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Cdk1 Negatively Regulates Midzone Localization of the Mitotic Kinesin Mklp2 and the Chromosomal Passenger Complex

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Summary

The survival of eukaryotes depends on the accurate coordination of mitosis with cytokinesis. Key for the coordination of both processes is the chromosomal passenger complex (CPC) comprising Aurora-B, INCENP, survivin, and borealin [1–5]. The translocation of the CPC from centromeres to the spindle midzone, a structure composed of antiparallel microtubules, at anaphase onset is critical for the completion of cytokinesis [6–12]. In mammalian cells, the mitotic kinesin Mklp2 is essential for recruitment of the CPC to the spindle midzone [13]. However, the mechanism regulating the binding of Mklp2 to microtubules has remained unknown. Here, we demonstrate that Mklp2 and the CPC mutually depend on each other for midzone localization; i.e., Mklp2 is mislocalized in INCENP-RNAi cells and vice versa. Remarkably, INCENP is required for localization of Mklp2 to the ends of stable microtubules in cells with low Cdk1 activity. In vitro assays revealed that the association between the CPC and Mklp2 is negatively regulated by Cdk1. Collectively, our data suggest that anaphase onset triggers the association between the CPC and Mklp2 and that this association targets the CPC-Mklp2 complex to the ends of stable microtubules in the spindle midzone.

Results and Discussion

Midzone localization of the CPC depends on the kinesin-6 family member Mklp2 [13]. Intriguingly, we observed that not only did INCENP fail to localize to the midzone in Mklp2-depleted cells, but Mklp2 was not detectable at the midzone in INCENP-RNAi cells (Figure 1A), suggesting that the CPC and Mklp2 are interdependent for proper midzone localization in anaphase. As previously shown [14], depleting INCENP resulted in the simultaneous destabilization of Aurora-B, whereas Mklp2 protein levels were not affected in INCENP-RNAi cells (Figure 1B), excluding the possibility that an off-target effect of the INCENP siRNA accounts for the lack of Mklp2 at the midzone.

To rule out the possibility that mislocalization of Mklp2 was an indirect consequence of defects occurring during early mitosis in INCENP-RNAi cells, we sought after an approach for separation of the late mitotic function of the CPC from the early one. Because INCENP is phosphorylated at T59 by Cdk1 until the onset of anaphase and the function of T59 phosphorylation was unknown [15], we speculated that the phosphorylation state of T59 regulates the translocation of the CPC to the midzone. To test this, we analyzed the localization of GFP-tagged wild-type (WT), a nonphosphorylatable mutant (T59V), or a phospho-mimic mutant (T59E) of INCENP in INCENP-RNAi cells (Figure S1, available online). Immunoblot analyses confirmed that endogenous INCENP was efficiently depleted and the rescue constructs were expressed at similar levels (Figure S1). We observed that, in line with previous reports [15], T59 was not critical for the early mitotic function of INCENP, as indicated by the fact that all three variants—unlike the empty GFP vector control—(1) restored the localization of the CPC to centromeres, (2) rescued the timed onset of anaphase, and (3) were able to induce a mitotic delay upon treatment of INCENP-RNAi cells with the microtubule poison taxol (Figure 1C and Figure S1). A strikingly different result was obtained when cells in anaphase were analyzed. GFP-INCENP^{WT} and the nonphosphorylatable T59V mutant localized correctly to the midzone in INCENP-depleted cells, whereas the phospho-mimic T59E variant remained on segregating chromosomes (Figure 1D). Likewise, Aurora-B and survivin were detected at the midzone in INCENP-RNAi cells expressing WT or T59V, as well as on the chromatin in the case of GFP-INCENP^{T59E} (Figure 1E and Figure S1). Consistent with the ability of WT and T59V to rescue CPC localization in anaphase, live-cell recording demonstrated that WT and T59V, but not T59E or the GFP vector control, efficiently complemented cytokinesis (Figure 1F). Thus, INCENP^{T59E} cannot support the postmetaphase function of the CPC, enabling us to study the role of the CPC in Mklp2 localization uncoupled from its function in early mitosis. As shown in Figure 2A, endogenous Mklp2 efficiently accumulated at the midzone of INCENP-RNAi cells expressing GFP-INCENP^{WT} or T59V, but localized diffusely in the cytoplasm of cells rescued with T59E. The failure of Mklp2 to localize correctly was not mediated by a gross defect in midzone microtubule organization, as indicated by the correct localization of PRC1 (Figure 2B). Collectively, these results suggest that the phosphorylation of INCENP at T59 acts in an inhibitory fashion on the transfer of the CPC to the spindle midzone, and they further corroborate our model of necessity of CPC and Mklp2 interdependence for correct localization.

Next, we analyzed the timing of the interaction between Mklp2 and the CPC in more detail. To this end, Mklp2 was immunopurified from cells released from a nocodazole block and associated proteins were analyzed by Western blotting. As shown in Figure 2C, INCENP and Aurora-B coprecipitated with Mklp2 as soon as cyclin-B levels began to decline. This interaction was specific, because INCENP and Aurora-B did not detectably coprecipitate when Mklp1 was immunopurified from cells in meta- or anaphase (Figure 2D). The interaction between Mklp2 and the CPC was mediated by the C-terminal cargo-binding domain of Mklp2, as demonstrated by Western blot analyses of GFP-tagged fragments of Mklp2 immunopurified from HeLa cells (Figure S1). Thus, anaphase onset triggers the association of the CPC with Mklp2.

Previously, Mklp2 has been shown to interact directly with Aurora-B [13]. Using recombinant Aurora-B purified from insect cells, we were able to confirm this interaction (data not shown). However, because the solubility of recombinant

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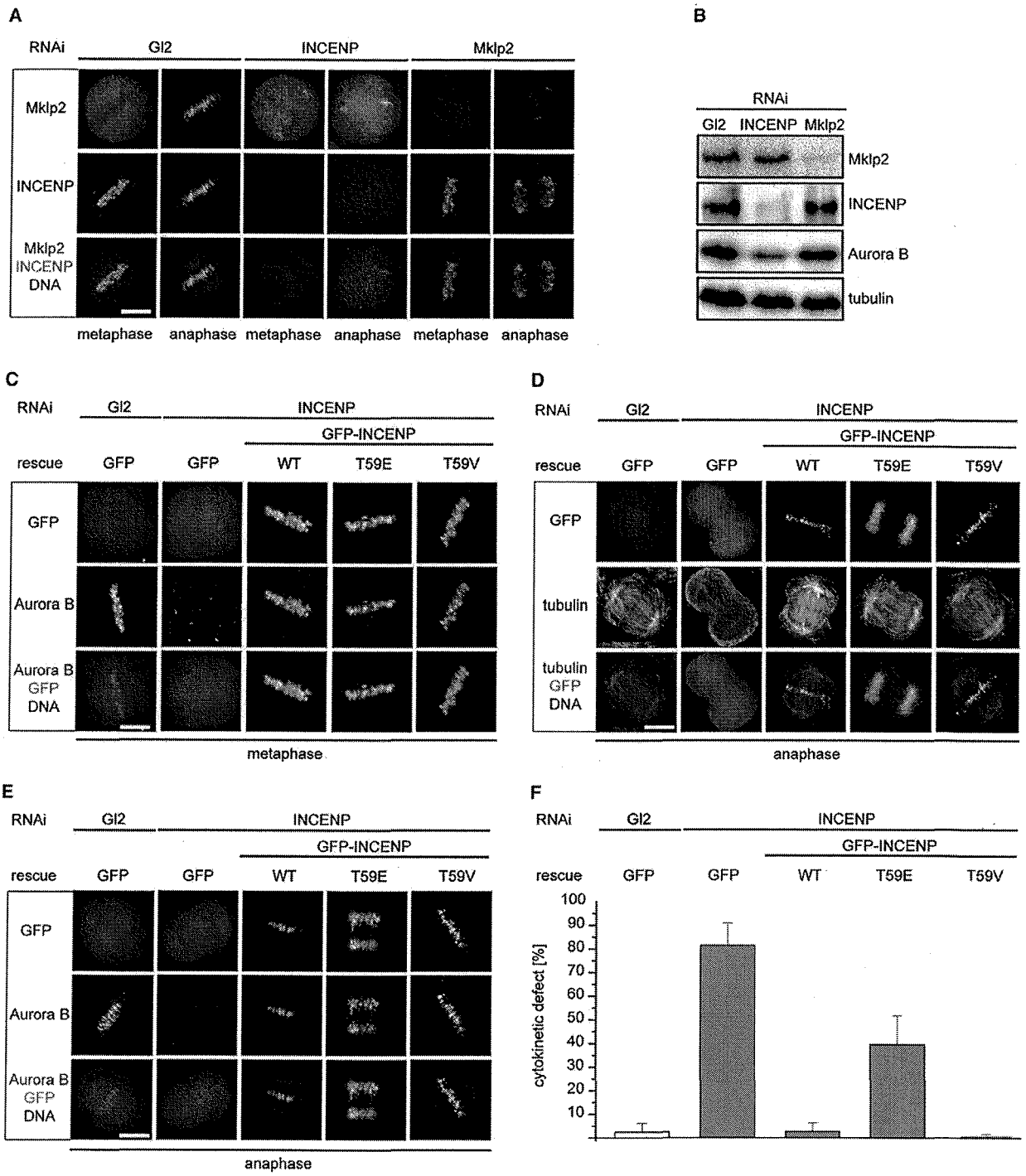

Figure 1. Mklp2 and the CPC Mutually Depend on Each Other for Midzone Localization

(A) HeLa cells transfected with the indicated siRNA duplexes were fixed and stained for Mklp2 (red), INCENP (green), and DNA (blue). Scale bar represents 10 μm.

(B) HeLa cells treated as described in (A) were processed for Western blot analyses. The α-tubulin Western blot served as a loading control.

(C) Immunofluorescence images of metaphase HeLa cells transfected with siRNA duplexes targeting INCENP or GI2 and transfected with empty GFP vector or the indicated INCENP variants. DNA, GFP-constructs, and Aurora-B are shown in blue, green, and red, respectively. As reported previously, INCENP depletion caused the destabilization of Aurora-B. Scale bar represents 10 μm.

(D) Immunofluorescence images of anaphase HeLa cells transfected with the indicated constructs and stained for DNA (blue), GFP (green), and tubulin (red). Scale bar represents 10 μm.

(E) Immunofluorescence images of anaphase HeLa cells treated as in (D). DNA, GFP-constructs, and Aurora-B are shown in blue, green, and red, respectively. Scale bar represents 10 μm.

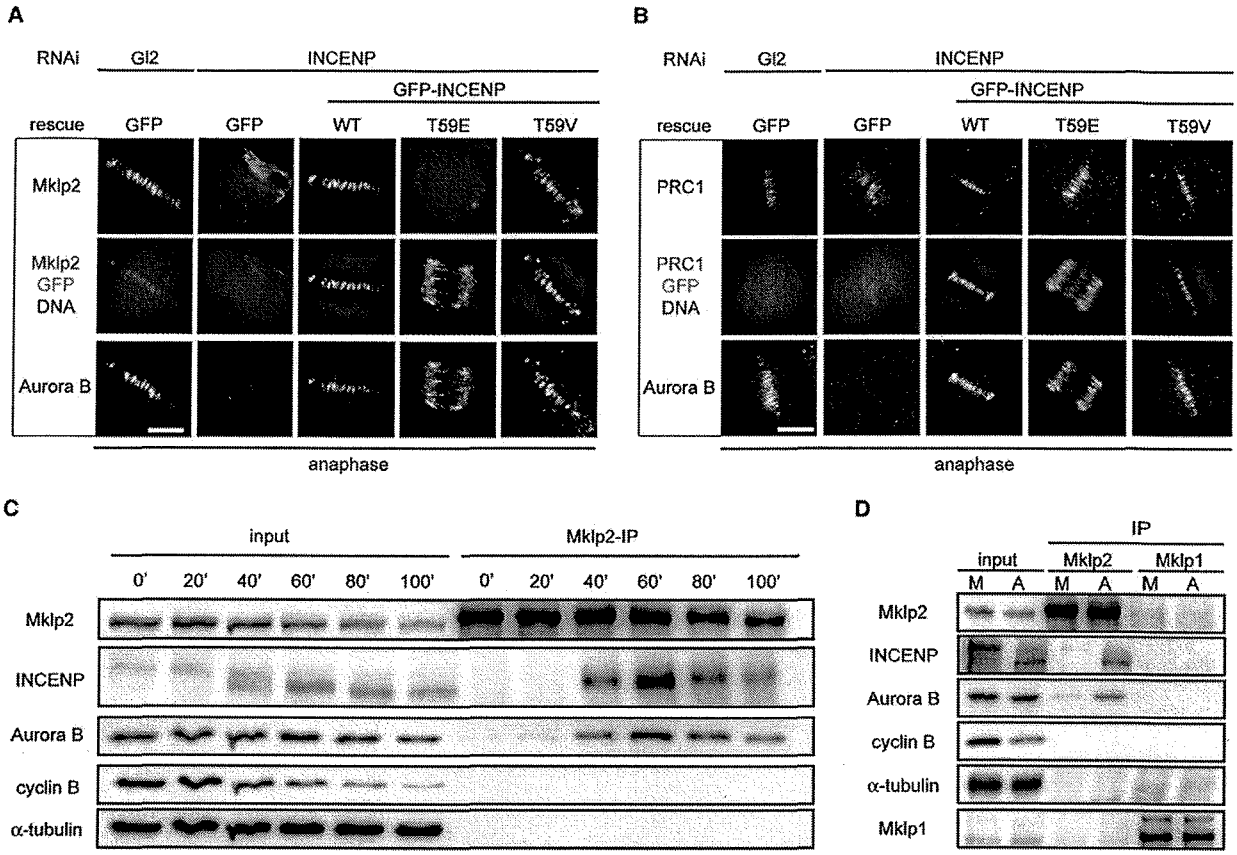

Figure 2. Anaphase Onset Triggers the Association between the CPC and Mklp2

(A) Immunofluorescence images of anaphase HeLa cells transfected with siRNA duplexes targeting INCENP or Gl2 and transfected with empty GFP vector or the indicated INCENP variants. Mklp2 is shown in red, DNA in blue, and GFP in green. The merged images do not show Aurora-B, for the purpose of clarity. Scale bar represents 10 μm.

(B) Anaphase HeLa cells treated like in A. PRC1 is shown in red, GFP in green, and DNA in blue. Scale bar represents 10 μm.

(C) Immunoblot analyses of Mklp2 immunopurified from synchronized HeLa cells. Cells arrested in prometaphase were collected by shake-off and released from the arrest for the indicated times. Lysates (input) and immune complexes (IP) were probed with Mklp2, Aurora-B, and INCENP antibodies. Cell-cycle progression was monitored by immunoblotting for cyclin B. The immunoblot for tubulin served as a loading control.

(D) Mklp2 or Mklp1 was immunopurified from extracts (input) of HeLa cells arrested at metaphase by nocodazole treatment (M, metaphase) or released from the arrest for 60 min (A, anaphase). Immunoprecipitates were analyzed by immunoblotting as indicated.

full-length INCENP depends on the coexpression of Aurora-B, we could not determine whether Mklp2 can also directly bind to INCENP. To analyze whether the phosphorylation state of T59 might be critical for the association of Mklp2 with the CPC, we immunopurified GFP-INCENP^{WT}, T59V, and T59E from anaphase cells. Intriguingly, compared to the WT and nonphosphorylatable form of INCENP, T59E was significantly less efficient in coprecipitating Mklp2, whereas, as expected, all three variants of INCENP were equally capable of binding Aurora-B (Figure 3A). To demonstrate that Cdk1 indeed accounts for the dissociation of Mklp2 and the CPC, we tested whether recombinant Cdk1 can dissociate the CPC-Mklp2 complex purified from HeLa extracts. To this end, Mklp2 was immunopurified from anaphase cells and the bead-bound Mklp2 precipitate was treated with recombinant active or heat-inactivated Cdk1/cyclin B, followed by a centrifugation step for separation of the bead-bound fraction from the supernatant. As shown in Figure 3B, INCENP and Aurora-B remained bound to Mklp2 when the precipitate was treated with heat-inactivated Cdk1/cyclin B. In stark contrast, Aurora-B and INCENP dissociated and were detected in the supernatant (only 1/3 was loaded) when the Mklp2 precipitate was treated with active Cdk1/cyclin B (Figure 3B). Consistently, incubation of the Mklp2-beads with Cdk1/cyclin B in the presence of the Cdk inhibitor roscovitine prevented the dissociation of Aurora-B from Mklp2 (Figure S1). Aurora-B was not inhibited by roscovitine addition, as indicated by its electrophoretic upshift characteristic of autophosphorylated, active Aurora-B (Figure S1). Collectively, these data demonstrate that the association between the CPC and Mklp2 is

(F) Quantification of live-cell recordings of Gl2- or INCENP-RNAi HeLa cells transfected with empty GFP vector or the indicated INCENP variants. For each condition, at least four independent experiments were performed, analyzing 120 cells at minimum. Bars represent the percentage of transfected cells that failed to complete cytokinesis and exited mitosis as binucleated cells. Data were averaged, and error bars represent standard deviations.

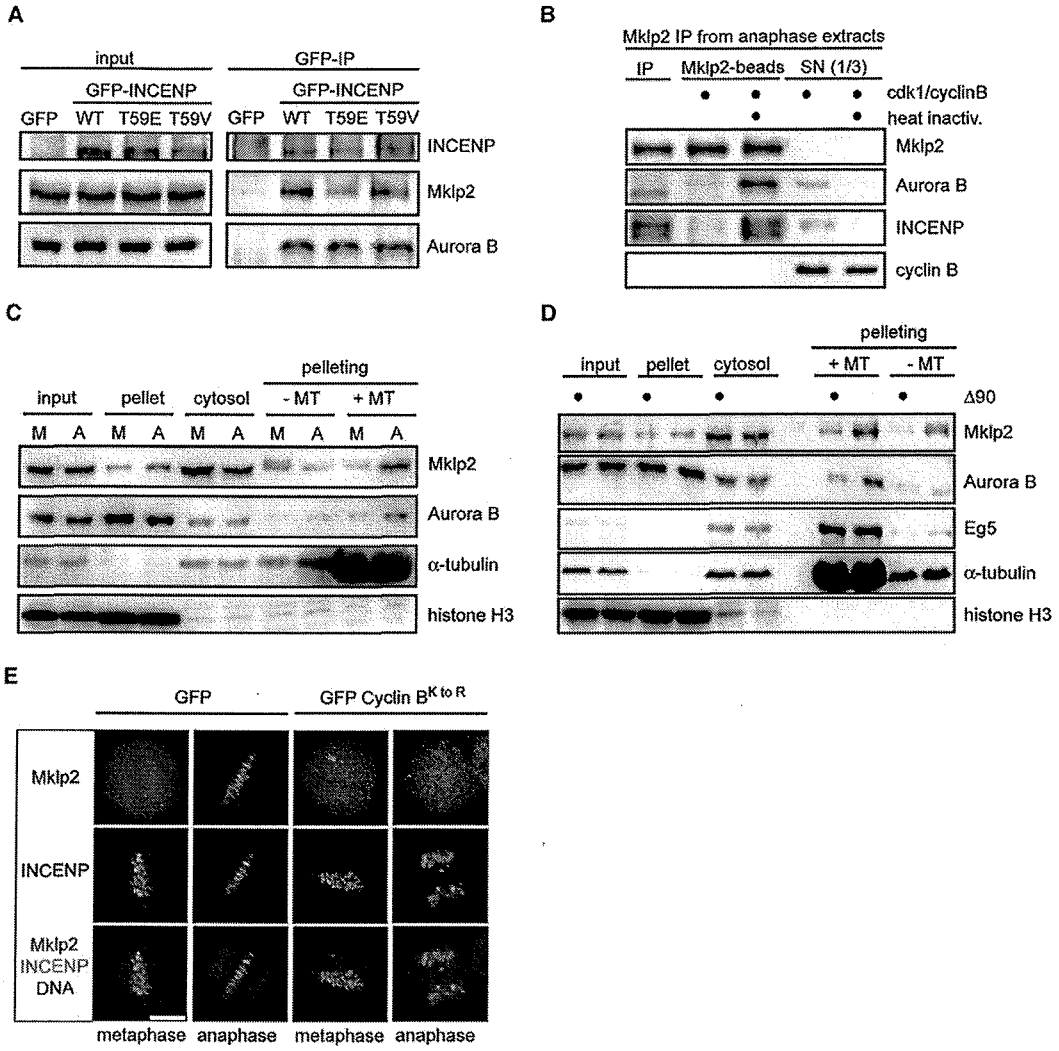

Figure 3. Cdk1 Negatively Regulates the Interaction between Mklp2 and the CPC

(A) Lysates (input) were prepared from anaphase HeLa cells transfected with empty GFP vector or the indicated INCENP variants followed by immunoprecipitation of the fusion proteins with GFP antibodies. Western blots were probed with INCENP, Aurora-B, and Mklp2 antibodies.

(B) Mklp2 was immunoprecipitated from anaphase extracts prepared as described in A (input), and the precipitate was incubated with ATP and recombinant active or heat-inactivated Cdk1/cyclin B for 30 min at 25°C. The reaction was separated in a bead-associated (Mklp2 beads) and supernatant (SN) fraction by centrifugation, followed by immunoblotting for Mklp2, Aurora B, INCENP, and cyclin B.

(C) Lysates (input) from HeLa cells arrested at metaphase by nocodazole treatment (M, metaphase) or released from the arrest for 60 min (A, anaphase) were separated by centrifugation into a pellet and cytosolic fraction. Taxol-stabilized microtubules were added to the cytosolic fraction and sedimented by centrifugation. Immunoblotting was carried out for Mklp2, Aurora B, and α-tubulin.

(D) Lysates of anaphase extracts (input) were supplemented with nondegradable cyclin B (Δ90) or buffer. Pellet and cytosolic fractions were separated by centrifugation, and taxol-stabilized microtubules were added to the cytosolic fraction. After centrifugation, microtubule-associated proteins were detected by immunoblotting.

(E) HeLa cells transfected with GFP-tagged nondegradable cyclin B (GFP-cyclin B^{KtoR}) or GFP vector control were fixed and stained for Mklp2 (red), INCENP (green), and DNA (blue). Scale bar represents 10 μm.

negatively regulated by Cdk1 activity and that T59, a reported Cdk1 phosphorylation site of INCENP, seems to be critical for this interaction.

To determine whether anaphase onset increases the affinity of Mklp2 and the CPC for microtubules, we performed spin-down assays with ectopically added microtubules. Specifically, we separated extracts prepared from metaphase or anaphase cells by centrifugation to obtain a pellet and supernatant fraction (cytosol), which we supplemented with taxol-stabilized microtubules, and upon sedimentation, we detected microtubule-associated proteins by Western blotting. Consistent with their cell-cycle-dependent localization in vivo, Mklp2 and the CPC efficiently associated with microtubules in anaphase extracts but not in metaphase extracts (Figure 3C). Immunoblotting for α-tubulin confirmed that the sedimentation of both Mklp2 and the CPC was dependent on microtubules. To verify that a decrease in Cdk1 activity accounts for the higher affinity of Mklp2 and the CPC for microtubules, we

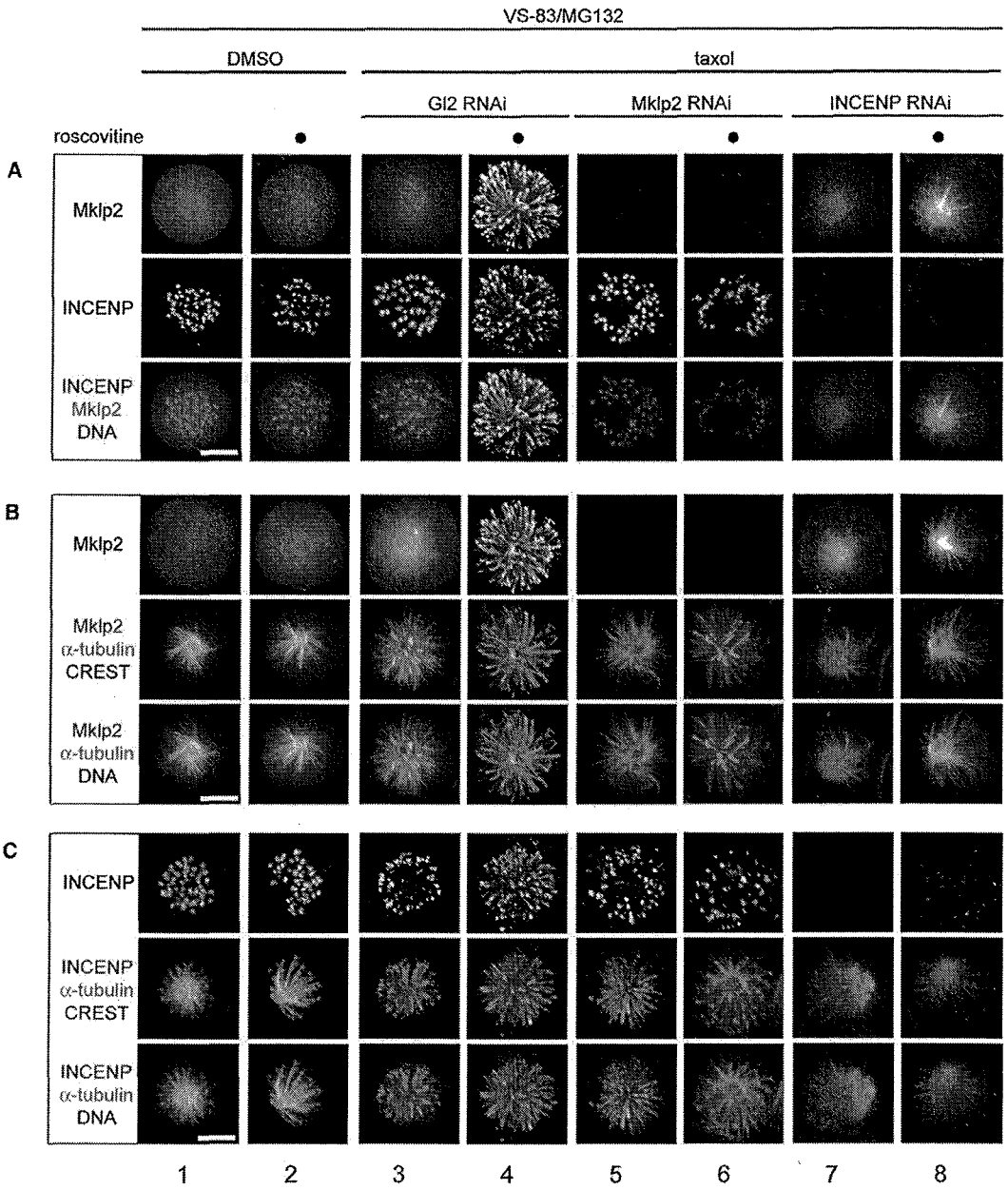

Figure 4. INCENP Is Required for Localization of Mklp2 Close to the Ends of Taxol-Stabilized Microtubules in Roscovitine-Treated Cells

Immunofluorescence images of HeLa cells arrested in mitosis with 100 μM VS-83 and 20 μM MG132 in the presence of 1 μM taxol and 100 μM roscovitine or DMSO as solvent control and transfected with the indicated siRNA duplexes targeting INCENP, Mklp2, or Gl2. Mklp2 is shown in green, INCENP in red, and DNA in blue (A). In (B) and (C), cells are stained for Mklp2 or INCENP in red, and these images were merged with either tubulin (green) and CREST (blue) or tubulin (green) and DNA (blue). Scale bar represents 10 μm.

repeated the spin-down assays from anaphase extracts in the presence of nondegradable cyclin-B (cycB$^{\Delta 90}$). Indeed, the addition of cycB$^{\Delta 90}$ to anaphase extracts prevented both Mklp2 and the CPC from efficiently cosedimenting with taxol-stabilized microtubules, whereas the control buffer had no effect (Figure 3D). This effect was specific, because the addition of cycB$^{\Delta 90}$ did not affect Eg5's affinity for microtubules (Figure 3D). Immunoblotting for histone H3 confirmed that the cytosolic fraction was efficiently depleted of chromosomes (Figure 3D), suggesting that Cdk1 might interfere with the interaction between the CPC and Mklp2 rather than with the dissociation of the CPC from chromatin. Consistently, we observed that the expression of nondegradable cyclin B (cyclin-B^{KtoR}) interfered with the association of the CPC with Mklp2 in anaphase, as indicated by the localization of the CPC to segregating chromosomes and the diffusely localized Mklp2 (Figure 3E). As expected, cyclin-B^{KtoR} had no effect on the localization of the CPC and Mklp2 in metaphase.

Our in vivo results demonstrate that the CPC and Mklp2 depend on each other for binding to midzone microtubules. Given that microtubule ends are concentrated at the midzone, we sought an approach that allowed us to analyze the binding of the CPC and Mklp2 to the tips of microtubules. To this end, cells arrested in metaphase with the proteasome inhibitor MG132 were treated with the potent Eg5 inhibitor VS-83 [16] for induction of monoasters with easily discernable microtubule tips. Consistent with previous reports [17], inactivation of Cdk1 by roscovitine was not sufficient to efficiently recruit the CPC or Mklp2 to microtubules (Figure 4A, panel 2). In contrast, cosuppression of microtubule dynamics by taxol enabled the CPC and Mklp2 to specifically accumulate at the tips of microtubules (Figure 4A, panel 4), a localization reminiscent of the situation in anaphase during which the CPC and Mklp2 accumulate in a narrow zone on midzone microtubules. The association of the CPC with microtubules was dependent on Mklp2, because INCENP was detected on chromatin in Mklp2-depleted cells (Figure 4A, panel 6). Intriguingly, in INCENP-depleted cells, Mklp2 failed to accumulate at the tips of microtubules but instead localized to the lattice of taxol-stabilized microtubules (Figure 4A, panel 8). Lattice binding of Mklp2 was dependent on the addition of taxol (compare panels 2 and 8 of Figure 4A). Thus, by the addition of taxol, we increased Mklp2's affinity for microtubules, enabling us to observe three phenotypes: no binding, tip binding, and lattice binding. Given that in the absence of INCENP, the efficient binding of Mklp2 to the lattice depends on the presence of roscovitine, we speculate that an additional Cdk1-regulated player is involved in the recruiting cascade of the CPC-Mklp2 complex to the spindle midzone. Although the molecular nature of this factor remains unknown, our data unambiguously demonstrate that the interaction between the CPC and Mklp2 is negatively regulated by Cdk1 dependent phosphorylation. Our phospho-mimic experiments suggest that the phosphorylation state of INCENP-T59 might be critical for the interaction between the CPC and Mklp2, an interaction that is required for their midzone localization, as shown by our RNAi-rescue experiments. The analyses of VS-83-treated cells suggest that stabilization of microtubules is necessary for the recruitment of the CPC and Mklp2 and, furthermore, that the CPC is essential for localization of Mklp2 close to the ends of taxol-stabilized microtubules. On the basis of these observations, it is tempting to speculate that the CPC might fulfill two functions: modulating Mklp2's affinity for microtubule ends and locally stabilizing microtubules. Notably, it has been shown that MCAK, a microtubule depolymerase, is specifically inactivated at the midzone by Aurora-B-mediated phosphorylation [18, 19]. Clearly, future research efforts will be required for elucidation of the exact underlying mechanism.

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