center, Philadelphia, PA; and rat polyclonal Centrin 3 antibodies were from Tomotoshi Marumoto, Kumamoto University School of Medicine, Japan. Antibodies to pApc1 have been described [26]. Other reagents and Experimental Procedures have been described in [52].

Calcium treatment to examine microtubule attachment to kinetochores was carried out as described by Kapoor et al. [47]. Briefly, cells were permeabilized for 90 s in a buffer containing 100 mM Pipes (pH 6.8), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.1% Triton X-100 and then fixed for 10 min with 4% formaldehyde in the same buffer, followed by immunofluorescence analysis.

Supplemental Data

Six Supplemental Figures and two Movies are available at http://www.current-biology.com/cgi/content/full/14/19/1712/DC1/.

Acknowledgments

We are grateful to T. Yen, and T. Marumoto for antibodies, to G. Wahl for EGFP-H2B cells, to J. van Meel and N. Kraut for Hesperadin, to M. Peter for generous support, to S. Hauf for comments on the manuscript, and to two anonymous reviewers for helpful suggestions. D.G. and I.G. are supported by European Molecular Biology Organization (EMBO) long term fellowships, and T.H. is supported by a fellowship from Japan Society for the Promotion of Science. Research in the laboratory of C.d.l.T. is supported by the Dirección General de Investigación del Ministerio de Ciencia y Tecnología (Project BMC2001-2195). Research in the laboratory of J.-M.P. is supported by Boehringer Ingelheim, Wiener Wirtschaftsförderungsfond, and EMBO.

Received: March 30, 2004
Revised: August 26, 2004
Accepted: August 31, 2004
Published: October 5, 2004

References

1. Mitchison, T., and Kirschner, M. (1984). Dynamic instability of microtubule growth. Nature *312*, 237–242.
2. Nicklas, R.B., and Ward, S.C. (1994). Elements of error correction in mitosis: microtubule capture, release, and tension. J. Cell Biol. *126*, 1241–1253.
3. King, J.M., and Nicklas, R.B. (2000). Tension on chromosomes increases the number of kinetochore microtubules but only within limits. J. Cell Sci. *113*, 3815–3823.
4. Maney, T., Ginkel, L.M., Hunter, A.W., and Wordeman, L. (2000). The kinetochore of higher eucaryotes: a molecular view. Int. Rev. Cytol. *194*, 67–131.
5. Rieder, C.L., Cole, R.W., Khodjakov, A., and Sluder, G. (1995). The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. J. Cell Biol. *130*, 941–948.
6. Nicklas, R.B., Waters, J.C., Salmon, E.D., and Ward, S.C. (2001). Checkpoint signals in grasshopper meiosis are sensitive to microtubule attachment, but tension is still essential. J. Cell Sci. *114*, 4173–4183.
7. Musacchio, A., and Hardwick, K.G. (2002). The spindle checkpoint: structural insights into dynamic signalling. Nat. Rev. Mol. Cell Biol. *3*, 731–741.
8. Waters, J.C., Chen, R.H., Murray, A.W., and Salmon, E.D. (1998). Localization of Mad2 to kinetochores depends on microtubule attachment, not tension. J. Cell Biol. *141*, 1181–1191.
9. Peters, J.-M. (2002). The anaphase-promoting complex: proteolysis in mitosis and beyond. Mol. Cell *9*, 931–943.
10. Blangy, A., Lane, H.A., d'Herin, P., Harper, M., Kress, M., and Nigg, E.A. (1995). Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. Cell *83*, 1159–1169.
11. Mayer, T.U., Kapoor, T.M., Haggarty, S.J., King, R.W., Schreiber, S.L., and Mitchison, T.J. (1999). Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. Science *286*, 971–974.
12. Shannon, K.B., and Salmon, E.D. (2002). Chromosome dynamics: new light on Aurora B kinase function. Curr. Biol. *12*, R458–R460.
13. Lampson, M.A., Renduchitala, K., Khodjakov, A., and Kapoor, T.M. (2004). Correcting improper chromosome-spindle attachments during cell division. Nat. Cell Biol. *6*, 232–237.
14. Hauf, S., Cole, R.W., LaTerra, S., Zimmer, C., Schnapp, G., Walter, R., Heckel, A., van Meel, J., Rieder, C.L., and Peters, J.M. (2003). The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. J. Cell Biol. *161*, 281–294.
15. Ditchfield, C., Johnson, V.L., Tighe, A., Ellston, R., Haworth, C., Johnson, T., Mortlock, A., Keen, N., and Taylor, S.S. (2003). Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. J. Cell Biol. *161*, 267–280.
16. Sunkel, C.E., and Glover, D.M. (1988). polo, a mitotic mutant of Drosophila displaying abnormal spindle poles. J. Cell Sci. *89*, 25–38.
17. Llamazares, S., Moreira, A., Tavares, A., Girdham, C., Spruce, B.A., Gonzalez, C., Karess, R.E., Glover, D.M., and Sunkel, C.E. (1991). polo encodes a protein kinase homolog required for mitosis in Drosophila. Genes Dev. *5*, 2153–2165.
18. Ohkura, H., Hagan, I.M., and Glover, D.M. (1995). The conserved Schizosaccharomyces pombe kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. Genes Dev. *9*, 1059–1073.
19. Lane, H.A., and Nigg, E.A. (1996). Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. J. Cell Biol. *135*, 1701–1713.
20. do Carmo Avides, M., Tavares, A., and Glover, D.M. (2001). Polo kinase and Asp are needed to promote the mitotic organizing activity of centrosomes. Nat. Cell Biol. *3*, 421–424.
21. Oakley, B.R., and Akkari, Y.N. (1999). Gamma-tubulin at ten: progress and prospects. Cell Struct. Funct. *24*, 365–372.
22. Barr, F.A., Sillje, H.H., and Nigg, E.A. (2004). Polo-like kinases and the orchestration of cell division. Nat. Rev. Mol. Cell Biol. *5*, 429–440.
23. Kumagai, A., and Dunphy, W.G. (1996). Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from Xenopus egg extracts. Science *273*, 1377–1380.
24. Qian, Y.W., Erikson, E., Li, C., and Maller, J.L. (1998). Activated polo-like kinase Plx1 is required at multiple points during mitosis in Xenopus laevis. Mol. Cell. Biol. *18*, 4262–4271.
25. Lindon, C., and Pines, J. (2004). Ordered proteolysis in anaphase inactivates Plk1 to contribute to proper mitotic exit in human cells. J. Cell Biol. *164*, 233–241.
26. Kraft, C., Herzog, F., Gieffers, C., Mechtler, K., Hagting, A., Pines, J., and Peters, J.-M. (2003). Mitotic regulation of the human anaphase-promoting complex by phosphorylation. EMBO J. *22*, 6598–6609.
27. Toyoshima-Morimoto, F., Taniguchi, E., Shinya, N., Iwamatsu, A., and Nishida, E. (2001). Polo-like kinase 1 phosphorylates cyclin B1 and targets it to the nucleus during prophase. Nature *410*, 215–220.
28. Liu, X., and Erikson, R.L. (2002). Polo-like kinase (Plk)1 depletion induces apoptosis in cancer cells. Proc. Natl. Acad. Sci. USA *100*, 5789–5794.
29. Takizawa, C.G., and Morgan, D.O. (2000). Control of mitosis by changes in the subcellular location of cyclin-B1-Cdk1 and Cdc25C. Curr. Opin. Cell Biol. *12*, 658–665.
30. Jackman, M., Lindon, C., Nigg, E.A., and Pines, J. (2003). Active cyclin B1-Cdk1 first appears on centrosomes in prophase. Nat. Cell Biol. *5*, 143–148.

31. Kanda, T., Sullivan, K.F., and Wahl, G.M. (1998). Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. Curr. Biol. *8*, 377–385.
32. Neef, R., Preisinger, C., Sutcliffe, J., Kopajtich, R., Nigg, E.A., Mayer, T.U., and Barr, F.A. (2003). Phosphorylation of mitotic kinesin-like protein 2 by polo-like kinase 1 is required for cytokinesis. J. Cell Biol. *162*, 863–875.
33. Bornens, M. (2002). Centrosome composition and microtubule anchoring mechanisms. Curr. Opin. Cell Biol. *14*, 25–34.
34. Donaldson, M.M., Tavares, A.A., Ohkura, H., Deak, P., and Glover, D.M. (2001). Metaphase arrest with centromere separation in polo mutants of Drosophila. J. Cell Biol. *153*, 663–676.
35. Merdes, A., Heald, R., Samejima, K., Earnshaw, W.C., and Cleveland, D.W. (2000). Formation of spindle poles by dynein/dynactin-dependent transport of NuMA. J. Cell Biol. *149*, 851–862.
36. Khodjakov, A., Cole, R.W., Oakley, B.R., and Rieder, C.L. (2000). Centrosome-independent mitotic spindle formation in vertebrates. Curr. Biol. *10*, 59–67.
37. Karsenti, E., and Vernos, I. (2001). The mitotic spindle: a self-made machine. Science *294*, 543–547.
38. Seong, Y.S., Kamijo, K., Lee, J.S., Fernandez, E., Kuriyama, R., Miki, T., and Lee, K.S. (2002). A spindle checkpoint arrest and a cytokinesis failure by the dominant-negative polo-box domain of Plk1 in U-2 OS cells. J. Biol. Chem. *277*, 32282–32293.
39. Howell, B.J., Hoffman, D.B., Fang, G., Murray, A.W., and Salmon, E.D. (2000). Visualization of Mad2 dynamics at kinetochores, along spindle fibers, and at spindle poles in living cells. J. Cell Biol. *150*, 1233–1250.
40. Kotani, S., Tugendreich, S., Fujii, M., Jorgensen, P.M., Watanabe, N., Hoog, C., Hieter, P., and Todokoro, K. (1998). PKA and MPF-activated polo-like kinase regulate anaphase-promoting complex activity and mitosis progression. Mol. Cell *1*, 371–380.
41. Golan, A., Yudkovsky, Y., and Hershko, A. (2002). The cyclin-ubiquitin ligase activity of cyclosome/APC is jointly activated by protein kinases Cdk1-cyclin B and Plk. J. Biol. Chem. *277*, 15552–15557.
42. Moshe, Y., Boulaire, J., Pagano, M., and Hershko, A. (2004). Role of Polo-like kinase in the degradation of early mitotic inhibitor *1*, a regulator of the anaphase promoting complex/cyclosome. Proc. Natl. Acad. Sci. USA *101*, 7937–7942.
43. Waizenegger, I.C., Hauf, S., Meinke, A., and Peters, J.-M. (2000). Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. Cell *103*, 399–410.
44. Cooke, C.A., Schaar, B., Yen, T.J., and Earnshaw, W.C. (1997). Localization of CENP-E in the fibrous corona and outer plate of mammalian kinetochores from prometaphase through anaphase. Chromosoma *106*, 446–455.
45. Hoffman, D.B., Pearson, C.G., Yen, T.J., Howell, B.J., and Salmon, E.D. (2001). Microtubule-dependent changes in assembly of microtubule motor proteins and mitotic spindle checkpoint proteins at PtK1 kinetochores. Mol. Biol. Cell *12*, 1995–2009.
46. Levesque, A.A., and Compton, D.A. (2001). The chromokinesin Kid is necessary for chromosome arm orientation and oscillation, but not congression, on mitotic spindles. J. Cell Biol. *154*, 1135–1146.
47. Kapoor, T.M., Mayer, T.U., Coughlin, M.L., and Mitchison, T.J. (2000). Probing spindle assembly mechanisms with monastrol, a small molecule inhibitor of the mitotic kinesin, Eg5. J. Cell Biol. *150*, 975–988.
48. Van Vugt, M.A., Van De Weerdt, B.C., Vader, G., Janssen, H., Calafat, J., Klompmaker, R., Wolthuis, R.M., and Medema, R.H. (2004). Polo-like kinase-1 is required for bipolar spindle formation but is dispensable for anaphase promoting complex/Cdc20 activation and initiation of cytokinesis. J. Biol. Chem. *279*, 36841–36854.
49. Gordon, M.B., Howard, L., and Compton, D.A. (2001). Chromosome movement in mitosis requires microtubule anchorage at spindle poles. J. Cell Biol. *152*, 425–434.
50. Arnaud, L., Pines, J., and Nigg, E.A. (1998). GFP tagging reveals human Polo-like kinase 1 at the kinetochore/centromere region of mitotic chromosomes. Chromosoma *107*, 424–429.
51. Logarinho, E., and Sunkel, C.E. (1998). The Drosophila POLO kinase localises to multiple compartments of the mitotic apparatus and is required for the phosphorylation of MPM2 reactive epitopes. J. Cell Sci. *111*, 2897–2909.
52. Gimenez-Abian, J.F., Sumara, I., Hirota, T., Hauf, S., Gerlich, D., de la Torre, C., Ellenberg, J., and Peters, J.-M. (2004). Regulation of sister chromatid cohesion between chromosome arms. Curr. Biol. *14*, 1187–1193.