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Article

The Small-Molecule Inhibitor BI 2536 Reveals Novel Insights into Mitotic Roles of Polo-like Kinase 1

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Summary

Background: The mitotic kinases, Cdk1, Aurora A/B, and Polo-like kinase 1 (Plk1) have been characterized extensively to further understanding of mitotic mechanisms and as potential targets for cancer therapy. Cdk1 and Aurora kinase studies have been facilitated by small-molecule inhibitors, but few if any potent Plk1 inhibitors have been identified.

Results: We describe the cellular effects of a novel compound, BI 2536, a potent and selective inhibitor of Plk1. The fact that BI 2536 blocks Plk1 activity fully and instantaneously enabled us to study controversial and unknown functions of Plk1. Cells treated with BI 2536 are delayed in prophase but eventually import Cdk1-cyclin B into the nucleus, enter prometaphase, and degrade cyclin A, although BI 2536 prevents degradation of the APC/C inhibitor Emi1. BI 2536-treated cells lack prophase microtubule asters and thus polymerize mitotic microtubules only after nuclear-envelope breakdown and form monopolar spindles that do not stably attach to kinetochores. Mad2 accumulates at kinetochores, and cells arrest with an activated spindle-assembly checkpoint. BI 2536 prevents Plk1's enrichment at kinetochores and centrosomes, and when added to metaphase cells, it induces detachment of microtubules from kinetochores and leads to spindle collapse.

Conclusions: Our results suggest that Plk1's accumulation at centrosomes and kinetochores depends on its own activity and that this activity is required for maintaining centrosome and kinetochore function. Our data also show that Plk1 is not required for prophase entry, but delays transition to prometaphase, and that Emi1 destruction in prometaphase is not essential for APC/C-mediated cyclin A degradation.

Introduction

In eukaryotes from yeast to men, orthologs of Polo-like kinase 1 (Plk1) control multiple essential steps of mitosis and meiosis (reviewed in [1]). In vertebrate cells, Plk1 has been implicated in the activation of Cdk1-cyclin B at mitotic entry [2–5], centrosome maturation [6], release of cohesin from chromosome arms [7–9], activation of the anaphase promoting complex/cyclosome (APC/C) by direct phosphorylation [10, 11] and by inactivation of the APC/C inhibitor Emi1 [12, 13], formation of bipolar spindles [14, 15], and accumulation of spindle assembly checkpoint (SAC) proteins at kinetochores [16–18]. Plk1 is believed to perform this multitude of functions by specific recruitment to substrates via its Polo-box domain (PBD). The PBD interacts with Plk1 substrates by binding to phosphosites, which are generated by "priming kinases" such as Cdk1 or by Plk1 itself [18–21]. It has been proposed that the interactions with multiple substrates result in Plk1's localization to diverse structures in the mitotic cell, where Plk1 is found at centrosomes, at unattached kinetochores, and in late mitosis at the cytokinetic furrow and midbody [1, 22].

In a number of cases, the study of protein kinases has been greatly facilitated by the identification of specific small-molecule inhibitors. For example, several compounds have been described that inhibit Aurora kinases [23–26] and Cdks (reviewed in [27]). In contrast, compounds that inhibit Plk1 have only been developed recently. However, in our analyses the first reported compound, called ON01910 [28], did not inactivate Plk1 efficiently in vitro or in vivo (see [29]; this issue of *Current Biology*). After the submission of this manuscript, two other compounds were reported to inhibit Plk1, but the specificity and potency of these inhibitors in human cells remain to be further investigated [30, 31].

We have developed a compound, BI 2536, that inhibits Plk1 with high potency (IC_{50} = 0.83 nM). This compound shows more than 1,000-fold selectivity against a large panel of protein kinases, but it affects the activities of other Polo-kinase family members, such as Plk2/Snk (IC_{50} = 3.5 nM) and Plk3/Fnk (IC_{50} = 9 nM; for technical reasons we have not yet been able to determine the effect of BI 2536 on Plk4/SAK). However, with the exception of Plk1, all other Polo family members are thought to function during G1 and S phases of the cell cycle (reviewed in [22]), and therefore BI 2536 is expected to specifically target Plk1 in mitotic cells. To directly test this, we first compared cellular effects of BI 2536 to published Plk1 RNAi data. Because BI 2536 mirrored phenotypes of Plk1 RNAi, we used this compound to analyze Plk1 functions that could not or not reliably be addressed by RNAi experiments.

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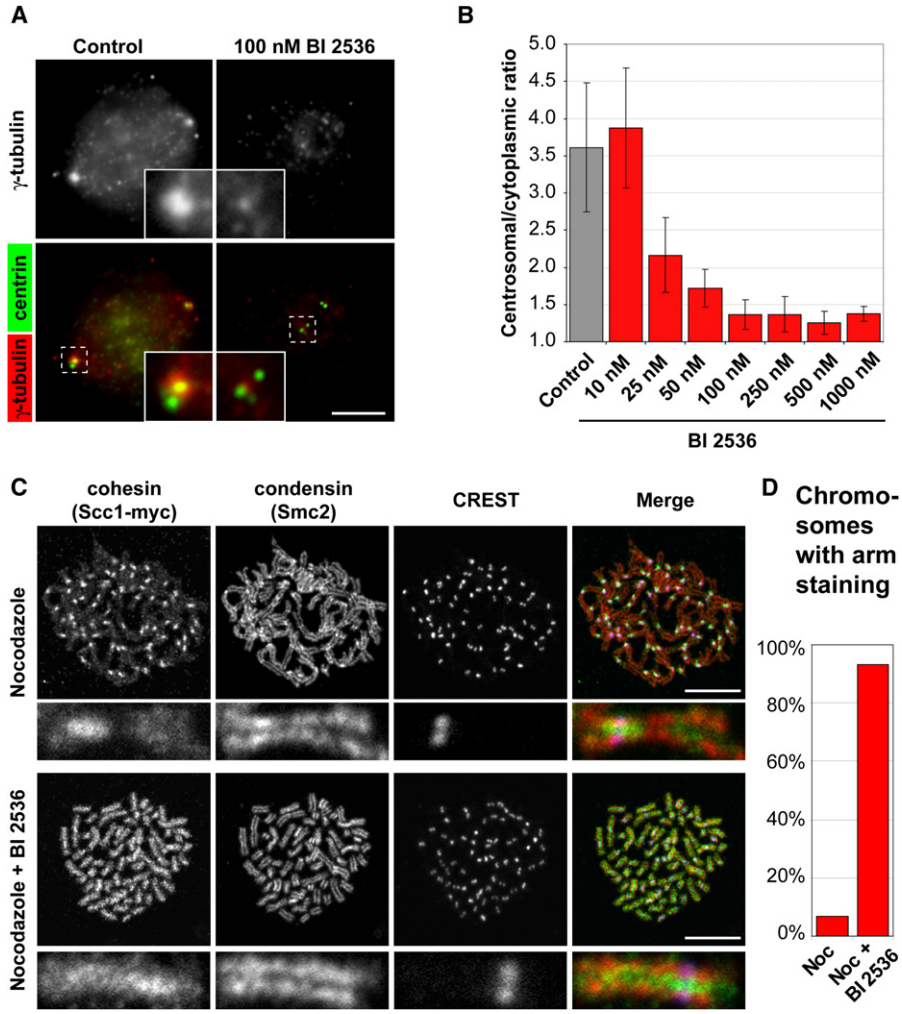

Figure 1. BI 2536 Specifically Blocks Plk1-Dependent Mitotic Events in a Dose-Dependent Manner

(A) Asynchronous populations of HeLa cells were treated for 2 hr with or without BI 2536 and stained for γ-tubulin and the centriole marker centrin (cen3). γ-tubulin is shown in red, and centrin is shown in green. The scale bar represents 10 μm.

(B) Quantification of centrosomal γ-tubulin levels at different concentrations of BI 2536 for mitotic cells processed as in (A). Total centrosomal intensity relative to the intensity in a same-size cytoplasmic region is shown (n > 20 each, average values ± SD).

(C) HeLa cells stably expressing the Myc-tagged cohesin subunit Scc1 were treated for 3 hr with 100 ng/ml Nocodazole or a combination of Nocodazole and 100 nM BI 2536; mitotic cells were shaken off and immunostained with the antibodies indicated. Scale bars represent 10 μm.

(D) Quantification of Scc1-9myc expressing cells of (C).

Results

BI 2536 Specifically Inhibits Plk1 Functions in Mitosis

The high potency and selectivity of BI 2536 in vitro and the mitotic arrest caused by BI 2536 in cultured cell lines and mouse tumor xenografts implied that BI 2536 also inhibits Plk1 in vivo [29]. To further test the selectivity of BI 2536, we investigated, in HeLa cells, several mitotic processes that are known to be dependent on Plk1.

First, we quantified the Plk1-dependent accumulation of γ-tubulin and the pApc6 phosphoepitope at mitotic centrosomes in cells treated with increasing concentrations of BI 2536 [6, 11, 32]. We found an almost identical dose-dependent reduction of both centrosomal γ-tubulin and pApc6 staining with full efficacy at 100 nM (Figures 1A and 1B; see also Figures S1A and S1B in the Supplemental Data available with this article online).

Second, we followed the release of cohesin from chromosome arms, a process that also depends on Plk1 [7–9]. Cohesin remained associated with chromosome arms, and arm cohesion was maintained even after prolonged mitotic arrest in BI 2536-treated cells, whereas in Nocodazole-treated control cells, cohesin was removed from chromosomes except for centromeric regions (Figures 1C and 1D; Figures S1C and S1D).

Taken together, these observations indicate that BI 2536 is a potent inhibitor of Plk1 in human cultured cells. Because in dose-response experiments, 100 nM BI 2536 induced a well-defined set of fully penetrant phenotypes, which faithfully recapitulated the effects of Plk1 RNAi, our data further suggest that Plk1 is maximally and specifically inhibited by treatment with 100 nM BI 2536 [29].

BI 2536-Treated Cells Spend Prolonged Periods of Time in Prophase and Enter Prometaphase at a Reduced Rate

In *Xenopus* oocyte extracts, depletion of the Plk1 ortholog Plx1 prevents activation of Cdk1 and mitotic entry [33], whereas depletion of Plk1 by RNAi in HeLa cells delays but does not prevent mitotic entry [12, 15]. Because the latter result could be caused by incomplete Plk1 depletion, we analyzed mitotic entry in HeLa cells that were treated with BI 2536.

We synchronized HeLa cells by double thymidine arrest and released them in the absence or presence of BI 2536 or Monastrol, a compound that arrests cells in prometaphase by inhibiting the kinesin Eg5 [34]. At different time points, cells were fixed, DNA was stained with DAPI, and mitotic stages were classified by chromosome morphology.

In both control and Monastrol-treated populations, cells began to enter mitosis 4 hr after thymidine release, and Monastrol-treated cells accumulated in prometaphase, whereas untreated cells exited mitosis (Figure 2A). BI 2536-treated cells accumulated in mitosis at a very similar rate to Monastrol-treated cells. Interestingly however, up to 30% of BI 2536-treated cells were in prophase, compared to only 5%–10% in the control or Monastrol-treated populations (Figure 2A). BI 2536 did not cause this delay in prophase indirectly by interfering with progression through S or G2 phase because we observed a similar increase in the occurrence of prophases when we added the compound 3 or 6 hr after release from thymidine (Figure S2A). We also found an almost identical increase of prophase populations by using histone 3 phosphorylated on Ser10 (H3S10ph), a marker that labels prophase cells in a specific dotted pattern (Figures S2B and S2C). Taken together, these data suggest that in human cells Plk1 inhibition does not interfere with entry into prophase but that Plk1 is required for timely entry into prometaphase.

We confirmed these observations at the single-cell level by filming HeLa cells that stably express a GFP-tagged version of the core histone H2B. These experiments revealed that nuclear-envelope breakdown (NEBD) only occurred several hours after chromosome condensation in BI 2536-treated cells, whereas control cells spent only 15–30 min between the initiation of condensation and NEBD (Figure 2C and Movie S1). As a consequence, the rate of entry into prometaphase was decreased by half in BI 2536-treated populations as compared to untreated controls (Figure 2B).

Entry into prometaphase is driven by activation of Cdk1-cyclin B. Plk1 has been proposed to activate Cdk1 through phosphorylation of the phosphatase Cdc25C [33], degradation of the kinase Wee1 [2], and by phosphorylation of cyclin B. Cyclin B phosphorylation by Plk1 was suggested to be required for nuclear translocation of Cdk1-cyclin B [3] and for activation of Cdk1-cyclin B at the centrosomes [5].

It is unclear how Plk1's functions—many of which have only been studied in vitro—are coordinated in cells. Therefore, we prepared total extracts of HeLa cells at different times after thymidine release into media containing Nocodazole with or without BI 2536 and analyzed these samples by immunoblotting. As expected, Cdk1 activation (detected by increased electrophoretic mobility of Cdk1) was delayed in BI 2536-treated cells by approximately 4 hr, (Figure 2D). BI 2536 also prevented timely Wee1 degradation and Cdc25C phosphorylation (which retards the electrophoretic mobility of Cdc25C) (Figure 2D). To analyze the effects of Plk1 activity on cyclin B, we also filmed cells expressing cyclin B-YFP. In Monastrol-treated control cells, cyclin B first accumulated at centrosomes and entered the nucleus approximately 12 min before NEBD (as previously reported for untreated cells [35], Figure 3A). In contrast, cyclin B did not accumulate at centrosomes in BI 2536-treated cells, but cyclin B eventually entered the nucleus with normal kinetics (Figure 3A). Taken together, these data indicate that Plk1 is required for phosphorylation of Cdc25C, degradation of Wee1, and centrosomal recruitment of cyclin B but not for nuclear import of Cdk1-cyclin B. Our results further imply that these functions of Plk1 are not absolutely essential for entry into prometaphase, although they are required for timely progression to this stage of mitosis.

BI 2536-Treated Cells Arrest in Prometaphase and Initiate Cyclin A Destruction without Degrading Emi1

Degradation of the APC/C inhibitor Emi1 in mitotic cell extracts has been shown to depend on Plk1 activity [12, 13], and it has been proposed that Emi1 destruction is required for degradation of cyclin A in prometaphase. However, controversial results have been obtained when Plk1 was depleted from HeLa cells by RNAi because a delay in cyclin A degradation was reported in one [12] but not in two other studies [11, 14]. We therefore analyzed the kinetics of both Emi1 and cyclin A degradation in BI 2536-treated cells by transfecting them with either cyclin A-YFP [36] or Emi1-YFP. We analyzed Monastrol-treated cells, which showed Emi1 and cyclin A degradation kinetics similar to untreated cells, as controls (Figure S3D; [36]).

In control cells, Emi1 degradation was initiated 10–20 min before NEBD and was complete 20 min after NEBD (Figure 3B), whereas the destruction of cyclin A was initiated at NEBD (Figure 3C). In BI 2536-treated cells, Emi1 degradation was delayed by at least 1 hr, but cyclin A levels nevertheless decayed with kinetics similar to those seen in Monastrol-treated cells (Figures 3B and 3C). Immunoblot experiments showed that BI 2536 treatment also resulted in increased levels of endogenous Emi1 in mitotic cells (Figure S3C).

Our data are therefore consistent with the notion that Plk1 activity is required for degradation of Emi1, but our results indicate that Emi1 degradation is not essential for cyclin A destruction. Thus, in vivo the properties of Emi1 may be similar to those of its *Drosophila* ortholog Rca1. This protein has been shown to inhibit APC/C during interphase when APC/C is activated by Cdh1, but Rca1 does not antagonize the activity of APC/C^{Cdc20} in mitosis [37]. A recent, detailed study of nondegradable Emi1 mutants led to similar conclusions (B.D.F. and J. Pines, unpublished data).

BI 2536 Treatment Leads to Spindle-Assembly-Checkpoint-Induced Prometaphase Arrest

Depletion of Plk1 by RNAi has previously been shown to cause a prometaphase arrest in which cyclin A is degraded but cyclin B is not [14, 15]. Similarly, we observed

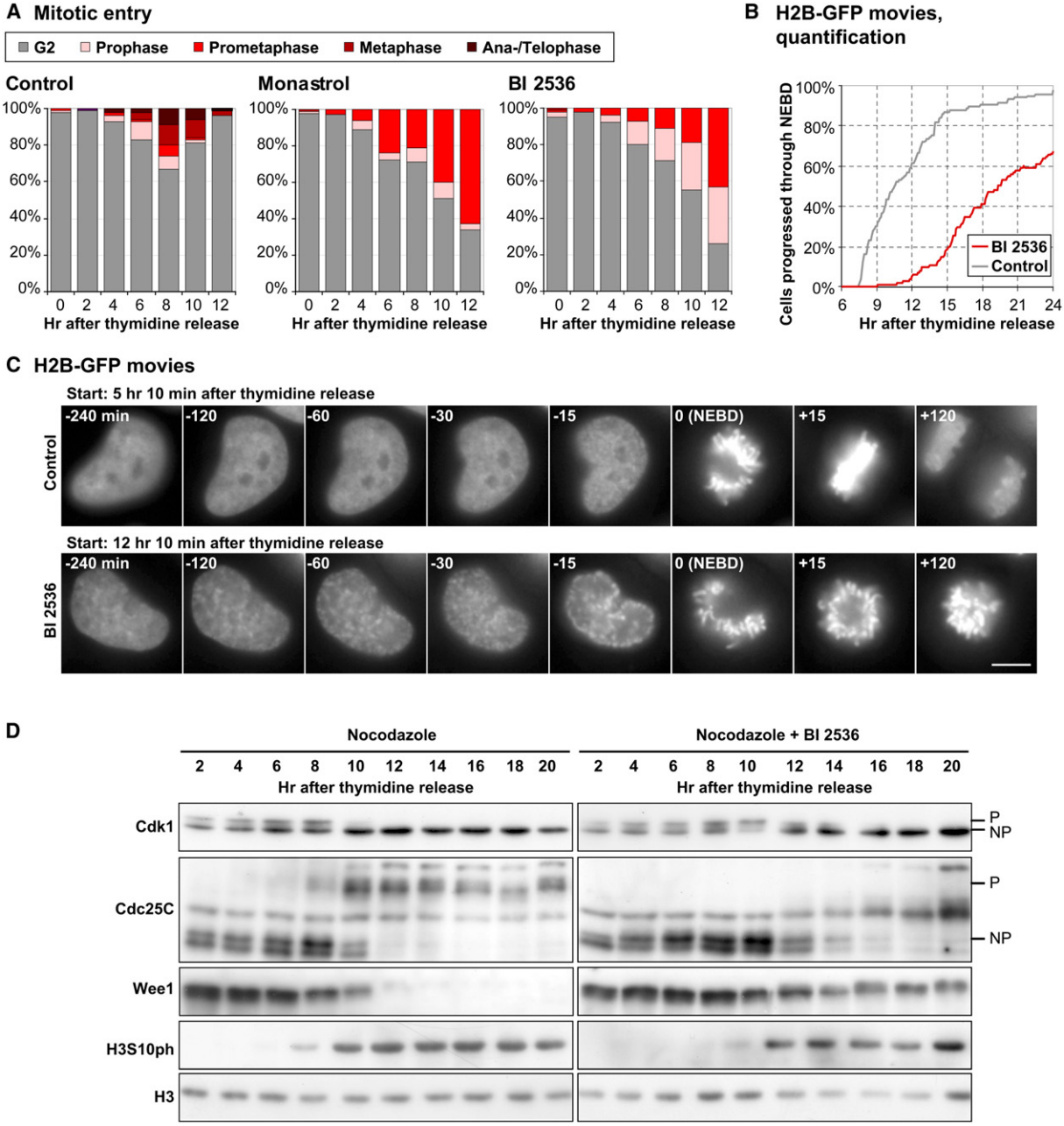

Figure 2. BI 2536-Treated Cells Enter Prophase Normally, but Their Transition into Prometaphase Is Delayed

(A) HeLa cells were synchronized by double thymidine arrest, fixed, and stained with DAPI at the indicated times after the second release. Drugs were added at t = 0, and 100 cells were staged at each time point.

(B) Quantification of the experiment shown in (C). A cumulative plot of cells that progressed through NEBD is shown in percentage of the total cell number (n = 113 and 115 for the control and BI 2536 treatment, respectively).

(C) Selected frames from a time-lapse series of HeLa cells expressing H2B-GFP after thymidine release in the presence or absence of BI 2536. Time is shown in minutes, and t = 0 is set to NEBD. The scale bar represents 10 μm. For the full movie, see Movie S1.

(D) Immunoblots of total cell lysates of synchronized HeLa cells. Either Nocodazole or Nocodazole and BI 2536 were added at the time of thymidine release and extracts were prepared at the indicated times after release. Abbreviations are as follows: P, phosphorylated form; NP, non-phosphorylated form.

that BI 2536-treated cells arrested in prometaphase and that neither cyclin B-YFP nor endogenous cyclin B were degraded in these cells, although both cyclin A-YFP and endogenous cyclin A were destroyed (Figures 3A and 3C; Figures S3A and S3B). These observations are consistent with the possibility that BI 2536-treated cells arrest in prometaphase because of activation of the SAC.

To further test this possibility, we treated BI 2536 arrested cells with Hesperadin, a compound that can override the SAC by inhibiting the kinase Aurora B [24]. When we filmed untreated HeLa cells that express H2B-GFP,

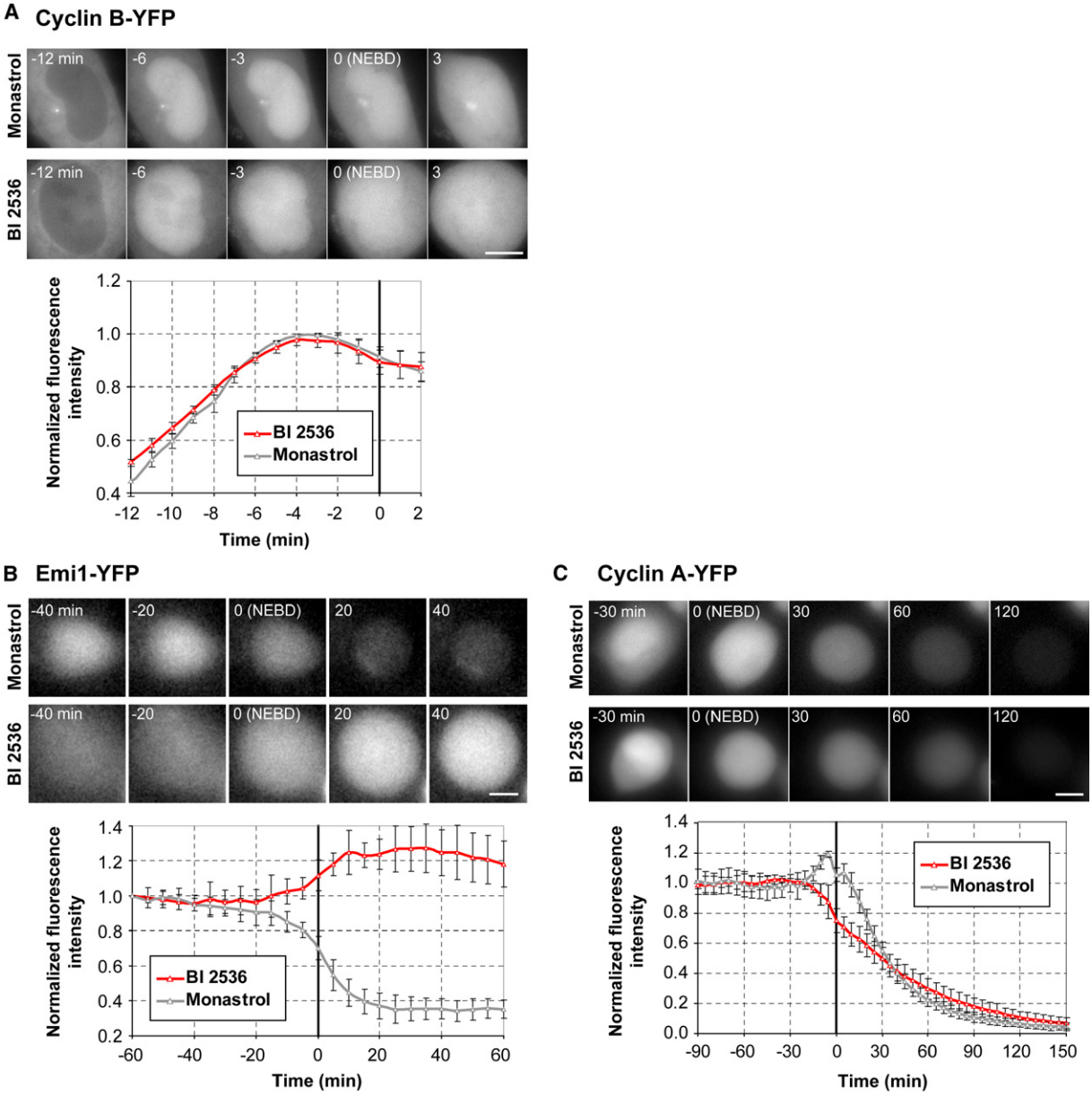

Figure 3. Inhibition of Plk1 by BI 2536 Prevents Degradation of Emi1 but Does Not Affect Kinetics of Cyclin A and Cyclin B Degradation

(A) HeLa cells were transfected with cyclin B-YFP and synchronized by a single thymidine treatment. Imaging was started 4 hr after the thymidine release, and 100 nM BI 2536 or 100 μM Monastrol was added to the medium at the same time. Hoechst 33342 was added to stain DNA for identifying prophase cells with condensed chromatin. Stacks of six images were acquired every minute; selected single frames and time points are shown. Time is shown in minutes relative to NEBD. The chart shows quantification of average fluorescence intensities measured in the nuclear region. Intensities were normalized between the maximal intensity and background outside the cells. Averages and standard deviations are plotted (n = 5).

(B) HeLa cells were transfected with Emi1-YFP and synchronized as in (A). Average projections for selected time points are shown ($n \geq 5$, each). Quantification, as in (A), is shown. Note that the apparent increase in Emi1 intensity after NEBD in BI 2536-treated cells is caused by rounding up of mitotic cells.

(C) Cells were transfected with cyclin A-YFP and imaged and quantified as in (A), $n \geq 5$ each.

Scale bars for all panels represent 10 μm.

all cells entered and rapidly exited mitosis (Figures 4A and 4B). Similarly, most Hesperadin-treated cells exited mitosis, but these cells formed multilobed nuclei because of failure of chromosome segregation (Figures 4A and 4B). In contrast, all BI 2536-treated cells arrested in mitosis, and most entered apoptosis directly from the mitotic arrest (Figures 4A and 4B). However, when cells were treated with a combination of Hesperadin and BI 2536, cells exited mitosis and formed multilobed nuclei similar to cells treated with Hesperadin alone (Figures 4A and 4B). Consistently, cyclin B levels were high in total lysates of cells arrested in mitosis by Nocodazole or BI 2536 (Figure 4D, lanes 2–4), but low levels were found in cells treated with Hesperadin and Hesperadin in

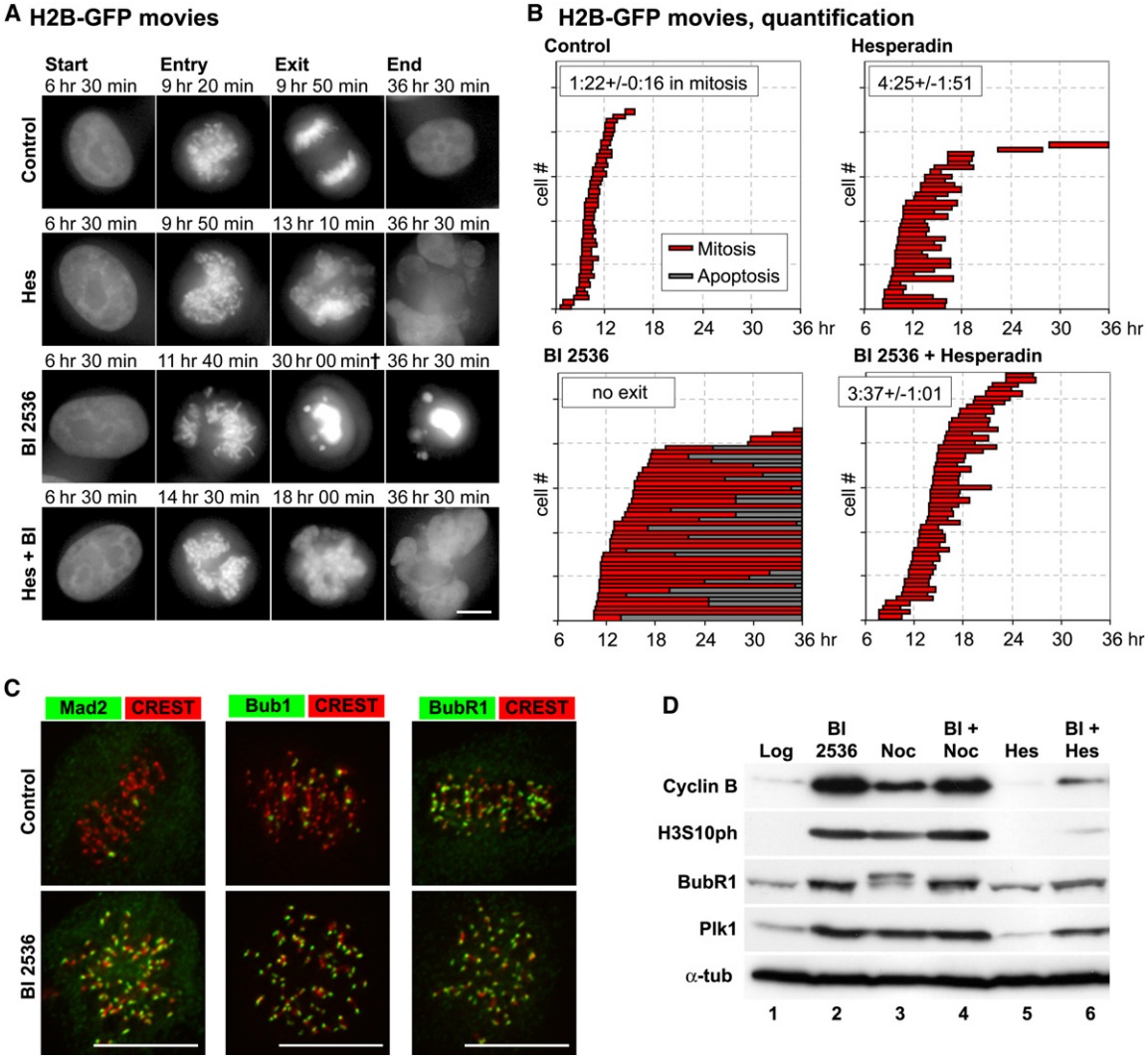

Figure 4. BI 2536 Treatment Leads to Spindle-Assembly-Checkpoint-Induced Prometaphase Arrest

(A) HeLa cells stably expressing H2B-GFP were synchronized by thymidine arrest and imaged after release in the presence of 100 nM Hesperadin, 100 nM BI 2536, a combination of the two drugs, or an equal amount of DMSO as the control. Selected cells representing the typical behavior of the population are shown at the start of recording, at the time of mitotic entry, at exit from mitosis or apoptosis, and at the end of the experiment. The scale bar represents 10 μm.

(B) Quantification of (A). For each cell filmed, the red bar represents the time spent in mitosis (defined by the time from NEBD to chromosome decondensation), and the gray bars indicate apoptosis. The numbers indicate the average and standard deviation of the time spent in mitosis (hh:mm).

(C) Asynchronous populations of HeLa cells were treated for 2 hr with 100 nM BI 2536, fixed, and stained for the indicated checkpoint proteins, and CREST was used as a centromeric marker. Maximum-intensity projections of three deconvolved 150 nm image sections are shown. Checkpoint protein is shown in green; CREST is shown in red. Scale bars represent 10 μm.

(D) Immunoblots of total cell lysates of cells 13 hr after the second thymidine release. 100 nM BI 2536, 50 ng/ml Nocodazole, 100 nM Hesperadin, or combinations of those were added at release from the second thymidine arrest.

combination with BI 2536 (Figure 4D, lanes 5 and 6). Thus, our data imply that BI 2536 treatment leads to activation of the SAC and that Plk1 activity is not essential for APC/C activation and mitotic exit.

Although Plk1 depletion was known to activate the SAC, the behavior of SAC proteins in Plk1 depleted cells has remained controversial. Depletion of Plk1 did not prevent the enrichment of Mad2 and BubR1 at kinetochores in some studies [14, 15], whereas others reported that depletion of Plk1 or Plx1 did prevent these interactions [16–18]. We therefore analyzed Mad2, BubR1, and also Bub1 by immunofluorescence microscopy in BI 2536-treated cells. In control prometaphase cells, we could clearly distinguish kinetochores that were either brightly or only dimly stained (Figure 4C and Figure S4B). Previous work has shown that the dim and bright signals represent kinetochores that are fully or not fully attached to microtubules, respectively [38, 39]. In cells arrested by BI 2536, more than 90% of kinetochores were brightly and homogeneously stained for all three SAC proteins (Figure 4C and Figure S4A). Quantification of these signals showed that the intensities on these kinetochores were similar to brightly labeled kinetochores of control cells, with the possible exception of

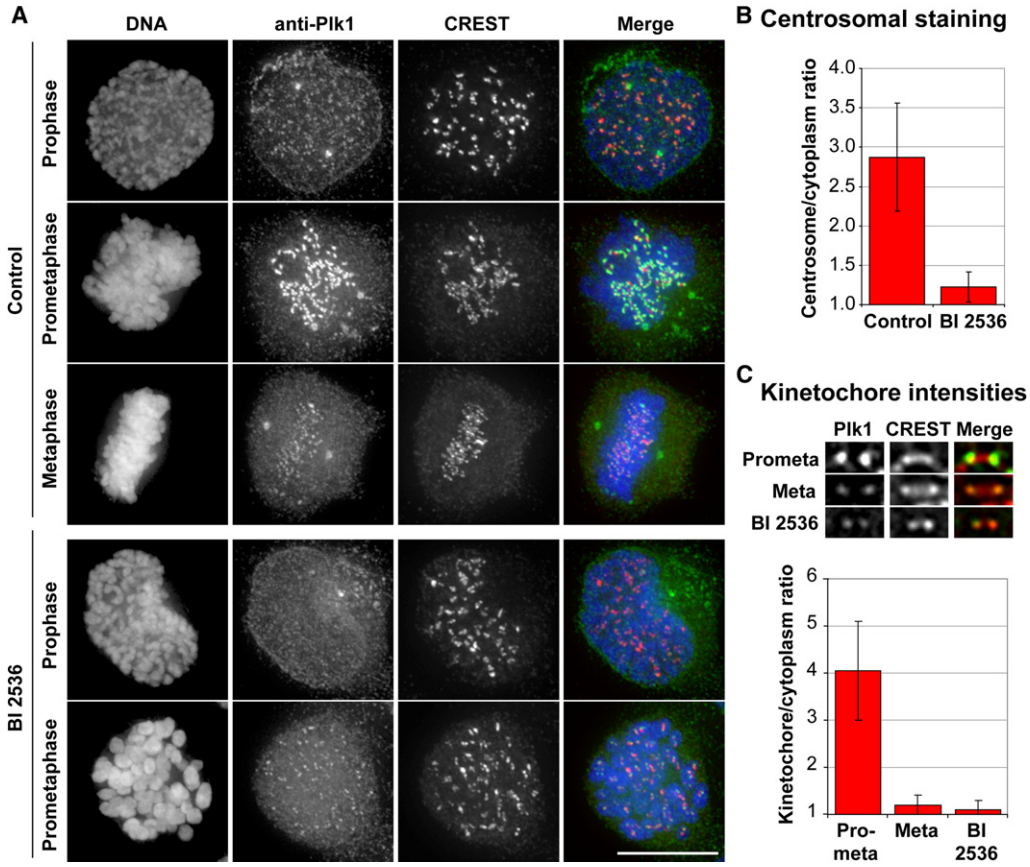

Figure 5. BI 2536 Prevents Plk1 Recruitment to Mitotic Centrosomes and Unattached Kinetochores

(A) Asynchronous populations of HeLa cells were treated with 100 nM BI 2536 for 2 hr, fixed, and processed for immunofluorescence. Maximum-intensity projections of stacks of deconvolved 150 nm sections are shown. The scale bar represents 10 μm.

(B) Centrosomal Plk1 staining was compared to the cytoplasmic signal in 10 prometaphase cells treated with or without BI 2536. Ratios and standard deviations are shown.

(C) Examples showing outer kinetochore staining of Plk1. For quantification of intensities, 100 kinetochores from at least five different cells were measured each in control prometaphase, control metaphase, and BI 2536 prometaphase arrest. Ratios to cytoplasmic staining and standard deviations are shown.

BubR1, whose kinetochore levels were slightly reduced by BI 2536 (Figure S4B). In immunoblot experiments, we also noticed that BubR1's mitotic mobility shift, which is caused by phosphorylation [40], was abolished when cells entered mitosis in the presence of BI 2536 (Figure 4D, compare lanes 3 and 4). Our data therefore indicate that Plk1 activity is not required for the recruitment of Mad2, Bub1, and BubR1 to unattached kinetochores, although Plk1 may be needed for BubR1 phosphorylation.

BI 2536 Prevents Plk1 Recruitment to Centrosomes and Kinetochores

The PBD of Plk1 is required for the recruitment of Plk1 to mitotic centrosomes and for enrichment of Plk1 at unattached kinetochores [41]. The PBD has been proposed to mediate these interactions by binding to phosphosites that are generated by Cdk1 [20, 42], but in some cases, it has also been reported that Plk1 can create its own phosphosite for PBD binding [18, 21, 43].

We therefore tested whether Plk1 activity is required for the correct subcellular localization of Plk1 in early mitosis. In control cells, Plk1 antibodies clearly stained centrosomes and unattached kinetochores (Figure 5A). In contrast, after BI 2536 treatment, centrosomes were labeled weakly and only dim kinetochore signals were observed in prometaphase arrested cells (Figures 5A–5C). Although BI 2536-treated cells arrest with kinetochores that are not occupied by a full complement of microtubules (see below), the intensity of Plk1 staining at these kinetochores was reduced more than 10-fold, to a level similar to the intensity of Plk1 staining at kinetochores in control metaphase cells (Figures 5A and 5C).

Our data therefore suggest that Plk1 activity is essential for the proper recruitment of Plk1 to mitotic centrosomes and unattached kinetochores. We cannot exclude the possibility that BI 2536 prevents Plk1 recruitment indirectly; however, recent findings by others have shown that Plk1 itself can create phosphoepitopes that are recognized by the PBD at kinetochores [18]. It is therefore conceivable that once recruited to these locations, Plk1 activity may initiate a positive feedback loop that could result in rapid and high levels of local accumulation of Plk1 at the sites where it performs its functions.

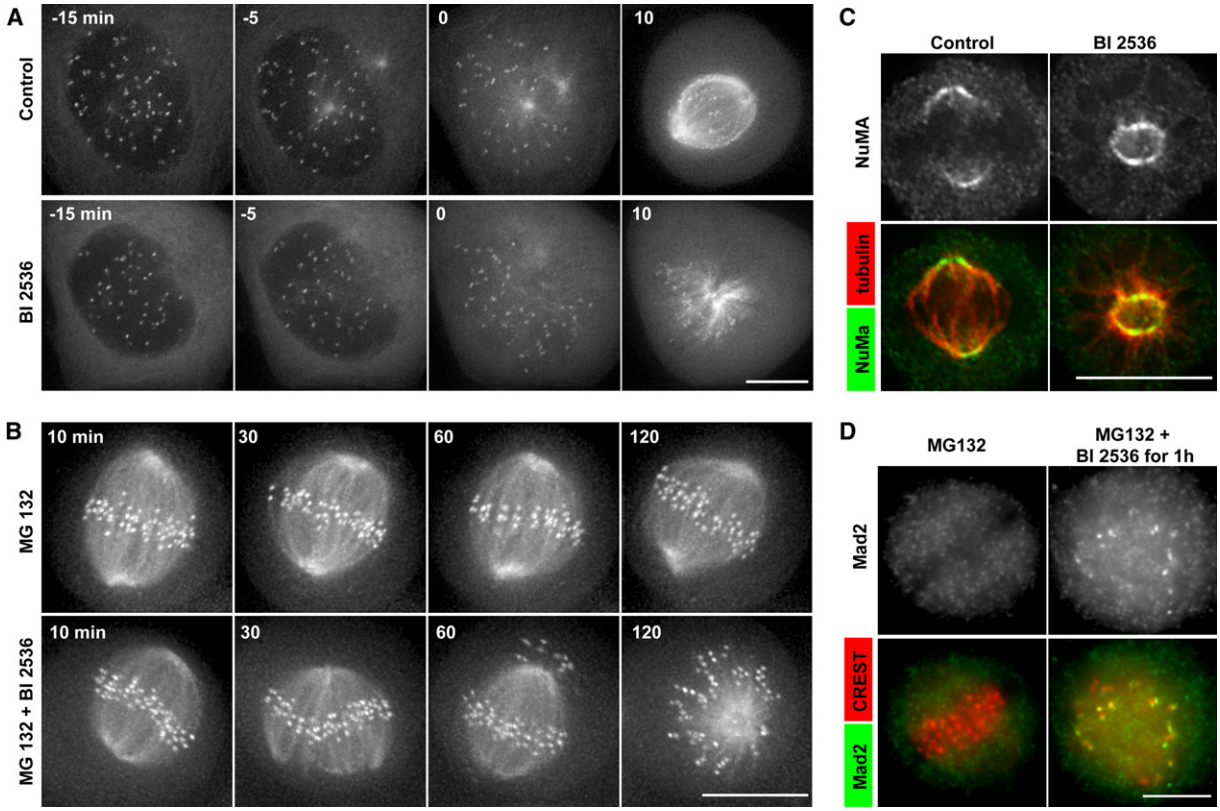

Figure 6. BI 2536 Prevents Formation and Maintenance of Centrosomal Microtubule Asters

(A) TC21 cells expressing α-tubulin-GFP and CENP-A-GFP were synchronized by a single thymidine arrest, treated with or without 100 nM BI 2536 4 hr after release, and imaged. Hoechst 33342 was added for identifying cells in prophase. Six z planes were acquired 1 μm apart every minute. Maximum-intensity projections of deconvolved images are shown for selected time points. Time is shown in minutes; t = 0 is set to NEBD. The scale bar represents 10 μm. For the full movie, see Movie S2.

(B) As in (A), except that cells were arrested for 2 hr with 10 μM MG 132 and BI 2536 was added at t = 0. Images were taken every 5 min. For the full movie, see Movie S3.

(C) Cells treated with or without 100 nM BI 2536 for 2 hr, fixed, and stained for NuMa (green) and tubulin (red). The scale bar represents 10 μm.

(D) In an experiment similar to (B), cells were stained for Mad2 so that the status of the spindle assembly checkpoint could be assessed. The scale bar represents 10 μm.

BI 2536 Inhibits Formation and Maintenance of Centrosomal Microtubule Asters

Plk1 is known to be required for the recruitment of γ-tubulin to mitotic centrosomes and for the formation of functional bipolar spindles [6, 14, 15]. However, it has not been analyzed in kinetic terms how microtubules behave in the absence of Plk1 activity and whether this activity is continuously required once bipolar spindles have been formed.

To address these questions, we generated a HeLa cell line (TC21) that stably coexpresses GFP-tagged versions of α-tubulin and of the centromere-specific histone CENP-A. Live cell imaging of these cells revealed that centrosomes started to nucleate short and dynamic mitotic microtubule asters approximately 10 min before NEBD (Movie S2). Thereafter, microtubules rapidly captured kinetochores and aligned them on a bipolar spindle in less than 10 min (Figure 6A and Movie S2). In contrast, prophase microtubule asters were undetectable in BI 2536-treated cells (Figure 6A and Movie S2; for immunofluorescence data, see Figure S5). In these cells, mitotic microtubules appeared only after NEBD, and these microtubules were instantaneously organized into a monopolar spindle (Figure 6A and Movie S2). Because this spindle configuration is very similar to the phenotype of the first described *polo* mutants in *Drosophila* [44], we refer to these structures as "Polo" spindles. The microtubule minus-end cross-linker NuMa [45] was enriched at the center of these "Polo" spindles, suggesting that NuMa and other microtubule associated proteins might organize microtubules in the absence of centrosomal nucleation (Figure 6C). The monopolar phenotype was not caused by unspecific inhibition of Eg5, in light of the fact that the ATPase activity of Eg5 was unaffected by BI 2536 in vitro (data not shown).

Taken together, our data imply that Plk1 activity is essential for centrosomal microtubule nucleation and that these centrosomal microtubule asters might have a prominent role in prophase, but our data also imply that centrosome independent mechanisms are able to mediate microtubule polymerization after NEBD as has been observed in noncentrosomal cells.

To address whether Plk1 is also required for maintaining centrosome function, we treated TC21 cells with MG132, a proteasome inhibitor that arrests cells with bipolar spindles in which all kinetochores are attached to microtubules (Figures 6B and 6D). Live cell imaging

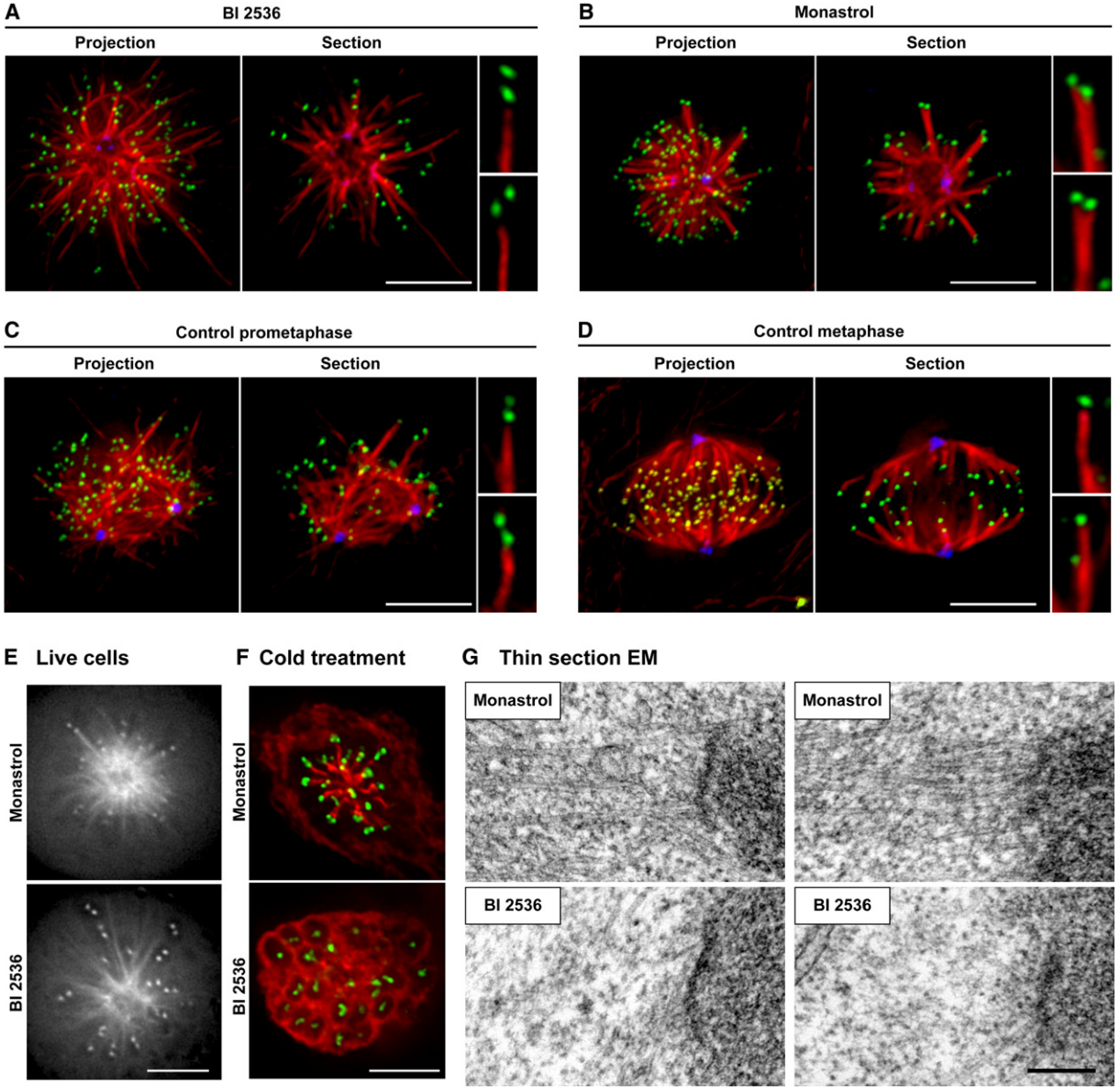

Figure 7. Plk1 Inhibition by BI 2536 Prevents Establishment and Maintenance of Stable Microtubule-Kinetochore Attachments

(A–D) Asynchronous populations of HeLa cells stably expressing CENPA-GFP were treated for 2 hr with 100 nM BI 2536, 100 μM Monastrol, or left untreated and were fixed and processed for immunofluorescence. Maximum-intensity projections of deconvolved stacks ("projection") or maximum-intensity projections of three 150 nm sections ("section") are shown. Insets show magnified views of kinetochore attachments. Centromeres (CENPA-GFP) are shown in green, α-tubulin is shown in red, and γ-tubulin is shown in blue. Scale bars represent 5 μm.

(E) TC21 cells were treated with BI 2536 or Monastrol. Deconvolved single optical sections are shown. The scale bar represents 5 μm.

(F) HeLa cells were treated with BI 2536 or Monastrol for 2 hr, put on ice for 15 min, fixed, and stained for α-tubulin (red) and CREST (green). Maximum-intensity projections of three deconvolved 150 nm sections are shown. The scale bar represents 5 μm.

(G) Thin section electron micrographs of kinetochores in cells treated with BI 2536 or Monastrol. The scale bar represents 200 nm.

revealed that spindles remained in this state for at least 2 hr (Figure 6B). However, when BI 2536 was added, spindles lost astral microtubules and focused poles within 10–30 min (Figure 6B and Movie S3). Thereafter, spindle poles often broadened (Figure 6B, 30 min) and several kinetochore pairs lost biorientation (Figure 6B, 60 min). Finally, spindles shortened and suddenly collapsed into a "Polo" spindle (Figure 6B, 120 min; Movie S3). We also performed immunofluorescence microscopy on fixed cells at different times after the addition of BI 2536. As in live cells, we found that spindles first lost focused poles (Figures S6A and S6B, 30 min) and shortening and collapse followed (Figures S6A and S6B, 60 and 120 min). Concomitantly, centrosomes detached from the spindle, moved toward the spindle equator, and gradually lost γ-tubulin staining (Figure S6A).

Taken together, our results indicate that Plk1 activity is required for maintaining the association of γ-tubulin with centrosomes and is necessary for continuous nucleation of microtubules from centrosomes. Similar findings have recently been obtained in experiments involving purified *Drosophila* centrosomes [30].

BI 2536 Inhibits the Formation and Maintenance of Kinetochore Fibers

It has been suggested that Plk1 [15] or Plk1 recruitment to kinetochores by NudC is required for generation of stable microtubule-kinetochore attachments [43]. However, it has not been analyzed in detail how microtubules interact with kinetochores in cells lacking Plk1 activity.

To understand which of the spindle abnormalities in BI 2536-treated cells might be indirectly caused by the monopolar nature of these spindles, we analyzed Monastrol-treated cells as controls because these cells also form monopolar spindles. Monastrol-treated cells possessed very distinguishable kinetochore fibers (K-fibers), which held kinetochore pairs within a narrow range of 3.45 ± 0.16 μm from the spindle center. In contrast, kinetochore pairs in BI 2536-treated cells were scattered and on average located further away from the spindle center (4.57 ± 0.54 μm). Moreover, Monastrol spindles were composed mostly of K-fibers, much like untreated metaphase cells (Figures 7B and 7D). In contrast, "Polo" spindles contained many thinner and longer microtubules, most of which did not end at kinetochores (Figure 7A), reminiscent of prometaphase cells where chromosomes are being captured and K-fibers are not yet established (Figure 7C). We could visualize similar structures in live cells (Figure 7E), indicating that microtubule structures were preserved by our fixation protocol.

To confirm that BI 2536-treated cells cannot establish stable K-fibers, we used cold treatment to depolymerize microtubules that are not part of K-fibers [46]. In BI 2536-treated cells, we could not visualize any microtubule structures after cold treatment, whereas K-fibers persisted in Monastrol-treated cells (Figure 7F).

To directly visualize microtubule-kinetochore attachments, we examined thin sections of BI 2536-treated cells by transmission electron microscopy. Also in this case, we compared "Polo" spindles with Monastrol-treated cells. Two major differences were apparent: In Monastrol-treated cells, "end-on" attachments of microtubule bundles were visible at many kinetochores (Figure 7G) and the majority of kinetochore plates were arranged on a tangent relative to the spindle center (Figure S7). In contrast, kinetochores in BI 2536-treated cells were only connected to a few, if any, microtubules, and these attachments were often lateral (Figure 7G). Furthermore, in BI 2536-treated cells, the kinetochores were oriented in various directions and often faced the opposite side of the chromosomes relative to the spindle center (Figure S7).

Taken together, our data suggest that Plk1 activity is essential for the establishment of stable microtubule-kinetochore attachments. It is possible that kinetochores can be captured by microtubules in the absence of Plk1 activity, but these attachments may not be stable and proper K-fibers may therefore not be formed. Depletion of BubR1 by RNAi also causes defects in microtubule-kinetochore attachments [23, 47]. Because BI 2536-treated cells lack the mitotic hyperphosphorylated form of BubR1 (see above, Figure 4D), Plk1 might contribute to K-fiber formation by phosphorylation of BubR1 at kinetochores.

To address whether Plk1 activity is also required for the maintenance of K-fibers, we analyzed TC21 cells that had been arrested in MG132 and subsequently treated with BI 2536. As described above, bipolar spindles collapsed 1–2 hr after the addition of BI 2536. At the same time, thick K-fibers disappeared and were replaced by thinner microtubule bundles (Figure 6B). Moreover, we also observed that Mad2 was rerecruited to kinetochores in cells fixed in similar conditions, indicating that many of the microtubule-kinetochore attachments had been dissolved after treatment with BI 2536 (Figure 6D). Our data therefore suggest that Plk1 activity is required for not only the establishment but also the maintenance of K-fibers.

Conclusions

Previous experiments have clearly established that Plk1 and its orthologs have a variety of essential roles in mitosis. However, description of Plk1 inactivation phenotypes has been limited by the possibility that late mitotic functions could have been obscured by earlier defects. Furthermore, in many experimental systems, it has been difficult to assess whether Plk1 had been inactivated completely. We have therefore used the small-molecule inhibitor BI 2536 to reinvestigate functions previously ascribed to Plk1 and to gain novel insights to mitotic roles of Plk1. Our data imply that Plk1 has specific functions in Emi1 degradation, activation of Cdk1-cyclin B, cohesin release, centrosome function, and regulation of microtubule-kinetochore attachments. A comparison of our results with previous data from RNAi and biochemical experiments indicate strongly that BI 2536 is a potent and specific inhibitor of Plk1 in live human cells. BI 2536 will therefore be likely to serve as a powerful tool in mitosis research.

Supplemental Data

Supplemental Data include Experimental Procedures, seven figures, and three movies and can be found with this article online at http://www.current-biology.com/cgi/content/full/17/4/304/DC1/.

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