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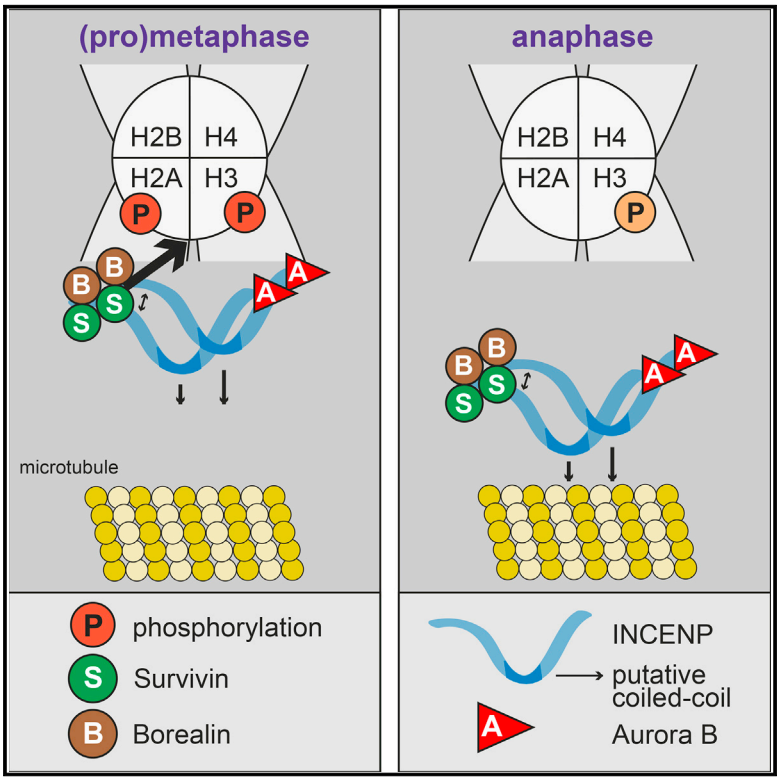
Inter-domain Cooperation in INCENP Promotes Aurora B Relocation from Centromeres to Microtubules

Graphical Abstract



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In Brief

Anaphase midzone localization of Aurora B, the enzymatic subunit of the chromosomal passenger complex, is essential for cytoplasmic division. Van der Horst et al. show that this localization depends on microtubule binding by the putative coiled-coil domain of INCENP and that microtubule binding is outcompeted by centromere binding before anaphase.

Highlights

- Microtubule binding by the CC domain of INCENP promotes CPC midzone localization
- The CPC multimerizes via the CEN box of INCENP
- Multimerization of the CPC increases the microtubule binding affinity of INCENP
- Centromere binding outcompetes microtubule binding of the CPC before anaphase

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Inter-domain Cooperation in INCENP Promotes Aurora B Relocation from Centromeres to Microtubules

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SUMMARY

The chromosomal passenger complex is essential for error-free chromosome segregation and proper execution of cytokinesis. To coordinate nuclear division with cytoplasmic division, its enzymatic subunit, Aurora B, relocalizes from centromeres in metaphase to the spindle midzone in anaphase. In budding yeast, this requires dephosphorylation of the microtubule-binding (MTB) domain of the INCENP analog Sli15. The mechanistic basis for this relocalization in metazoans is incompletely understood. We demonstrate that the putative coiled-coil domain within INCENP drives midzone localization of Aurora B via a direct, electrostatic interaction with microtubules. Furthermore, we provide evidence that the CPC multimerizes via INCENP's centromere-targeting domain (CEN box), which increases the MTB affinity of INCENP. In (pro)metaphase, the MTB affinity of INCENP is outcompeted by the affinity of its CEN box for centromeres, while at anaphase onset—when the histone mark H2AT120 is dephosphorylated—INCENP and Aurora B switch from centromere to microtubule localization.

INTRODUCTION

During cell division the genome needs to be faithfully transmitted over two new daughter cells. This requires error-free chromosome segregation during mitosis followed by proper execution of cytokinesis. The evolutionary conserved chromosomal passenger complex (CPC), consisting of Aurora B kinase and the non-enzymatic subunits INCENP, Survivin and Borealin, is thought to coordinate successful nuclear division with cytokinesis through its dynamic subcellular localization. In early mitosis, the CPC localizes to the inner centromere where it regulates the mitotic checkpoint and ensures that sister chromatids become connected to microtubules emanating from opposite spindle poles. After anaphase onset, the CPC localizes to the spindle midzone, equatorial cortex, and midbody where it mediates cytokinesis (Carmena et al., 2012). Relocation of the mammalian CPC from centromeres in metaphase to the spindle midzone in anaphase depends on multiple inputs. A Cul3-containing ubiquitin ligase complex releases Aurora B from chromatin in anaphase and mediates its subsequent recruitment to midzone microtubules by binding to the Kelch protein KLHL21 (Maerki et al., 2009). In addition, the mitotic kinesin MKlp2 interacts with the CPC in anaphase and is required for its midzone localization (Gruneberg et al., 2004; Hümmer and Mayer, 2009). In budding yeast, a central MTB domain in Sli15 (the budding yeast analog of INCENP) plays an essential role in midzone localization. Phosphorylation of this domain by Cdc28/Cdk1 and Ipl1/Aurora B prevents premature binding to the spindle before anaphase, while dephosphorylation of these sites by Cdc14 mediates the relocation to the spindle midzone in anaphase (Cormier et al., 2013; Mirchenko and Uhlmann, 2010; Nakajima et al., 2011; Pereira and Schiebel, 2003). Similar to Sli15, metazoan INCENP contains a central MTB domain, known as the putative coiled-coil (CC) domain (Mackay et al., 1993, 1998; Tseng et al., 2010). However, it is unclear whether this domain is required for midzone localization of the CPC in anaphase (Mackay et al., 1993, 1998; Vader et al., 2007). Moreover, whether this domain forms a coiled-coil or a stable single alpha helix (SAH) is currently debated (Peckham and Knight, 2009). We therefore refer to it as "putative CC" domain. The putative CC domain of INCENP shows only low sequence identity to the MTB domain of Sli15 (24%, Clustal O multiple sequence alignment). In addition, it harbors no serine residues and only a single threonine that does not fit a Cdk1 or Aurora B consensus motif (Cormier et al., 2013; Makrantoni et al., 2014; Nakajima et al., 2011), suggesting a different mode of regulation. We here demonstrate that the putative CC domain of INCENP is required for midzone localization of INCENP and Aurora B in anaphase, and we uncover a mechanism by which INCENP and Aurora B switch from centromere binding in (pro)metaphase to microtubule-binding in anaphase.

RESULTS

The Putative CC Domain of INCENP Mediates Relocation of the CPC to the Anaphase Midzone

Our initial observations suggested that relocation of the human CPC to the spindle midzone might depend on the putative CC

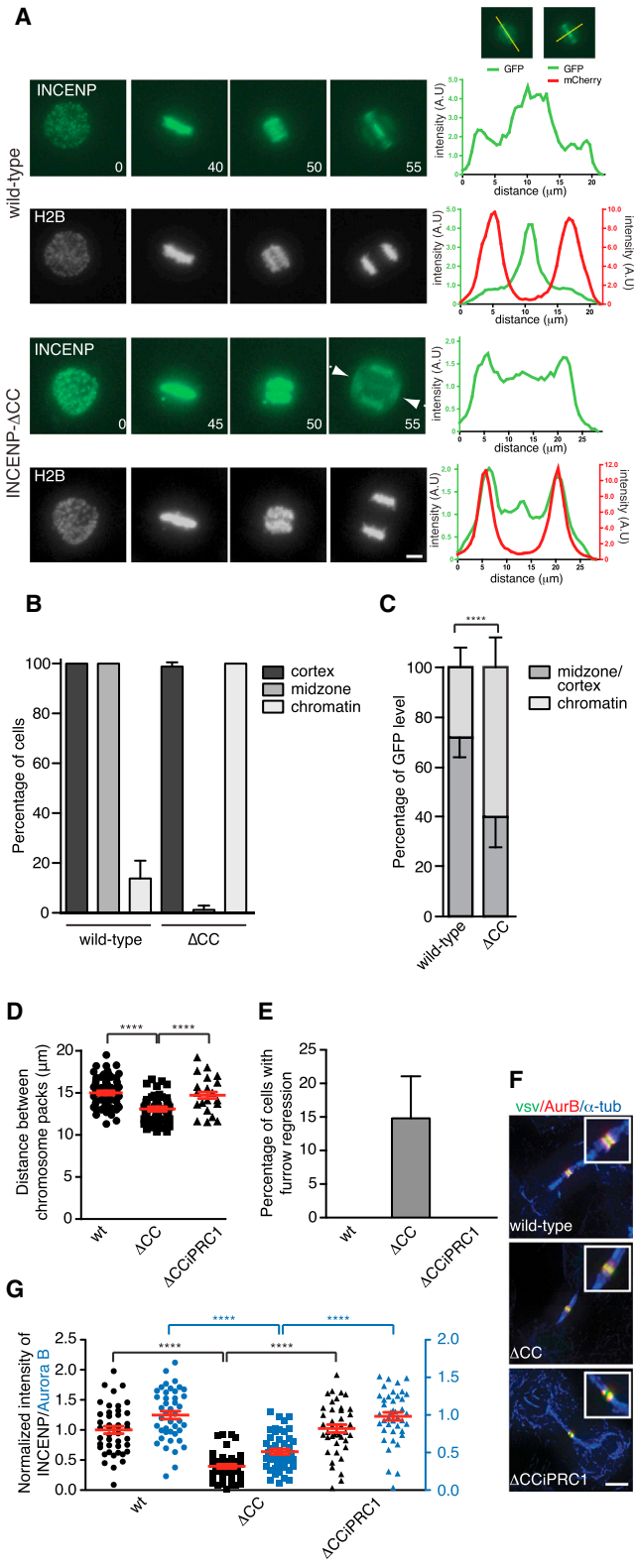


Figure 1. INCENP-ΔCC Fails to Relocate to the Spindle Midzone in Anaphase and Causes Cytokinesis Failure

(A) Live-cell imaging of HeLa Flp-In T-Rex cells depleted of endogenous INCENP and expressing H2B-mCherry and INCENP-GFP (wild-type, WT) or INCENP-ΔCC-GFP. Lineplots of INCENP-GFP, INCENP-ΔCC-GFP, and H2B-mCherry (at t = 55 min).

(B) Quantification of live-cell imaging experiments. INCENP localization was analyzed in 60–90 cells from two to three experiments. Results are mean ± SD.

(C) Relative GFP intensity levels in the midzone/cortex and on chromatin. Results are mean ± SD.

(D) Distance between chromosome masses was measured in the last frame before chromatin decondensation (Figure S1D). n = 60–90 cells (20 for INCENP-ΔCCiPRC1) from two to three experiments. Red lines depict mean ± SEM.

(E) Percentage of cells displaying cleavage furrow regression. >80 cells were analyzed (17 for INCENP-ΔCCiPRC1) in two to four experiments. Results are mean ± SD.

(F) Cells expressing the indicated constructs were processed to visualize INCENP (vsv), Aurora B, and α-tubulin. Telophases were imaged.

(G) Levels of INCENP-GFP (black) and Aurora B (blue) at the midbody were quantified and normalized to the amount in cells expressing wild-type INCENP. Red lines depict the mean ± SEM of at least 40 cells from two experiments.

****p < 0.0001, unpaired t test. Scale bar represents 5 μm. See also Figure S1.

domain of INCENP (Vader et al., 2007). To substantiate this, we analyzed the localization of INCENP-GFP and a deletion mutant lacking this domain (INCENP-ΔCC-GFP) by live-cell imaging of stable HeLa Flp-In T-Rex cells with inducible expression of these constructs and depleted of the endogenous protein by INCENP small interfering RNA (Figures 1A–1C and S1A–S1C; Movies S1 and S2). INCENP-GFP and INCENP-ΔCC-GFP displayed similar centromere localization during early mitosis (Figures 1A and 2G). However, when cells entered anaphase INCENP-ΔCC failed to efficiently relocate to the spindle midzone, and a substantial fraction of the protein remained associated with chromatin (Figures 1A–1C and 2H; Movie S2). Notably, INCENP-ΔCC was also detectable at the equatorial cortex near the site of furrow ingression suggesting that INCENP's cortical localization does not depend on its putative CC domain (Figures 1A–1C; Movies S1 and S2).

Midzone Localization of the CPC Is Required for Spindle Elongation and Stable Furrow Ingression

To evaluate the consequences of absence of INCENP from midzone microtubules, we analyzed living cells expressing INCENP or INCENP-ΔCC (Figures S1A–S1C). The distance between the chromosome masses right before chromosome decondensation was shorter in cells expressing INCENP-ΔCC than in INCENP-expressing cells, while timing of chromosome decondensation was comparable (Figures 1D and S1D). This suggested that spindle elongation was impaired, in line with earlier data showing that perturbation of CPC function in anaphase disturbs spindle elongation (Ahonen et al., 2009; Uehara et al., 2013; Xu et al., 2010). Second, in ~15% of the INCENP-ΔCC-expressing cells (n = 88) the furrow regressed after initial and timely furrow ingression (Figures 1E, S1E, and S1F), resulting in cytokinesis failure. In contrast, in none of the cells expressing wild-type INCENP (n = 87) was furrow regression or cytokinesis failure observed (Figures 1E and S1E).

Despite its failure to localize to the midzone in anaphase, in telophase INCENP-ΔCC did appear at the midbody (Figure 1F). We hypothesized that the midbody fraction was most likely derived from the small pool of INCENP-ΔCC that localized to

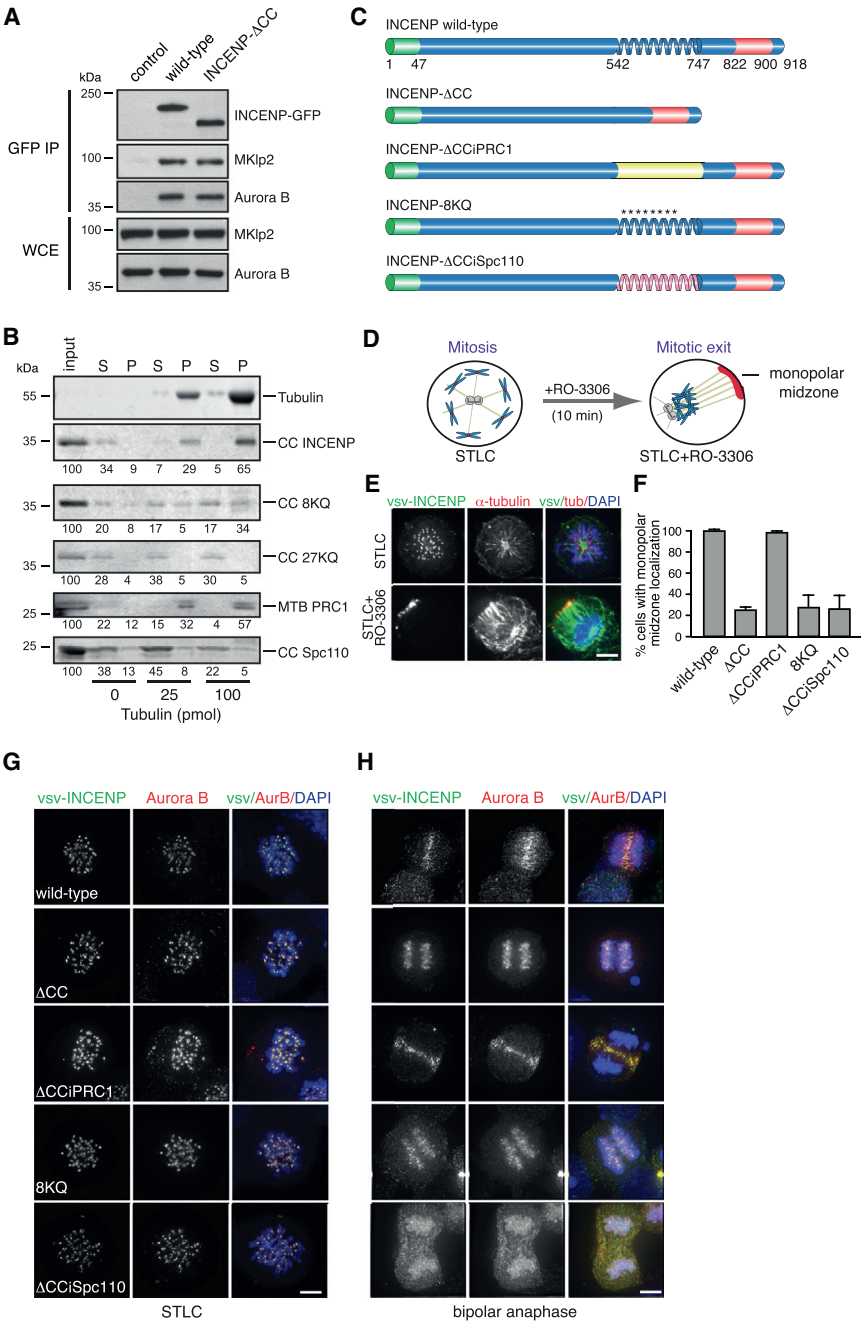


Figure 2. The MTB Capacity of INCENP's Putative CC Domain Is Required for Midzone Localization

(A) GFP-tagged INCENP was IP'ed from HeLa Flp-In T-Rex cells synchronized in mitosis using STLC followed by a 15-min treatment with 10 μM RO-3306. IP and whole-cell extracts (WCE) were analyzed for MKlp2 and Aurora B.

(B) Taxol-stabilized microtubules were incubated with the indicated recombinant protein domains and pelleted. Supernatant (S) and pellet (P) fractions were analyzed using SDS-PAGE and InstantBlue staining. Numbers indicate the fraction of protein bound to microtubules relative to input. Note that in the samples without microtubules we could not fully recover our input material, due to protein being present in the cushion after ultracentrifugation.

(C) Scheme of the used INCENP variants. CEN box (green), putative CC domain, and IN box (red) are indicated.

(D) Synchronization protocol used to analyze CPC relocation in a large number of cells (adapted from Hu et al., 2008).

(E) HeLa Flp-In T-Rex cells depleted of endogenous INCENP and transiently expressing vsv-INCENP were treated as in (D). INCENP and α-tubulin were visualized.

(F) HeLa Flp-In T-Rex cells depleted of endogenous INCENP and transiently expressing the indicated INCENP variants were treated as in (D). Results are mean ± SD of 150 cells from two to four experiments.

(G and H) Prometaphases (STLC, G) and bipolar anaphases (H) of cells transiently transfected with the indicated INCENP variants and depleted of endogenous INCENP.

Scale bar represents 5 μm. See also Figures S1 and S2.

the equatorial cortex in anaphase, and that the level of this INCENP variant at the midbody would therefore be lower than the midbody levels of wild-type INCENP derived from both the spindle midzone and the cortex. Indeed, INCENP-ΔCC and Aurora B levels at the midbody were reduced by ~50% compared to wild-type INCENP (Figure 1G). This suggests that a failure of the CPC to localize to the midzone microtubules causes central spindle and midbody defects that can result in furrow regression. However, the presence of the complex at the equatorial cortex appears to be sufficient to allow initial furrow ingression.

The MTB Property of INCENP's Putative CC Domain Is Required for Midzone Localization of Aurora B

To understand why INCENP-ΔCC was unable to relocate to the midzone in anaphase, we first tested whether INCENP-ΔCC was capable of binding MKlp2 (Gruneberg et al., 2004; Hümmer and Mayer, 2009). INCENP-GFP and INCENP-ΔCC-GFP were immunoprecipitated (IP'ed) from HeLa Flp-In T-Rex cells forced into mitotic exit using a successive treatment with the Eg5 inhibitor S-Trityl-L-cysteine (STLC) and the Cdk1 inhibitor RO-3306 (Hu et al., 2008). Both INCENP and INCENP-ΔCC interacted with MKlp2 (Figure 2A). Moreover, MKlp2 depletion prevented cortical localization of INCENP-ΔCC confirming that the interaction between INCENP and MKlp2 does not depend on the putative CC domain and that cortical localization of INCENP is dependent on MKlp2 (Figures S1C and S2A–S2C) (Kitagawa et al., 2013).

The putative CC domain of metazoan INCENP was suggested to either directly or indirectly interact with microtubules (Mackay et al., 1993, 1998; Tseng et al., 2010). To substantiate this, we purified recombinant CC domain (aa 543–746 of human INCENP) from *E. coli* and performed microtubule co-sedimentation assays. The microtubule-binding (MTB) domain of human protein regulator of cytokinesis 1 (PRC1; aa 279–482 (Mollinari et al., 2002; Tseng et al., 2010) and the CC domain of the yeast protein Spc110 (aa 216–452) (Kilmartin et al., 1993) were included as positive and negative control, respectively. As expected, the MTB domain of PRC1 pelleted with taxol-stabilized microtubules, whereas the Spc110 CC domain did not. Importantly, the putative CC domain of INCENP also pelleted with microtubules (Figure 2B). Most proteins that interact with microtubules, bind to the negatively charged E hook of tubulin (Steinmetz and Akhmanova, 2008). Interestingly, the putative CC domain of INCENP contains a high percentage of charged amino acids (>60%), and we could lower its affinity for microtubules by mutating 8 or 27 positively charged lysine residues to an amino acid with a neutral side chain (glutamine, 8KQ, and 27KQ respectively; Figure 2B). Notably, the 8KQ mutations are not expected to affect the predicted coiled coil propensity (MARCOIL), nor impair formation of a SAH, because Q is likely to substitute for K in the latter structure (Peckham and Knight, 2009). This demonstrates that INCENP's putative CC domain can directly interact with microtubules, most likely in an electrostatic manner, reinforcing recent findings by Noujaim et al. using a larger fragment of INCENP (aa 491–873) (Noujaim et al., 2014).

To determine whether the MTB capacity of the putative CC domain is required for midzone localization of INCENP, the domains used in the microtubule co-sedimentation assay were inserted into human INCENP-ΔCC to generate INCENP-ΔCCiPRC1-GFP, INCENP-8KQ-GFP, and INCENP-ΔCCiSpc110-GFP (Figure 2C). These were transiently expressed in HeLa cells in which endogenous INCENP was knocked down, and the relocation of INCENP and Aurora B from centromeres to the midzone was assessed. To obtain high cell numbers for the analysis, cells were synchronized in mitosis with STLC and forced into mitotic exit by chemical Cdk1 inhibition (Figures 2D and 2E; Hu et al., 2008). Relocation of INCENP and Aurora B from centromeres to the "monopolar midzone" is microtubule dependent (Hu et al., 2008; Figure S2D), and the MTB domain of PRC1 was capable of restoring both monopolar and bipolar midzone localization of INCENP-ΔCC and Aurora B (Figures 2F, 2H, and S2E). A similar rescue of INCENP relocation was observed in HeLa Flp-In T-Rex cells stably expressing INCENP-ΔCCiPRC1 (Figures S1A, S1B, and S2F; Movies S3 and S4). Importantly, INCENP-ΔCCiPRC1 also rescued spindle elongation and midbody accumulation of Aurora B and promoted stable furrow ingression comparable to wild-type INCENP (Figures 1D–1G and S1D–S1F). In contrast to INCENP and INCENP-ΔCCiPRC1, INCENP-ΔCC, INCENP-8KQ, and INCENP-ΔCCiSpc110 failed to localize Aurora B to the midzone of monopolar and bipolar anaphases (Figures 2F, 2H and S2E). The observed relocation defects were not due to abnormal localization of these proteins in (pro)metaphase since centromere localization appeared normal (Figure 2G). Together, this suggests that INCENP requires the MTB capacity of its putative CC domain for relocation to the central spindle in anaphase.

Multimerization of the CPC Increases Its MTB Affinity

Because the putative CC domain of INCENP can directly bind microtubules in vitro and its MTB capacity is required for localization of INCENP and Aurora B to the central spindle, we predicted that a GFP-CC domain would be recruited to the mitotic spindle before anaphase. However, GFP-CC domain localized weakly to the spindle in only ~9% of the cells (Figures 3A and 3B). We reasoned that the affinity of this domain for microtubules is probably low, which could be (1) an intrinsic property of the putative CC domain or (2) due to shielding by post-translational modifications in cells (Cormier et al., 2013; Nakajima et al., 2011). To discriminate between these two options, we tested whether we could increase the MTB affinity by introducing a second putative CC domain in tandem. If its MTB potential is shielded in cells, we predicted that a second domain would not enhance microtubule binding. However, if intrinsically low, a second domain would increase binding. In line with the latter option, we observed clear spindle localization of GFP-2xCC in >80% of the cells (Figures 3A and 3B). Neutralizing positively charged lysine residues in the double CC (2x8KQ) abolished spindle localization (Figures 3A and 3B). This implied that the MTB affinity of the CPC might be increased by multimerization of INCENP, and thereby juxtaposition of multiple INCENP MTB domains. Two pieces of evidence suggested that INCENP may indeed multimerize in cells. First, we found that a fraction of INCENP-ΔCC was capable of relocalizing to the monopolar midzone when endogenous INCENP was present (Figure 3C), implying that INCENP-ΔCC can piggyback on the endogenous CPC to localize to the central spindle. Second, we could pull down vsv-tagged INCENP from transfected HEK293T cells after immunoprecipitation of the GFP-tagged INCENP CEN box (aa 1–48) (Figure 3D), suggesting the involvement of the CEN box of INCENP in multimerization.

INCENP MTB Is Outcompeted by the Affinity of the CEN Box for Centromeres before Anaphase

If multimerization is required for MTB, then introduction of an additional putative CC domain into INCENP (Figure S3A) would cause INCENP and Aurora B to prematurely localize to microtubules in prometaphase. However, this was not the case; INCENP-2xCC localized to centromeres indistinguishably from wild-type INCENP (Figure 4A). Given that GFP-2xCC did localize to the mitotic spindle (Figures 3A and 3B), we hypothesized (1) that MTB may compete with centromere binding, (2) that before anaphase the affinity of the CPC for centromeres is higher than for microtubules, and (3) that the CPC:centromere affinity has to decrease upon anaphase onset. The interaction of the CPC with centromeres depends on two histone marks: histone H3 phosphorylated by Haspin on threonine 3 (H3T3ph) and histone H2A phosphorylated on threonine 120 (H2AT120ph) by Bub1 (Kawashima et al., 2010; Kelly et al., 2010; Wang et al., 2010; Yamagishi et al., 2010). To test whether these histone marks are lost when cells enter anaphase, we quantified H3T3ph and H2AT120ph levels at different stages of anaphase discriminated by an increased distance of the

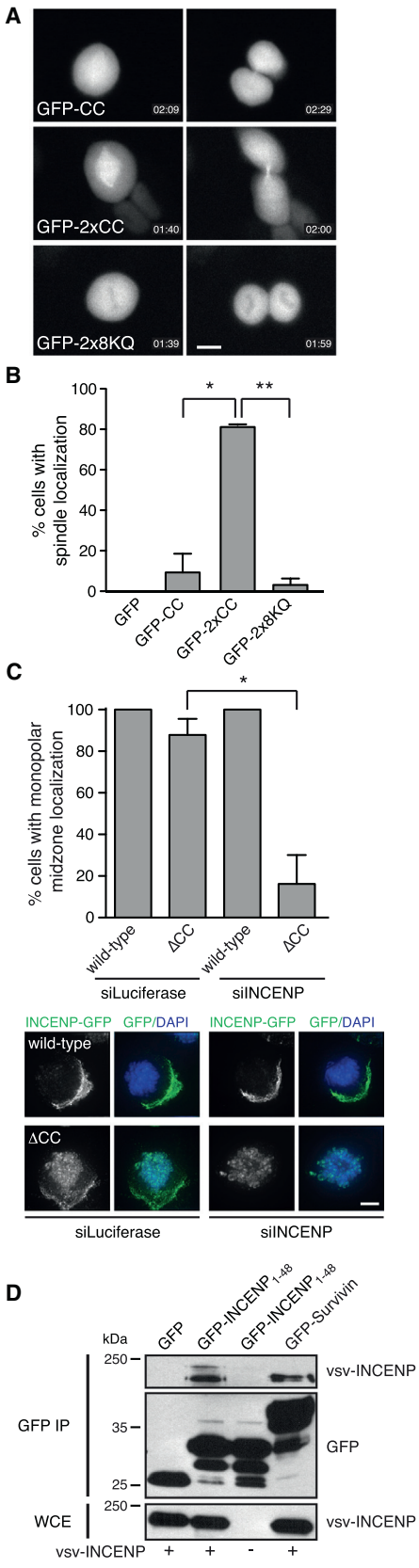


Figure 3. INCENP Can Multimerize in a CEN Box-dependent Manner

(A) U2OS cells transiently expressing GFP-tagged INCENP putative CC domain (one or two domains in tandem) were imaged live. Stills show localization of the GFP-tagged proteins during mitosis and cytokinesis (h:min). Note: For GFP-CC a cell was selected that represents the minor fraction with weak microtubule localization. Scale bar represents 10 μm.

(B) Quantification of (A). n = 25–53 cells from two experiments. Results are mean ± SD. *$p < 0.05$, **$p < 0.01$, unpaired t test.

(C) Quantification of INCENP localization in HeLa Flp-In T-Rex cells with or without endogenous INCENP and treated as in Figure 2D. Results are mean ± SD from two experiments (140 cells). *$p < 0.05$. Representative images are shown below the graph. Scale bar represents 5 μm.

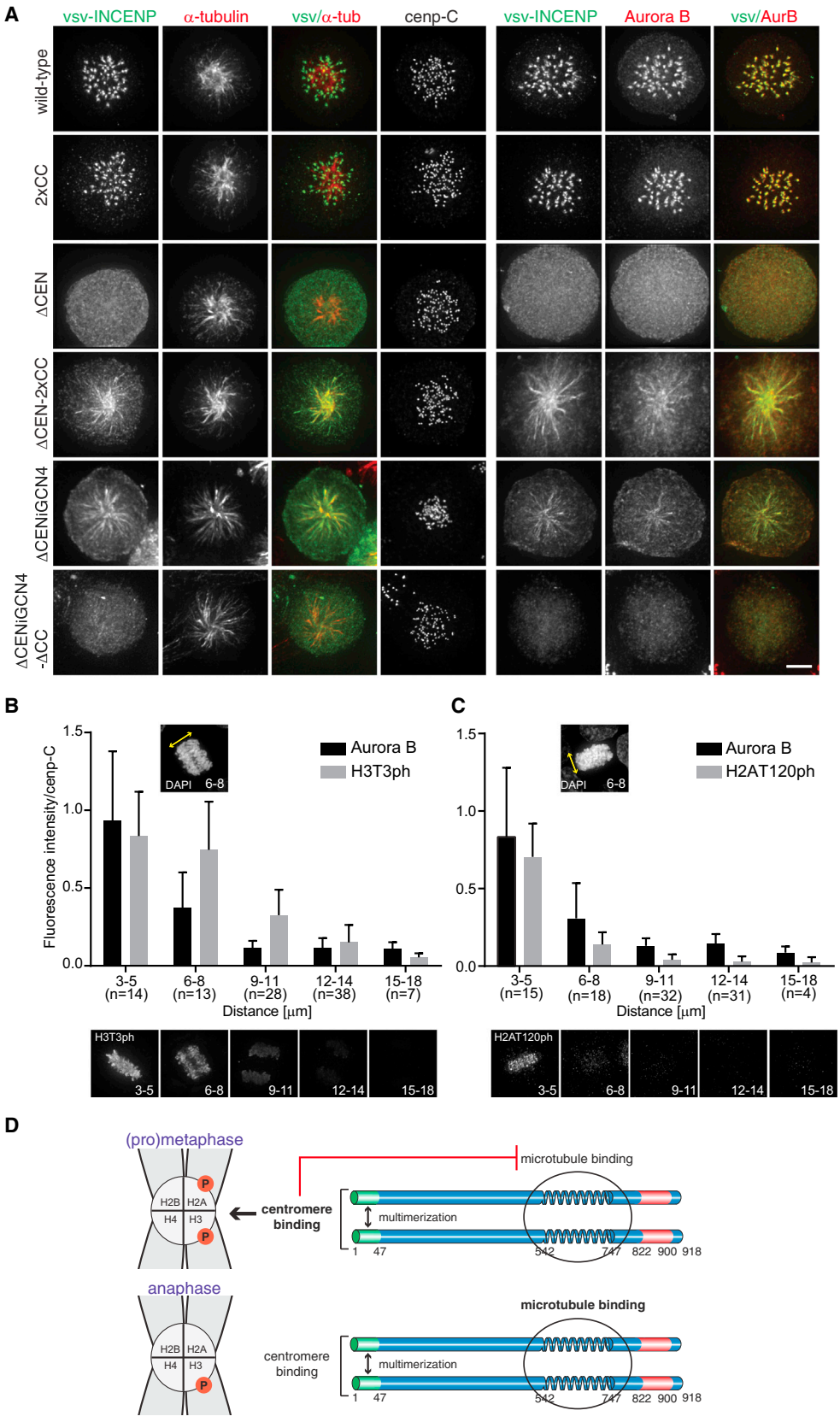
(D) GFP-INCENP$_{1-48}$, or GFP-Survivin were immunoprecipitated from HEK293T cells expressing vsv-INCENP and synchronized in mitosis using STLC. Anti-GFP-IP and WCE were probed for vsv and GFP.

separating chromosome masses (Figures 4B, 4C, S3B, and S3C). Interestingly, we observed a rapid loss of H2AT120ph in early anaphase followed by a more gradual disappearance of H3T3ph. Relocation of Aurora B coincided with the loss of H2AT120ph (Figures S3B and S3C).

Centromere binding of the CPC requires the N-terminal CEN box of INCENP (Ainsztein et al., 1998). To overcome the potential affinity competition by the INCENP N terminus in prometaphase, we removed the CEN box from INCENP-2xCC (Figure S3A). This resulted in localization of both INCENP-ΔCEN-2xCC and Aurora B to the mitotic spindle in prometaphase (Figure 4A). In contrast, INCENP-ΔCEN (with only one putative CC domain) did not visibly localize to spindle microtubules in prometaphase or anaphase (Figure 4A) (Mackay et al., 1993). Given that we found that INCENP CEN box could form a complex with full-length INCENP (Figure 3D), we inferred that removal of the CEN box might not only impair centromere localization, but also multimerization of the CPC thereby decreasing its MTB affinity. In line with this reasoning, we were able to force INCENP-ΔCEN onto microtubules in prometaphase when we replaced the CEN box of INCENP for the dimerization domain of GCN4 (aa 250–281) (Tseng et al., 2010). Importantly, mitotic spindle localization of this fusion protein was alleviated when the putative CC domain was removed (Figure 4A).

DISCUSSION

Based on our findings, we propose a model for how CPC relocation from centromeres to the midzone microtubules in anaphase is promoted (Figure 4D). At anaphase onset, the affinity of the CPC for centromeres declines due to dephosphorylation of H2AT120, and this allows the CC domain of INCENP to interact with microtubules. Multimerization of the CPC involving the CEN box of INCENP likely increases its MTB affinity. In addition, dephosphorylation of MKlp2 and of threonine-59 in INCENP promotes the interaction between MKlp2 and INCENP (Hümmer and Mayer, 2009; Kitagawa et al., 2014). Since the MKlp2 C terminus also has the capacity to directly bind microtubules in vitro (Kitagawa et al., 2014; Echard et al., 1998), the INCENP putative CC domain and MKlp2 C terminus may act together in efficient microtubule binding in anaphase, allowing the CPC to move along microtubules toward the spindle midzone. We presume



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that INCENP-ΔCC forms a cargo that cannot be delivered efficiently to the overlapping microtubules in the anaphase midzone by MKlp2.

Our findings imply that the CEN box of INCENP has a dual function during mitosis. First, it is crucial for centromere localization of the CPC by mediating recognition of phosphorylated histone marks via Borealin and Survivin. Second, upon anaphase onset when the centromere phosphorylation marks disappear, the CEN box remains required for multimerization of the CPC to allow efficient microtubule binding and thereby midzone localization. How the CEN box mediates multimerization of the complex requires further studies. Since it associates with Survivin and Borealin, and Borealin was shown to dimerize, the latter is a potential candidate to facilitate multimerization of the CPC (Bourhis et al., 2009; Jeyaprakash et al., 2007; Klein et al., 2006). Alternatively, recent studies demonstrated that the CEN box of INCENP can interact with MKlp2 and that MKlp2 has the capacity to oligomerize (Kitagawa et al., 2014).

Despite the lack of sequence conservation between the MTB domain of Sli15 and the putative CC domain in mammalian INCENP, the function of this central domain in INCENP appears to be evolutionary conserved: it is required for microtubule binding and thereby for central spindle localization in anaphase. In addition, both in budding yeast and humans the MTB potential of this domain is suppressed before anaphase. However, the mode of regulation of this suppression appears to be very different. In budding yeast, MTB of Sli15 before anaphase is prevented by Cdc28/Cdk1- and Ipl1/Aurora B-mediated phosphorylation, while we here provide evidence that in human cells MTB of INCENP via its putative CC domain is outcompeted by the centromere-binding affinity of its N-terminal CEN box (Figure 4D).

EXPERIMENTAL PROCEDURES

Stable Cell Lines

HeLa Flp-In T-Rex cells stably expressing doxycycline-inducible INCENP constructs were generated by transfection with pcDNA5/FRT/TO and pOG44 (Life Technologies). Cells were grown under 200 μg/ml hygromycin (Roche), and 4 μg/ml blasticidin (PAA Laboratories) selection and single colonies were picked. Protein expression was induced with 1 μg/ml doxycycline (Sigma-Aldrich). Cells were synchronized in mitosis with 20 μM STLC (Tocris Bioscience) for 16 hr and mitotic exit was induced by a 10- to 15-min treatment with 10 μM RO-3306 (Merck Millipore). For synchronization in G1/S, cells were treated with 2 mM thymidine (Sigma-Aldrich) for 24 hr.

Recombinant Protein Production and Microtubule Co-sedimentation

GST-tagged protein domains were produced in Rosetta (DE3) bacteria. Untagged domains were eluted from the glutathione beads using PreScission protease. Purified protein domains were used in microtubule co-sedimentation assays according to the manufacturer's instructions (Cytoskeleton). Supernatant and pellet samples were analyzed by SDS-PAGE and InstantBlue staining.

Live-Cell Microscopy

Cells were imaged on a Personal DeltaVision system (Applied Precision) with a 40 × /1.35 numerical aperture (NA) UPlanSApo objective (Olympus) using a CoolSNAP HQ camera (Photometrics). Six planes, 2.0 μm apart, were imaged every 5 min. Images are maximum intensity projections. Average fluorescence intensities were calculated in midzone/cortex and chromatin areas for GFP-INCENP or GFP-INCENP-ΔCC using ImageJ.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.06.038.

AUTHOR CONTRIBUTIONS

A.v.d.H. and M.J.M.V. performed most experiments. K.B. performed experiments for Figures 2B, 3A, and 3B. M.S.v.d.W. generated INCENP-ΔCCiPRC1, and M.A.H. generated H2B-mCherry baculovirus. Experimental design, data interpretation, and manuscript writing was performed by A.v.d.H. and S.M.A.L.

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Figure 4. CEN Box-Dependent Multimerization of the CPC Allows Microtubule Localization of the CPC in (Pro)metaphase, and INCENP MTB Is Outcompeted by Centromere-Binding Affinity before Anaphase

(A) HeLa cells transiently expressing the indicated INCENP variants were synchronized in mitosis using STLC. Cells were processed to visualize INCENP (vsv), α-tubulin and cenp-C, or INCENP (vsv) and Aurora B (right panel). Scale bar represents 5 μm.

(B and C) Levels of H3T3ph and H2AT120ph in anaphase. HeLa cells were synchronized in G2 with 10 μM RO-3306 for 24 hr. Cells were fixed 40–45 min after release and processed to visualize Aurora B, cenp-C, and H3T3ph (B) or H2AT120ph (C). Fluorescence intensities were measured in the cenp-C areas. Anaphase stages were discriminated based on the distance of the separating chromosome masses (inset). The number of cells analyzed per distance group is indicated. Representative images of H3T3ph (B) and H2AT120ph (C) are shown below the graphs. Results are mean ± SD.

(D) Model illustrating how relocation of the CPC from centromeres to the spindle midzone during anaphase may be accomplished. p, phosphorylation. See also Figure S3.

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