Plk1 negatively regulates PRC1 to prevent premature midzone formation before cytokinesis

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ABSTRACT To achieve mitosis and cytokinesis, microtubules must assemble into distinct structures at different stages of cell division—mitotic spindles to segregate the chromosomes before anaphase and midzones to keep sister genomes apart and guide the cleavage furrow after anaphase. This temporal regulation is believed to involve Cdk1 kinase, which is inactivated in a switch-like way after anaphase. We found that inhibiting Plk1 caused premature assembly of midzones in cells still in metaphase, breaking the temporal regulation of microtubules. The antiparallel microtubule-bundling protein PRC1 plays a key role in organizing the midzone complex. We found that Plk1 negatively regulates PRC1 through phosphorylation of a single site, Thr-602, near the C-terminus of PRC1. We also found that microtubules stimulated Thr-602 phosphorylation by Plk1. This creates a potential negative feedback loop controlling PRC1 activity. It also made the extent of Thr-602 phosphorylation during mitotic arrest dependent on the mechanism of the arresting drug. Unexpectedly, we could not detect a preanaphase regulatory role for Cdk1 sites on PRC1. We suggest that PRC1 is regulated by Plk1, rather than Cdk1 as previously proposed, because its activity must be spatiotemporally regulated both preanaphase and postanaphase, and Cdk1 activity is too binary for this purpose.

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INTRODUCTION

Mitosis can be conceptually divided into molecular events that occur preanaphase, when Cdk1 levels are high, and those that occur postanaphase, when Cdk1 levels drop dramatically. In concert with this view, many cell division proteins function predominantly during prophase–metaphase or anaphase–telophase but not both. For example, jinesin-5 promotes bipolarity of the mitotic spindle preanaphase, whereas kinesin-6 (also known as MKLP1) promotes bipolarity of the midzone microtubule complex postanaphase. We might expect the activity of such proteins to be regulated by Cdk1, as is believed to be the case for kinesin-5 and -6 (Mishima *et al.*, 2004; Cahu *et al.*, 2008). However, other cell division proteins function both preanaphase and postanaphase. In this case, spatiotemporal regulation by Cdk1 might be inappropriate, because its activity is roughly binary, that is, high in metaphase and low in anaphase. This may be one reason Plk1 and Aurora kinases, which are active both preanaphase and postanaphase, evolved to regulate cell division in conjunction with Cdk1 (Petronczki *et al.*, 2008; Carmena *et al.*, 2009). However, their activity is not uniform throughout cell division; rather, they are subjected to complicated and poorly understood spatiotemporal regulation (Fuller *et al.*, 2008).

PRC1 is a microtubule-bundling protein that is part of a conserved module that organizes an antiparallel microtubule array that forms in the center of the cell after anaphase onset, called the midzone complex or central spindle (Eggert *et al.*, 2006; Glotzer, 2009). Functions of the midzone complex include keeping separated sister chromatids apart after kinetochore fibers (k-fibers) disassemble (Straight *et al.*, 2003) and signaling the cortex to position the cleavage furrow (Murata-Hori and Wang, 2002; Bringmann, 2005). Genetic removal of PRC1 or its homologues in several organisms leads to failed cytokinesis without obvious effects on mitotic spindle assembly or chromosome segregation (Mollinari *et al.*, 2005). PRC1 is phosphorylated by Cdk1, and given its postanaphase function, it was assumed that Cdk1 phosphorylation blocks its activity during metaphase (Mollinari *et al.*, 2002, 2005). Consistent with this

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Abbreviations used: CENPE, centrosome-associated protein E; KIF4, kinesin family member 4; MKLP1, mitotic kinesin–like protein 1; MKLP2, mitotic kinesin–like protein 2; Plk1, polo-like kinase 1; PRC1, protein regulator of cytokinesis 1.

hypothesis, the yeast PRC1 homologue Ase1 was shown to be negatively regulated by Cdk1 phosphorylations (Khmelinskii *et al.*, 2007; Fu *et al.*, 2009). However, these negative regulatory Cdk1 sites are not evolutionally conserved, even between budding and fission yeasts. In the absence of functional data on Cdk1 phosphorylation sites on PRC1, it remains unclear whether PRC1 is regulated through a similar mechanism in mammalian cells. PRC1 is also phosphorylated by Plk1 on Thr-602 (Neef *et al.*, 2007), but the functional role of this regulation was unclear. Plk1 typically recognizes substrates through a priming mechanism (Elia *et al.*, 2003), docking on prephosphorylated primer either on the target protein or on different proteins in the same complex (Park *et al.*, 2010). PRC1 Thr-602 appears to be a self-docking site of Plk1 (Neef *et al.*, 2007). Here we uncover an unexpected role of phosphorylation of PRC1 on Thr-602 by Plk1. We find that this phosphorylation causes strong negative regulation of PRC1 that inhibits premature midzone formation and that Plk1 phosphorylation of this site is positively controlled by microtubules. We hypothesize that this circuit (microtubules stimulate Plk1 to inhibit PRC1) evolved to allow optimal regulation of PRC1 both preanaphase and postanaphase.

RESULTS

Plk1 activity prevents premature midzone formation in metaphase

Inhibiting Plk1 in mitosis using the potent and specific small molecule BI-2536 leads to monopolar spindles (Lenart *et al.*, 2007). While confirming this result, we noticed an unreported, rapid response that occurred before the bipolar spindles collapse into monopoles. Within minutes after addition of 100 nM BI-2536 to metaphase HeLa cells, microtubules became unusually bundled in the equatorial region (Figure 1A, red arrows, and Supplemental Movie S1). Later, chromosomes flipped outward, and eventually spindles converted into monopoles within tens of minutes (Figure 1A, green arrows). Figure 1B illustrates the kinetic progression from normal metaphase through enhanced bundling at the equator to monopolarity in cells treated with drug at metaphase and scored by live imaging.

The equatorial microtubule bundles induced by BI-2536 in metaphase resembled midzone bundles that normally form in anaphase. Using thin-section electron microscopy (EM), we found microtubules in drug-treated metaphase cells organized as antiparallel bundles with electron-dense material coating the central overlap region (Figure 1C, yellow arrows). Morphologically similar buildup of electron-dense material on antiparallel overlaps is a diagnostic feature of cytokinesis midzones by EM (Mullins and Biesele, 1977). Drug-induced metaphase bundles also recruited several proteins that are normally recruited to midzone bundles during cytokinesis (Figure 1D). These included the midzone assembly proteins PRC1 and centralspindlin (MKLP1 complexed with RacGAP1), CENPE (a kinesin that normally relocalizes from kinetochores to midzones after cytokinesis onset), and CEP55 and ARF6, which are involved in furrow membrane trafficking during late cytokinesis. Not all midzone proteins were recruited to drug-induced metaphase bundles. Aurora-B, MKLP2, and Kif4 retained their normal metaphase associating with centromeres, and Plk1 itself was diffusely localized (Figure 1E). In addition, the furrow-specific cortical components RhoA and citron were not prematurely recruited.

Drug-induced metaphase bundles were spatially distinct from kinetochore-fibers (Supplemental Figure S1A), and their assembly did not block spindle assembly checkpoint in cells that progressed to anaphase before monopolar organization (Supplemental Figure S1B and Supplemental Movie S2). A structurally unrelated Plk1 inhibitor, GW-843682X (Johnson *et al.*, 2007; Lansing *et al.*, 2007), caused identical premature midzone assembly and protein recruitment, suggesting that the drug effect is specific to Plk1 inhibition (Supplemental Figure S1, C–E).

To summarize Figure 1, addition of BI-2536 to metaphase cells rapidly induced equatorial microtubule bundles that resembled midzones bundles in structure and recruitment of certain midzone assembly factors. We will refer to them as "metaphase midzones." We concluded that Plk1 activity in metaphase inhibits premature midzone formation. Blocking Plk1 activity relieves this inhibition and breaks the temporal constraint, triggering assembly of midzone bundles in metaphase.

Plk1 prevents premature midzone assembly in metaphase by phosphorylating PRC1 on Thr-602

To identify which proteins Plk1 phosphorylates to inhibit premature midzone formation we focused on the well-characterized assembly factors PRC1, centralspindlin (MKLP1/RacGAP1), and CENPE, which all responded to BI-2536 treatment by relocating to microtubule bundles in metaphase as they do in cytokinesis (Figure 1D). Among these, only PRC1 depletion blocked metaphase midzone formation in response to BI-2536 (Figure 2, A–C). When PRC1 was depleted and cells treated with BI-2536, microtubules no longer formed bundles across the equator, and other midzone proteins were not recruited. Thus PRC1 was our primary candidate whose Plk1 phosphorylation negatively regulates metaphase midzone assembly.

We next profiled PRC1 phosphorylations by mass spectrometry, seeking sites that were phosphorylated in normal metaphase but not in metaphase after BI-2536 treatment. This required cell synchronization in a metaphase-like state. For this we used a kinesin-5 inhibitor, *S*-trityl-L-cysteine (STLC; Skoufias *et al.*, 2006) so as to retain dynamic microtubules. In general we believe synchronization with STLC provides a better mimic of normal metaphase than synchronization with drugs that stabilize or destabilize microtubules, and in this case the presence of dynamic microtubules proved critical for the subsequent analysis (see later discussion). BI-2536 triggered premature recruitment of PRC1 to microtubule bundles in monopolar spindles (Figure 3A). Centralspindlin subunits were not stably recruited to these parallel bundles as assayed by fixed immunofluorescence, perhaps reflecting a lower affinity of centralspindlin for parallel compared with antiparallel bundles before cytokinesis onset (Figure 3B).

Peptides that corresponded to four phosphorylation sites on PRC1 (Ser-554, Ser-571, Ser-564, and Thr-602) were enriched after 15 min of BI-2536 treatment on top of STLC compared with STLC alone in two independent mass spectrometry experiments. Among them, only the phospho–Thr-602 peptide was recovered in both experiments (Figure 3C). PRC1 Thr-602 fits a consensus Plk1 phosphorylation motif LNXp(S/T) (Kettenbach *et al.*, 2011) and was previously shown to be phosphorylated by Plk1 in vitro (Neef *et al.*, 2007). As a control, the known Cdk1 site Thr-470 (Jiang *et al.*, 1998) was highly phosphorylated both before and after BI-2536 treatment (Figure 3C).

PRC1 Thr-602 point mutant mimics Plk1 inhibition and causes premature midzone assembly in metaphase

To test whether any of the candidate Plk1 sites in PRC1 were required for suppression of premature midzone assembly, we expressed green fluorescent protein (GFP)–fused PRC1 and corresponding point mutants. PRC1 isoform 2 was used because it is the only essential isoform among three (Neef *et al.*, 2007). We referred to its fusion as GFP-PRC1^{WT}. A common challenge when working with PRC1 is that its localization during metaphase is very dose sensitive. When a GFP fusion was expressed using a standard mammalian promoter it localized on metaphase spindles

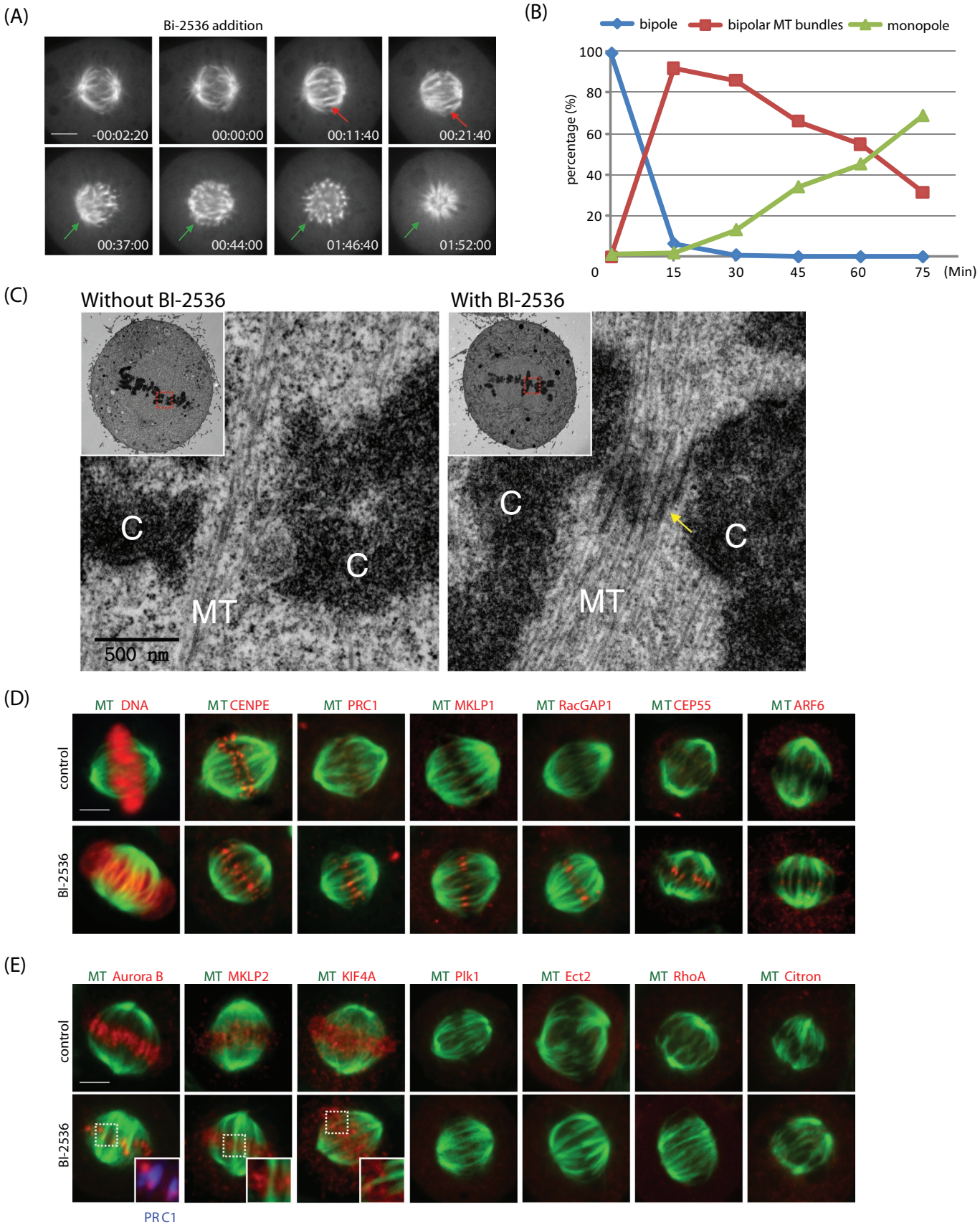

FIGURE 1: Effect of BI-2536 treatment on microtubules in metaphase. (A) HeLa cells stably expressing GFP–β-tubulin were imaged using spinning disk confocal microscope (Supplemental Movie S1). After BI-2536 was added at time zero, spindle microtubules bundled across the equator (red arrows) and then became monopolar spindles (green arrows) over

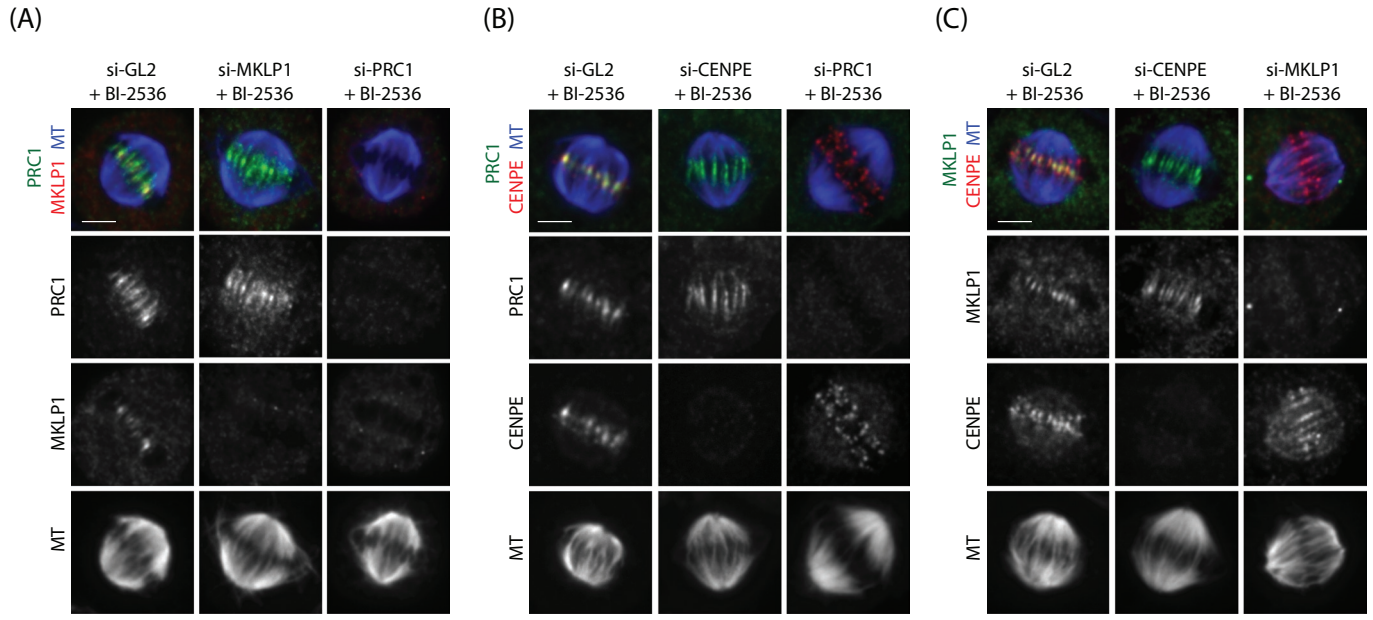

FIGURE 2: Identification of required proteins for premature metaphase midzone formation. (A–C) HeLa cells transfected with PRC1, MKLP1, or CENPE small interfering RNA were fixed for immunofluorescence after 15 min of BI-2536 treatment. PRC1 depletion blocked microtubule bundling and relocalization of MKLP1 and CENPE. MKLP1- or CENPE-depleted cells still assembled metaphase midzones. Bars, 5 μm.

(Figure 3D, leftmost column), as previously observed (Mollinari *et al.*, 2005). We tried and compared different approaches to overcome this overexpression artifact. A fusion of yellow fluorescent protein to endogenous PRC1 by insertion into the chromosome localized normally; it was recruited to microtubules in anaphase but not metaphase unless in the presence of BI-2536 (two stable cell lines provided by Uri Alon, Weizmann Institute, Rehovot, Israel; unpublished data). However, it was not feasible to engineer phospho-site mutants into this construct. Integrating a single additional copy of GFP-PRC1 gene expressed from its endogenous promoter in a bacterial artificial chromosome was sufficient to cause recruitment all over the metaphase spindle (MitoCheck database, www.mitocheck.org; Hutchins *et al.*, 2010), showing that it is very difficult to avoid this overexpression artifact using current technology. Instead, we analyzed whether the overexpressed GFP-PRC1^{WT} on spindles is still functionally regulated by Plk1 during metaphase. Resembling endogenous PRC1, GFP-PRC1^{WT} relocalized to tightly focused bundles at the equator in response to Plk1 inhibition (compare the first two columns in Figure 3D). Furthermore, MKLP1 was recruited to the same location as GFP-PRC1^{WT} only after drug treatment (Figure 3D, compare the first two columns). These observations argue that GFP-PRC1^{WT} retains negative regulation by Plk1 during metaphase even though it is overexpressed. Although the overexpressed GFP fusion binds uniformly to microtubules, only after Plk1 is inhibited does it concentrate at the equator and recruit MKLP1. Thus we could use this construct to analyze the effect of phosphorylation.

We next expressed point mutants of the candidate Plk1 sites identified by mass spectrometry. All GFP-PRC1s were expressed at a similar level by immunoblotting (unpublished data). The GFP-PRC1^{S554A,S571A,S574A} triple mutant behaved identically to GFP-PRC1^{WT}. Expression of GFP-PRC1^{T602A}, in contrast, caused both PRC1 focusing to bundles at the equator and MKLP1 recruitment to these bundles in metaphase without Plk1 inhibition (Figure 3D and Supplemental Figure S2A). This single point mutation appeared to completely mimic the effect of Plk1 inhibition by drug. However, unlike BI-2536 treatment, GFP-PRC1^{T602A} only blocked phosphorylation of PRC1 on Thr-602 and left other Plk1 phosphorylations intact, for example, RacGAP1 on Thr-170 (Supplemental Figure S2A, yellow arrow). These data show that PRC1 Thr-602 is the key regulatory target of Plk1 for inhibition of premature midzone assembly. Preventing phosphorylation of this single site alone on GFP-PRC1

time. (B) HeLa cells treated with BI-2536 were fixed at different time points for immunofluorescence. Kinetics of mitotic spindle transition with BI-2536 treatment showed that bipolar microtubule bundles peaked to >90% within 15 min. Mitotic cells with bipolar spindles were counted as bipoles, or bipolar microtubule bundles, if microtubules were bundled across the equator. Mitotic cells without clear bipolar spindle structures were counted as monopoles. Total cell number, 2133. (C) Thin-section EM of HeLa cells in metaphase showed that BI-2536 caused antiparallel microtubule bundles across the equator between chromosomes. Electron-dense materials coated the central interdigitated matrix of antiparallel microtubule bundles (yellow arrow). Views of the whole cell are shown as insets, with magnified areas boxed in red. C, chromosomes; MT, microtubules. (D, E) In response to BI-2536 treatment in metaphase, chromosomes stayed unsegregated and microtubules bundled in metaphase. CENPE, PRC1, MKLP1, RacGAP1, CEP55, and ARF6 relocalized on microtubule bundles as in cytokinesis; Aurora B, MKLP2, KIF4, Plk1, Ect2, RhoA, and Citron stayed at their metaphase localizations. Boxed regions are magnified and shown as insets. In this and all subsequent figures, except as otherwise noted, we characterized each cell in metaphase by DNA morphology but do not present its DNA image for clarity. Bars, 5 μm. See also Supplemental Figure S1.

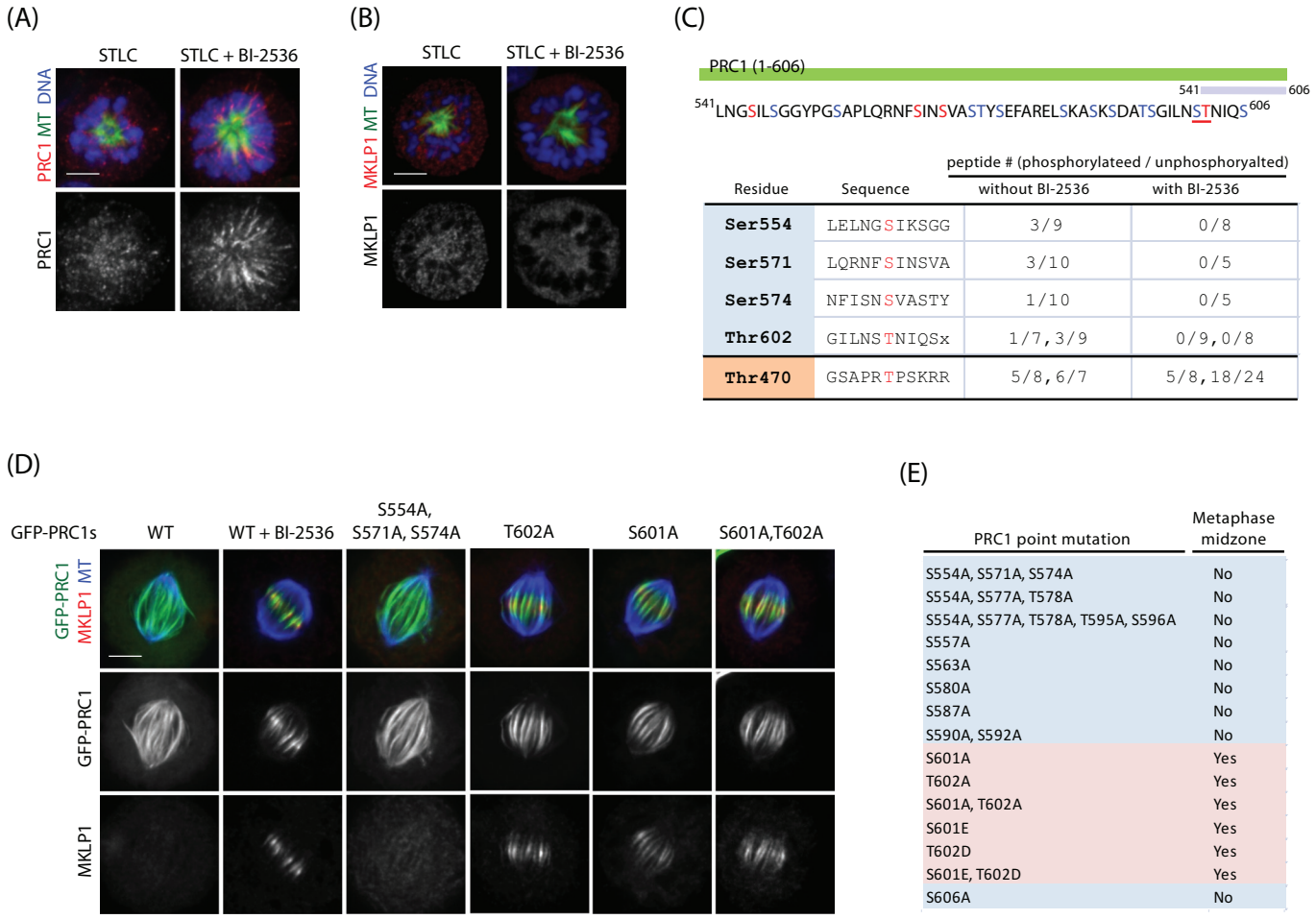

FIGURE 3: Characterization of Plk1 phosphorylation sites on PRC1 for midzone assembly inhibition. (A, B) BI-2536 triggered microtubule bundling in STLC arrested mitotic monopolar cells. HeLa cells were treated with STLC for 12 h, followed by 15 min of BI-2536 treatment. Monopolar microtubules were bundled with relocalized (A) PRC1 but not (B) MKLP1. (C) Phosphorylation profiles of PRC1 through mass spectrometry. Top, four sites losing their phosphorylations after BI-2536 treatment, shown in red, and 12 other, nearby serine/threonine residues, shown in blue. Residues that relieved the inhibition of midzone assembly after alanine replacements are underlined. Bottom, table showing residues dephosphorylated after BI-2536 treatment, shown shaded in blue, and Cdk1 site Thr-470 as control, shown in orange. (D) GFP-PRC1 point mutants were expressed in the absence of BI-2536. Alanine replacements of Ser-601 and/or Thr-602 mimicked BI-2536 effects on GFP-PRC1^{WT} to focus PRC1 and recruit MKLP1 on bundled microtubules in metaphase. (E) Summary of GFP-PRC1 point mutants of all 16 serine/threonine in the C-terminal region of PRC1. Mutants able to trigger midzone formation in metaphase are shaded in pink. Bars, 5 μm. See also Supplemental Figure S2.

triggers the midzone assembly program in metaphase but not other aspects of cytokinesis.

All our identified phosphorylation sites were in the PRC1 C-terminal region, which is rich in S/T residues (Figure 3C, 16 of the last 53 amino acids). To confirm the specificity of Thr-602 as the target of Plk1 regulation, we mutated all 12 other serine/threonine to alanine and found that only PRC1^{S601A} caused the same phenotypes as PRC1^{T602A} (Figure 3, D and E). We suspect that mutating Ser-601 may impede Plk1 docking or Thr-602 phosphorylation. None of S601E, T602D, or double mutants restored inhibition of midzone formation (Figure 3E). Thus a negative charge will not substitute for a phosphate group of Thr-602 for negative regulation of PRC1 by Plk1. A series of insertion and truncation mutants of GFP-PRC1^{WT} was also tested (summarized in Supplemental Figure S2B), showing that the region around Thr-602, and especially N-terminal to the site, is involved in metaphase inhibition of GFP-PRC1 (Supplemental Figure S2, C and D). We suspect that the PRC1 C-terminal region is involved in autoinhibition of the microtubule-bundling function PRC1 and/or its ability to recruit downstream midzone proteins such as MKLP1.

PRC1 Thr-602 is phosphorylated by Plk1 before anaphase

Our data so far showed that inhibition of phosphorylation on Thr-602 prematurely activates PRC1 during metaphase. Thus we expected endogenous PRC1 Thr-602 to be phosphorylated during metaphase. However, a previous report showed that PRC1 Thr-602 only becomes phosphorylated after anaphase (Neef *et al.*, 2007). We reexamined this question using the same phospho-epitope antibody described by Neef *et al.* (2007), which they generously provided as a gift. We first probed Thr-602 phosphorylation in cells arrested in mitosis with drugs. Mitotic arrest does not correspond exactly to any normal state, but it is at least preanaphase in terms of kinase regulation.

Thr-602 was detectably phosphorylated in cells arrested in mitosis with both STLC and nocodazole, although the signal was significantly stronger in STLC arrest (Figure 4A, lanes 1 and 2). The weak signal in nocodazole arrest probably explains why Neef *et al.* (2007) did not detect Thr-602 phosphorylation before anaphase. Thr-602 phosphorylation during mitotic arrest was abolished by BI-2563 treatment (Figure 4A, lanes 3 and 4), confirming it is a Plk1 site. Phosphorylation of Thr-481, a Cdk1 site on PRC1 (Jiang *et al.*, 1998), was equally strong in both mitosis-arresting drugs and was unaffected by BI-2563, as expected (Figure 4A, lanes 1–4). When cells were forced out of mitotic arrest with the Cdk1 inhibitor RO-3306, phosphorylation on the Cdk1 site Thr-481 was lost, whereas phosphorylation on the Plk1 site Thr-602 was retained (Figure 4A, lanes 5 and 6). The extent of phosphorylation on Thr-602 was still much stronger in STLC than nocodazole. These observations suggest a strong dependence of PRC1 Thr-602 phosphorylation on microtubule polymerization. Western blots released from a G2 block by washout of the CDK1 inhibitor RO3306 showed that PRC1 is phosphorylated on Thr-602 in the absence of drugs (Supplemental Figure S3A).

To avoid possible artifacts of mitotic arrest, we next probed Thr-620 phosphorylation in single cells by immunofluorescence. Thr-602 phosphorylation of endogenous PRC1 was not detected until furrow ingression in telophase (Figure 4B, yellow arrows). Coincident with this delayed phosphorylation, Plk1—the kinase that phosphorylates and docks on Thr-602—also significantly increased on midzones after furrow ingression, coincident with Thr-602 phosphorylation (Supplemental Figure S3B, C). These suggest that PRC1 Thr-602 phosphorylation during cytokinesis occurs as a late event, which may correspond to a maturation phase of midzone assembly we recently described (Hu *et al.*, 2011). Lack of staining by anti–Thr-602 antibody in metaphase was due to the diffuse localization in cytoplasm of endogenous PRC1 at this stage. To improve our sensitivity for detection of Thr-602 phosphorylation by immunofluorescence, we expressed GFP-PRC1^{WT}. This causes mild overexpression of PRC1 and localization to metaphase microtubules, as discussed earlier. Using this approach, we detected robust phosphorylation of PRC1 on both Thr-602 and Thr-481 during metaphase (Figure 4C).

Microtubules stimulate Plk1 phosphorylation of PRC1 on Thr-602

Our Western blot data (Figure 4A) showed that Thr-602 phosphorylation was much weaker in cells arrested in mitosis with nocodazole than with the kinesin-5 inhibitor STLC, suggesting that phosphorylation is stimulated by microtubules. We confirmed the drug difference by mass spectrometry. In nocodazole-arrested cells we identified zero peptides with Thr-602 phosphorylated compared with seven with it dephosphorylate, whereas in STLC we recovered three phosphorylated compared with nine dephosphorylated. To further test for a role of microtubules in Thr-602 phosphorylation, we arrested cells in STLC and then further treated them with Taxol or nocodazole at different concentrations to change the amount of polymerized tubulin. Taxol had no significant effect on to Thr-602 phosphorylation, whereas nocodazole caused a concentration-dependent decrease (Figure 4D). Cells treated with only nocodazole and not STLC showed the same trend, except that nocodazole at lower concentrations could not arrest cells in mitosis, leading to lower average level of phosphorylation (Supplemental Figure S3D). These results show that microtubules stimulate phosphorylation of PRC1 on Thr-602 by Plk1, but normal polymerization dynamics is not required for this stimulation. They may also explain the apparent discrepancy in our results with those of Neef *et al.* (2007), who used only nocodazole for mitotic arrest.

Cdk1 phosphorylation does not directly regulate PRC1 activity

Cdk1 is usually implicated as the negative regulator of cytokinesis events in metaphase (Glotzer, 2009). Three Cdk1 sites in PRC1 are phosphorylated during metaphase (Malik *et al.*, 2009; Ozlü *et al.*, 2010), and thus it is logical to expect that PRC1 is negatively regulated by Cdk1. Two studies proposed such regulation, but in both cases they tested it with a C-terminal–truncated PRC1 missing the last 35 amino acids (Mollinari *et al.*, 2002; Zhu *et al.*, 2006). Our results showed that C-terminal–truncated PRC1 is constitutively active independent of its Cdk1 sites (Supplemental Figure S2, B–D) and is thus inappropriate for analysis of regulation by kinase activity.

When we triggered midzone assembly in metaphase by adding BI-2536, Cdk1 activity was high and PRC1 could still relocalize on metaphase midzones with Thr-481 phosphorylated (Figure 5A, white arrow). This is consistent with our mass spectrometry data in Figure 3C. Our results indicate that Cdk1 does phosphorylate PRC1 on Thr-481 in metaphase, but this phosphorylation does not inhibit PRC1 binding to, or bundling, microtubules into midzones as previously proposed.

To test whether full-length PRC1 is negatively regulated by Cdk1, we mutated all the known Cdk1 sites (Thr-470, Thr-481, and Ser-513) to alanine (Malik *et al.*, 2009; Ozlü *et al.*, 2010). As before, we analyzed these mutants in the context of mildly overexpressed GFP-PRC1. Thr-470 and Thr-481 localize within the nuclear localization sequences of PRC1 (Mollinari *et al.*, 2002). However, Cdk1 phosphorylations do not regulate PRC1's nuclear sequestration in G2 phase (Supplemental Figure S4, A and B, and Supplemental Movies S3 and S4). In metaphase, GFP-PRC1^{T407A,T481A,S513A} triple mutant localized identically to GFP-PRC1^{WT}, both before and after BI-2536 addition (Figure 5B). These data suggest that the three known Cdk1 sites are not very important for regulation of PRC1 during metaphase, with the caveat that we had to ask this question using mildly overexpressed protein. PRC1 regulation by Cdk1 and Plk1 may be quite complex, given that different splice isoforms differ at the C-terminus (see *Discussion*). However, our data strongly favor negative regulation during metaphase by Plk1 and not by Cdk1.

Taken together, our data suggest the possibility that PRC1 can be activated in the presence of high Cdk1 activity in metaphase. Microtubules and Plk1, rather than Cdk1 as previously believed, play a critical role in PRC1 regulations throughout the whole of cell division.

DISCUSSION

Our work provides new insights into PRC1 regulation and function throughout cell division. Drugs that inhibit Plk1 or mutations that mimic loss of Plk1 phosphorylation broke the temporal constraint on PRC1 activity and promoted assembly of midzone bundles prematurely in metaphase while CDK1 activity was still high. We found that midzone formation is temporally gated by a single phosphorylation site on PRC1, Thr-602. Plk1 negatively regulates midzone formation in metaphase by phosphorylating this site, and microtubules promote this reaction. Cdk1 regulation, in contrast, appeared to be much less important.

Specificity of small-molecule inhibitors of Plk1

There are five Polo kinase family members in humans: Plk1, Plk2, Plk3, Plk4, and the recently identified Plk5 (de Carcer *et al.*, 2011). We tested two different Polo kinase small-molecule inhibitors—BI-2536 and GW-843682X—and observed identical phenotypes. Both caused premature midzone assembly in metaphase. BI-2536 and GW-843682X have unrelated structures. Both are potent Plk1

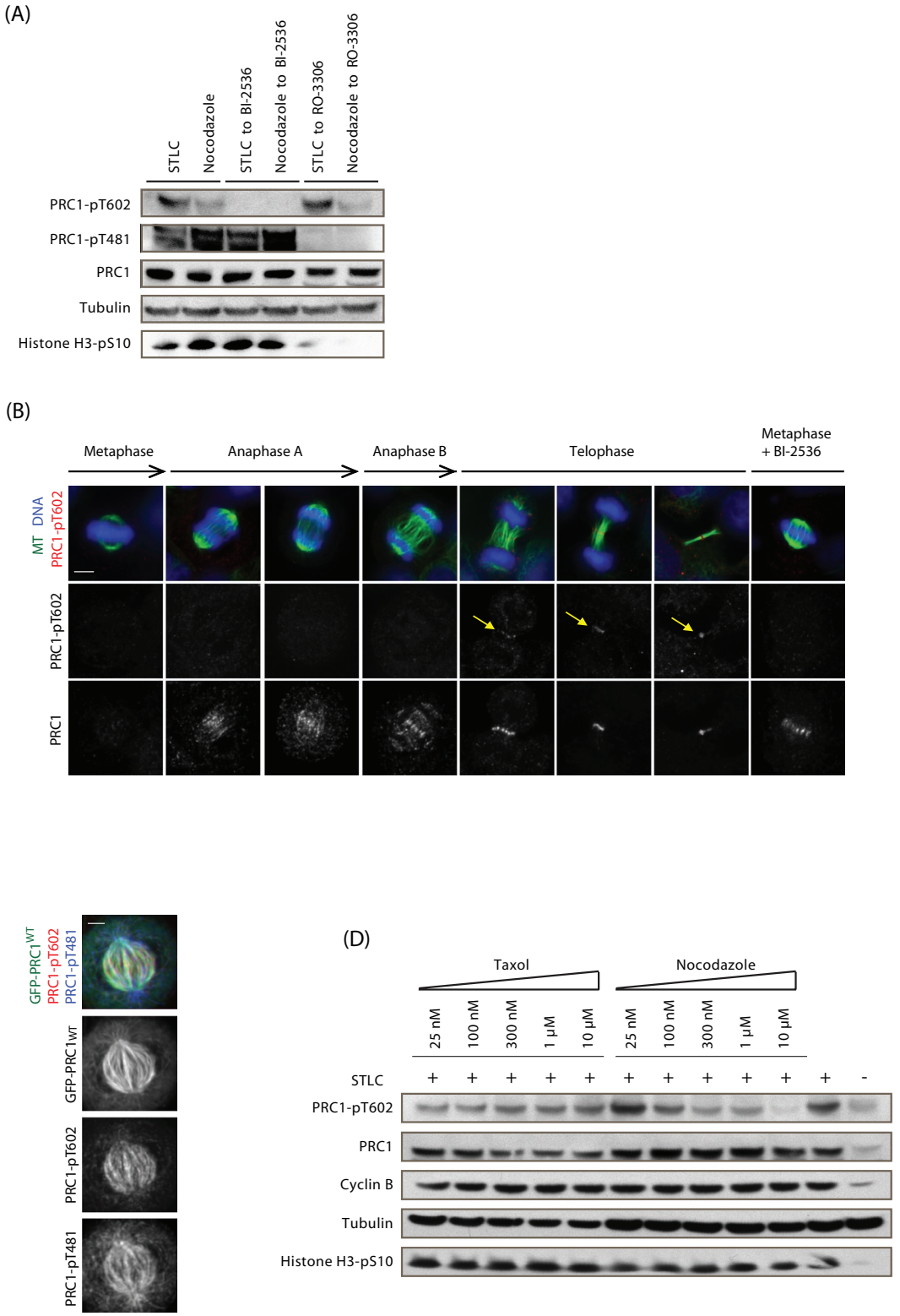

FIGURE 4: Spatiotemporal regulation of PRC1 Thr-602 phosphorylation in different phases of the cell cycle. (A) Western blots of drug-synchronized HeLa cell lysate. PRC1 Thr-602 was significantly less phosphorylated in nocodazole- than in STLC-treated cells; in both, drugs arrested metaphase or RO-3306–forced cytokinesis. (B) Immunofluorescence of endogenous PRC1 Thr-602 phosphorylation throughout cell division. PRC1 relocalized on midzone in early anaphase, but Thr-602 was not phosphorylated until furrow ingression in telophase (yellow arrows). (C) Cells expressing GFP-PRC1^{WT} were fixed for immunofluorescence. Thr-481 and Thr-602 were both phosphorylated in mitosis. (D) STLC-arrested mitotic cells were additionally treated with Taxol or nocodazole in different concentrations. PRC1 Thr-602 phosphorylation decreased with increasing nocodazole concentration but maintained the same level with different Taxol concentrations. Bars, 5 μm. See also Supplemental Figure S3.

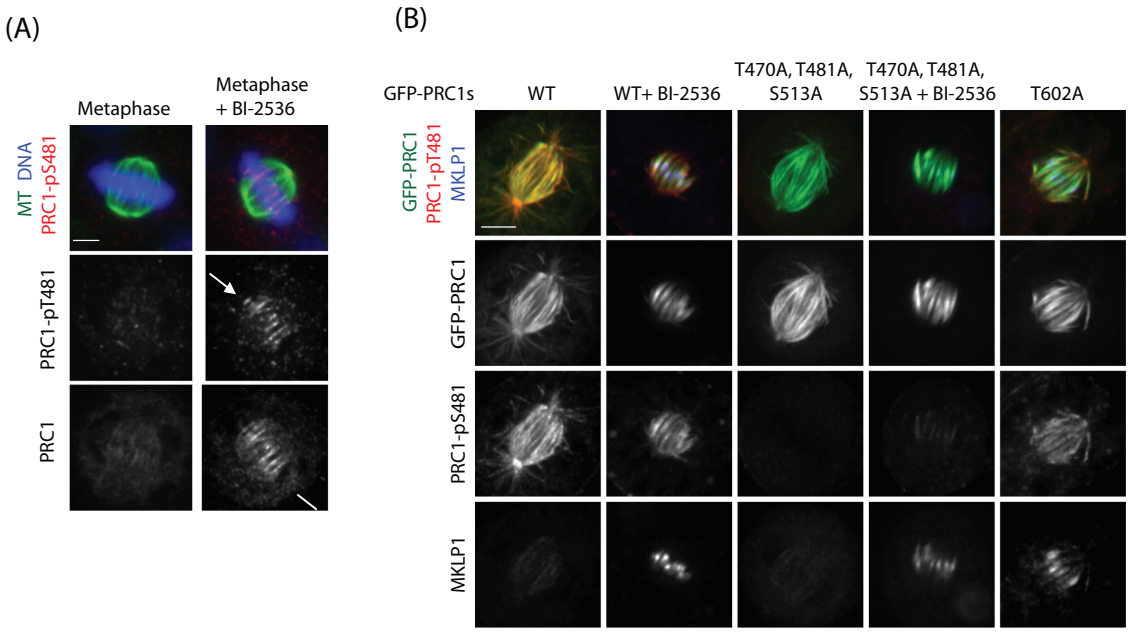

FIGURE 5: CDK1 phosphorylation does not directly regulate PRC1 activity and Thr-602 phosphorylation. (A) Immunofluorescence of PRC1 Thr481 phosphorylation in metaphase. Phosphorylation of PRC1 Thr-481 was not visible in metaphase unless BI-2536 was added to relocalize PRC1 to microtubules (white arrow). (B) GFP-PRC1^{T470A,T481A,S513A} behaved identically to GFP-PRC1^{WT}. Both were neither localized on microtubules nor recruited MKLP1 in metaphase unless BI-2536 was added. Bars, 5 μm. See also Supplemental Figure S4.

inhibitors (IC_{50} = 0.8 and 2.2 nM, respectively) with high selectivity over other kinase families. Although they are less selective among Polo-like kinases, all other members except Plk1 are believed to function during G1 and S phases. Thus both BI-2536 and GW-843682X are expected to specifically target Plk1 in mitotic cells.

Metaphase midzones as a system for dissecting cytokinetic networks

Our data showed that midzones can be assembled outside cytokinesis. Midzones provide a physical platform that localizes, and probably regulates, many crucial cytokinesis proteins (Glotzer, 2009). Midzones normally assemble when Cdk1 activity decreases in anaphase, but anaphase causes alterations in essentially every system in the cell. Forcing midzone formation in metaphase uncouples midzone assembly from anaphase onset. We believe that this provides a useful system for dissecting regulatory events that are specific for midzone formation away from the complex changes of anaphase. For example, PRC1 was shown to be the adaptor or scaffold protein for recruitment of several kinesins to midzones, including CENPE, MKLP1, MKLP2, KIF4, and KIF14 (Gruneberg *et al.*, 2006). When we induced midzones in metaphase, MKLP1 was recruited to them, but MKLP2 and KIF4 stayed at their normal metaphase location on chromosomes (Figure 1E). This suggests that other regulations, beyond PRC1 binding, are involved in midzone recruitment of the later two kinesins. MKLP2 (which brings CPC to midzones) and Ect2 (which signals RhoA to the furrow) are negatively regulated by Cdk1 phosphorylation (Hara *et al.*, 2006; Hummer and Mayer, 2009). This regulation may prevent the midzone CPC pathway and the cortical RhoA pathway being prematurely activated in metaphase even if midzone bundles do transiently form under specific preanaphase circumstances. Conversely, MKLP1 (kinesin-6), an essential component of the centralspindlin complex, is recruited to metaphase midzones. This casts some doubt on a previous conclusion that it must be dephosphorylated on a Cdk1 site at Thr-8 to promote midzone recruitment (Mishima *et al.*, 2004). Lack of recruitment of KIF4 to metaphase midzones is interesting, in that this kinesin is a key regulator of plus-end dynamics in anaphase midzones (Bieling *et al.*, 2010; Hu *et al.*, 2011). Investigating how plus-end dynamics is differentially regulated in metaphase versus anaphase midzones would be an interesting topic for further research.

Midzone assembly and midbody formation

Microtubules behave differently at different stages of cytokinesis. Midzones assemble and elongate in anaphase but lose their dynamics and remain at fixed length in telophase (Hu *et al.*, 2011). Phosphorylation of PRC1 Thr-602, which inhibits PRC1 bundling microtubules and recruiting midzone proteins, was detected in telophase (Figure 4B). Perhaps PRC1 activity is not required for PRC1 to stay on nondynamic telophase midzones or other proteins help to anchor PRC1 on midzones. The change of Thr-602 phosphorylation of PRC1 is also coincident with midbody formation, which starts during furrow ingression (Hu *et al.*, 2012). Unlike on midzones in anaphase, MKLP1 and CENPE do not colocalize with PRC1 on midbody (Hu *et al.*, 2012), suggesting that PRC1-mediated recruitment no longer exists. Among three PRC1 isoforms, isoform 2 is the only one essential for completing cytokinesis and the only isoform that can be inhibited by phosphorylation on Thr-602. Thus the rephosphorylation of PRC1 on Thr-602 might play crucial roles for midbody formation and integrity in late cytokinesis.

Inhibitory roles of PRC1 C-terminal region

PRC1 and its domains have been well studied. To form complete midzones, PRC1 not only bundles microtubules by its central region (Mollinari *et al.*, 2002) but also recruits essential midzone proteins through its N-terminal region (Kurasawa *et al.*, 2004). The structure of PRC1 shows how it bundles microtubules, but the C-terminal region was not included in this structure (Subramanian *et al.*, 2010). Although no full-length PRC1 has been crystallized,

the PRC1 C-terminal region is believed to be localized close to the N-terminal helical domains in the tertiary structure (Zhu *et al.*, 2006). Thus the C-terminal region is the only part of the protein without a defined function. Our data showed that Thr-602 phosphorylation, truncation, and some extensions of the C-terminal region all lead to constitutive activity (Figure 3D and Supplemental Figure S2D). We suspect that the C-terminal region causes autoinhibition by binding to its N-terminal and central regions, and this autoinhibition is blocked by Thr-602 phosphorylation. Whether it does so in-*cis* or in-*trans* (i.e., on the same or different polypeptides) remains to be determined.

Homeostatic model for PRC1 regulation

We found that PRC1 is regulated by Plk1, and not by Cdk1 as in budding and fission yeast. We suspect that this regulation evolved to avoid strictly binary regulation (off in metaphase, on in anaphase) and to allow for more subtle spatiotemporal regulation. The precise nature of this regulation and its biological purpose are not yet clear. PRC1 Thr-602 is phosphorylated by Plk1 in a reaction that depends on microtubule polymer and, at least at the cell population level, appears to show graded activity. This leads us to propose a model in which PRC1, Plk1, and microtubules together compose a homeostatic circuit that regulates antiparallel microtubule bundling throughout the whole of cell division. Under normal condition, when microtubule density is high, cells do not need PRC1 activity to build or maintain preanaphase spindle bipolarity; PRC1 is mostly inhibited by microtubule-stimulated Plk1 phosphorylation on Thr-602, and spindle bipolarization is driven by kinesin-5 and -15 (Tanenbaum *et al.*, 2009). If microtubule density is lowered, it may become harder for these kinesins to enforce antiparallel interactions. Under these conditions PRC1 becomes less phosphorylated in Thr-602 and more activated to bundle antiparallel microtubules. Another way to think about this is that mammalian cells may use multiple, parallel networks to drive bipolarization (kinesin-5, kinesin-15/TPX2, dephosopho-PRC1, and dynein acting on astral microtubules) to ensure that this critical aspect of spindle architecture is robust to variation in microtubule density, cell shape, and other as-yet-unknown variables.

MATERIALS AND METHODS

Cell culture and reagents

HeLa cells were cultured in DMEM with 10% fetal bovine serum at 37°C and 5% CO_2. HeLa cells stably expressing GFP–β-tubulin was a gift from P. Chang (MIT, Cambridge, MA). Cells were incubated with 100 nM BI-2536 (N. Gray, Harvard Medical School, Boston, MA) or 1 μM GW-843682X (Axon Medchem, Groningen, Netherlands) to inhibit Plk1 kinase activity. To arrest cell in mitosis, 2 μM *S*-trityl-L-cysteine (Merck, Darmstadt, Germany) or 3 μM nocodazole (Sigma-Aldrich, St. Louis, MO) was used for 12-h treatment. GFP-PRC1 isoforms were cloned from PCR products into pEGFP-C1 (Clontech, Mountain View, CA). SMARTpool siRNA (Dharmacon, Lafayette, CO) was used to silence specific genes.

Immunofluorescence

HeLa cells on coverslips were fixed by pure MeOH for 3 min or 10% trichloroacetic acid for 15 min and blocked and incubated with primary or secondary antibodies in AbDil buffer for 45 min. Sources of antibodies were as follows: Plk1 and Aurora B (N. Ozlü and C. Field, Harvard Medical School); PRC1-pT602 (Francis Barr, University of Liverpool, Liverpool, United Kingdom); Ect2 (M. Glotzer, University of Chicago, Chicago, IL); MKLP2 (T. Mayer, University of Konstanz, Konstanz, Germany). PRC1 (sc9342, sc8356), PRC1-pT481 (sc11768), MKLP1 (sc867), RhoA (sc179), ARF6 (sc7971), and Plk1 (sc17783) were from Santa Cruz Biotechnology (Santa Cruz, CA); RacGAP1 (ab2270), CENPE (ab5093), KIF4 (ab3815), centrin 1 (Ab11257), and cyclin B (Ab72) were from Abcam (Cambridge, MA). Citron (611376) was from BD Biosciences (San Jose, CA), CEP55 (H00055165-A01) from Abnova (Taipei City, Taiwan), histone H3 pS10 from Millipore (Billerica, MA), RacGAP1-pT170 (39266) from Active Motif (Carlsbad, CA), (fluorescein isothiocyanate–conjugated) monoclonal anti–α-tubulin (DM1A) and Hoechst from Sigma-Aldrich, and TOPO3 and Alexa Fluor 488–, 594–, or 647–labeled donkey anti-mouse, rabbit, and goat antibodies from Molecular Probes (Invitrogen, Carlsbad, CA).

Microscopy and imaging

Fluorescence images were done by a TE-2000 spinning-disk confocal microscope with a 1.3 numerical aperture/100× oil-immersion objective (Nikon, Melville, NY). Images were captured on a Photonic OrCA ER CCD camera (Hamamatsu, Hamamatsu City, Japan) for fixed samples or an Andor electron-multiplying charge-coupled device (EMCCD) camera (Andor, South Windsor, CT) for live imaging. Microscope control and image processing were through MetaMorph software (Universal Imaging, West Chester, PA). The protocol for thin-section EM was described in Hu *et al.* (2008).

Immunoprecipitation of PRC1 for mass spectrometry analysis

HeLa cells were lysed in buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 150 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 1 mM ethylene glycol tetraacetic acid, 0.5% NP-40, 1 mM dithiothreitol) including protease and phosphatase inhibitors (1 μM okadaic acid, 1 μM microcystine, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM β-glycerolphosphate, 1 mM sodium pyrophosphate). The extract was incubated with protein A beads (Bio-Rad, Hercules, CA) alone followed by incubation with anti-PRC1 antibody coupled with protein A to immunoprecipitate PRC1 at 4°C. The gel band corresponding to PRC1 was cut for the analysis. In-gel tryptic digest and mass spectrometry was performed according to Ozlü *et al.* (2010). ProteinPilot software 3 (AB Sciex, Framingham, MA) was used for protein identification, and phosphopeptides with 95% and higher confidences were reported (Shilov *et al.*, 2007).

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