INCENP–aurora B interactions modulate kinase activity and chromosome passenger complex localization

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Dynamic localization of the chromosomal passenger complex (CPC) during mitosis is essential for its diverse functions. CPC targeting to centromeres involves interactions between Survivin, Borealin, and the inner centromere protein (CENP [INCENP]) N terminus. In this study, we investigate how interactions between the INCENP C terminus and aurora B set the level of kinase activity. Low levels of kinase activity, seen in INCENP-depleted cells or in cells expressing a mutant INCENP that cannot bind aurora B, are sufficient for a spindle checkpoint response when microtubules are absent but not against low dose taxol. Intermediate kinase activity levels obtained with an INCENP mutant that binds aurora B but cannot fully activate it are sufficient for a robust response against taxol, but cannot trigger CPC transfer from the chromosomes to the anaphase spindle midzone. This transfer requires significantly higher levels of aurora B activity. These experiments reveal that INCENP interactions with aurora B in vivo modulate the level of kinase activity, thus regulating CPC localization and functions during mitosis.

Introduction

Successful cell division requires the temporal and spatial integration of chromosomal and cytoskeletal events. One key integrator is the conserved chromosomal passenger complex (CPC): inner centromere protein (CENP [INCENP]), aurora B kinase, Borealin/Dasra B, and Survivin (Vagnarelli and Earnshaw, 2004; Vader et al., 2006b; Ruchaud et al., 2007; Kelly and Funabiki, 2009). Spatially regulated activity of the CPC is essential for the correction of kinetochore microtubule attachment errors, bipolar spindle stability, and completion of cytokinesis. The requirement for CPC activity in the spindle checkpoint response is still actively debated (Rieder and Maiato, 2004; Musacchio and Salmon, 2007; Santaguida and Musacchio, 2009). Aurora B is not required for spindle assembly checkpoint arrest if microtubules are depolymerized; however, the checkpoint is compromised in low dose taxol if aurora B is inactive (Biggins and Murray, 2001; Ditchfield et al., 2003; Yue et al., 2008).

INCENP is the scaffold upon which the CPC assembles (Cooke et al., 1987). The INCENP N terminus is essential for centromere targeting (Mackay et al., 1993; Ainsztein et al., 1998). This region forms a three-helix bundle with the N terminus of Borealin and C terminus of Survivin (Klein et al., 2006; Jeyaprakash et al., 2007), both of which contribute to centromere targeting of the CPC (Klein et al., 2006; Vader et al., 2006a).

The INCENP C terminus binds aurora B through its highly conserved IN box (Adams et al., 2000; Kaitna et al., 2000; Bolton et al., 2002; Honda et al., 2003; Sessa et al., 2005) partly activating the kinase. Phosphorylation of a TSS motif near the INCENP C terminus leads to full kinase activation through a feedback mechanism (Bishop and Schumacher, 2002; Bolton et al., 2002; Honda et al., 2003).

CPC targeting to the spindle midzone during anaphase/telophase (Earnshaw and Cooke, 1991) requires association

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Abbreviations used in this paper: CENP, centromere protein; CPC, chromosomal passenger complex; H3S10ph, H3 phospho-Ser10; H3S28ph, H3 phospho-Ser28; INCENP, inner CENP; SBP, streptavidin-binding peptide; TrAP, triple affinity purification.

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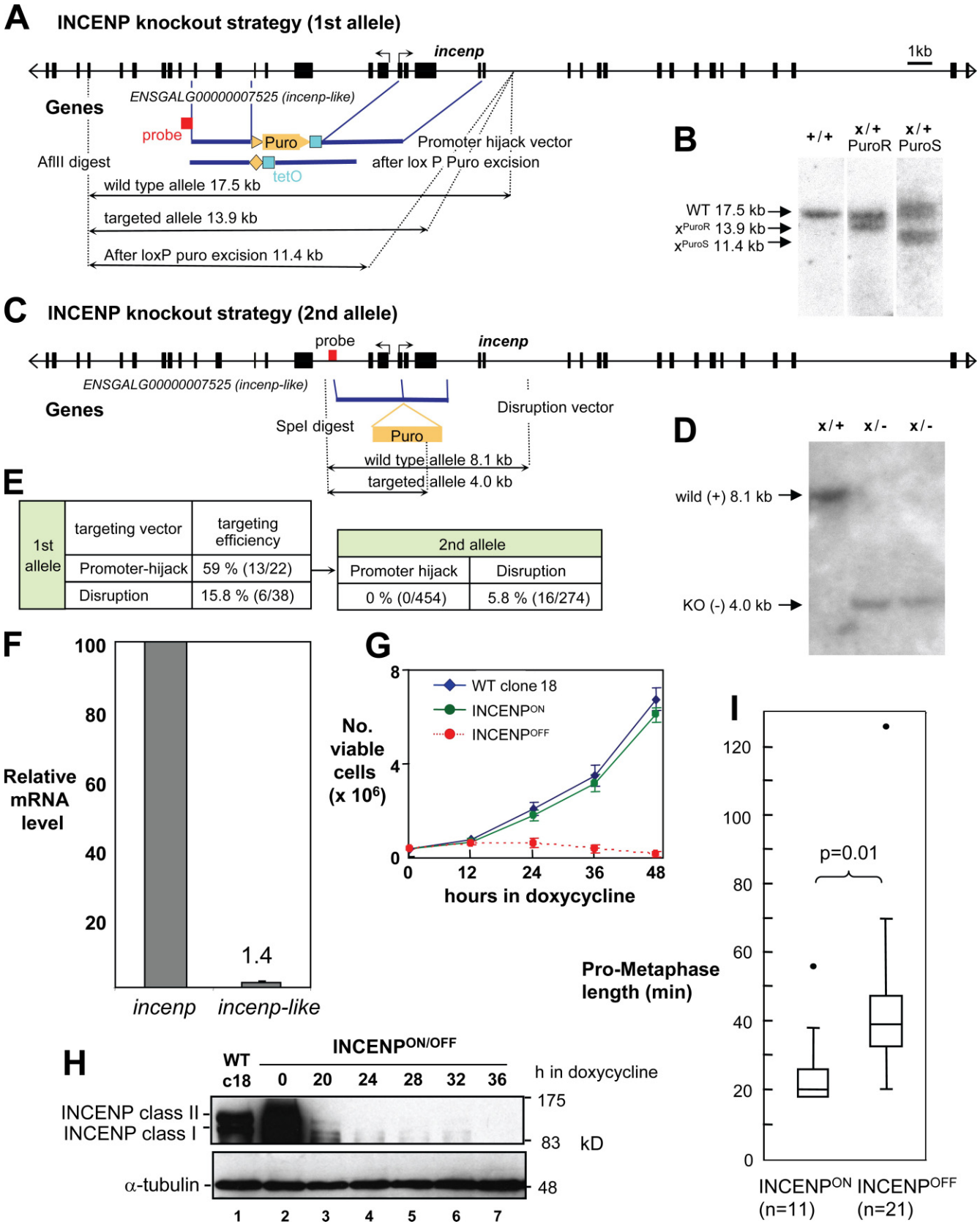

Figure 1. **Generation and characterization of an INCENP promoter hijack–conditional knockout cell line.** (A) Map of the chicken INCENP locus showing the region replaced in the promoter hijack, the size of the resulting restriction fragments, and the probe used in Southern analysis (red box). (B) Southern analysis showing the first targeting (PuroR) and the digestion after removal of the puromycin marker (PuroS; Samejima et al., 2008). (C) Map of the chicken INCENP locus showing the region targeted by the gene disruption construct. (D) Southern analysis demonstrating targeting of the gene disruption construct. Only one band is observed because the probe (red bar) recognizes a region deleted in the promoter hijack of the first allele. KO, knockout. (E) Statistical

with MKLP2 (Gruneberg et al., 2004) and is negatively regulated by Cdk1 phosphorylation (Hümmer and Mayer, 2009). In budding yeast, INCENP/Sli15 transfer to the anaphase spindle requires Cdc14 dephosphorylation of at least six residues, and a nonphosphorylated mutant transfers prematurely during metaphase (Pereira and Schiebel, 2003).

In this study, we describe the expression of mutant forms of INCENP differing in their ability to bind and activate aurora B in an INCENP-conditional knockout cell line. Our experiments reveal that modulation of INCENP–aurora B interactions results in different levels of kinase activity that correlate with different functional states of the CPC. Formation of an INCENP–Survivin–Borealin complex is sufficient for CPC targeting to centromeres regardless of the level of aurora B kinase activity. Low levels of kinase activity give a weak spindle checkpoint response against low dose taxol. This is considerably strengthened by slightly increasing the level of kinase activity. Finally, significantly higher levels of aurora B activity are required for CPC to transfer to the spindle midzone at anaphase onset.

Results

INCENP is essential for cell division

We used a "promoter hijack" strategy (Samejima et al., 2008) to obtain a conditional INCENP knockout, with expression of one *incenp* allele under control of a *kif4A* promoter fragment and the other allele having a disruption of the open reading frame, truncating the protein after amino acid 28 (Fig. 1, A–D). A fragment from the INCENP promoter (Samejima et al., 2008) exhibited only low transcriptional activity and was not used further. In these cells, INCENP is ∼20 times overexpressed (Fig. 1 H). We refer to this conditional cell line as INCENP$^{ON/OFF}$.

Chickens, like several other model organisms, but not humans, express paralogues of INCENP, Borealin, and Survivin termed INCENP-like, Dasra A/Australin (Sampath et al., 2004; Gao et al., 2008), and Survivin2 (Ma et al., 2009), respectively. These paralogues may function primarily in meiosis (Gao et al., 2008), and Survivin2 is not expressed in DT40 cells (unpublished data). *incenp-like* transcripts were detected in DT40 cells, but at extremely low levels: only 1.4% of canonical *incenp* (Fig. 1 F). Furthermore, in INCENP$^{ON/OFF}$ cells, doxycycline addition had no significant effect on *incenp-like* expression. Thus, INCENP-like, even if it is translated as a functional protein in DT40 cells, cannot scaffold formation of a functional CPC. Its function remains unknown and will not be further considered in this study.

INCENP^{ON} cells are phenotypically indistinguishable from wild-type DT40 clone 18 cells with regard to growth rate (Fig. 1 G), mitotic index (Fig. 2 B), and ratio of individual mitotic phases (Fig. 2, C–J). In contrast, INCENP^{OFF} cultures cease proliferating within 12 h, and the majority of cells die within 48 h of doxycycline addition (Fig. 1 G). Levels of INCENP class I and II (two alternative splice variants of INCENP; Mackay et al., 1993) expressed from the hijacked allele dropped 12–20 h after the addition of doxycycline, becoming essentially undetectable by 24 h (Fig. 1 H). INCENP was undetectable on chromosomes by indirect immunofluorescence in INCENP^{OFF} cells after 26 h in doxycycline (Fig. 2 A, panel 7; and Fig. S1 B), and H3 phospho-Ser10 (H3S10ph) and H3 phospho-Ser28 (H3S28ph) levels were also substantially reduced (Fig. 2 A, panels 14 and 15).

INCENP^{OFF} mitotic cells exhibited a typical loss of CPC function phenotype with delays in chromosome alignment (Fig. 1 I) leading to an accumulation of cells in prometaphase (Fig. 2, C and D). INCENP^{OFF} cells eventually entered anaphase but failed in cytokinesis, resulting in massive increases in bi- and multinucleated cells (Fig. 2, H and I) and multipolar spindles (Fig. 2 J). This system for eliminating INCENP function in DT40 cells allowed us to evaluate INCENP–aurora B interactions during vertebrate cell division.

Rescue of INCENP function by the class I splice variant

Triple affinity purification (TrAP)–tagged INCENP$^{WT\ class\ I}$ (hereafter referred to as TrAP-INCENP^{WT}) under control of an SV40 promoter that is insensitive to doxycycline repression efficiently rescued the life of the INCENP^{OFF} cells. INCENP^{OFF}/TrAP-INCENP^{WT} cells grew with kinetics indistinguishable from wild-type or INCENP^{ON} cells (Fig. 3 B). The TrAP tag incorporates His, streptavidin-binding peptide (SBP), and S tags and can be monitored by immunoblotting and immunofluorescence using a monoclonal antibody recognizing the SBP tag (Hudson et al., 2008; Samejima et al., 2008). TrAP-INCENP$^{WT\ class\ II}$ could also support the growth of INCENP^{OFF} cells (unpublished data). Thus, alternative splicing of INCENP is not essential for life in DT40 cells.

Immunoblots confirmed that the TrAP-INCENP^{WT}–expressing clone selected for study stably expresses the tagged protein at a level comparable with endogenous wild-type INCENP I (Fig. 3 F, lanes 6 and 1, respectively). The TrAP tag caused a minor mobility shift, and TrAP-INCENP^{WT} comigrated with endogenous INCENP class II. A faster migrating band that comigrated near INCENP class I on mini gels is a proteolytic fragment of TrAP-INCENP that does not comigrate with endogenous INCENP class I on full-sized SDS gels (Fig. 3 H).

As expected, TrAP-INCENP^{WT} exhibited a typical CPC distribution pattern in INCENP^{OFF} cells. It was concentrated in centromeres from prophase to metaphase, where it colocalized

summary of the targeting efficiencies. (F) Relative mRNA level of *incenp* and *incenp-like* gene expressed in wild-type clone 18 cells. Three independent RNA preparations were used in three independent experiments. (G) Growth curves of DT40 wild-type (WT; clone 18), INCENP^{ON}, and INCENP^{OFF} cells. Wild-type and INCENP^{ON} cells have a similar proliferation rate. INCENP^{OFF} cells cease proliferation 12 h after doxycycline addition. (H) Immunoblot showing that endogenous INCENP is efficiently depleted by 20 h after the addition of doxycycline. α-Tubulin was used as a loading control. (I) INCENP^{OFF} cells are defective in completion of chromosome alignment. Live cell imaging of INCENP$^{ON/OFF}$ cells stably expressing histone H2BRFP revealed that INCENP^{OFF} cells stay for a statistically longer period in prometaphase before entering to anaphase. Images were taken every 2 min. (B and D) X indicates the promoter hijack allele. Error bars indicate SD.

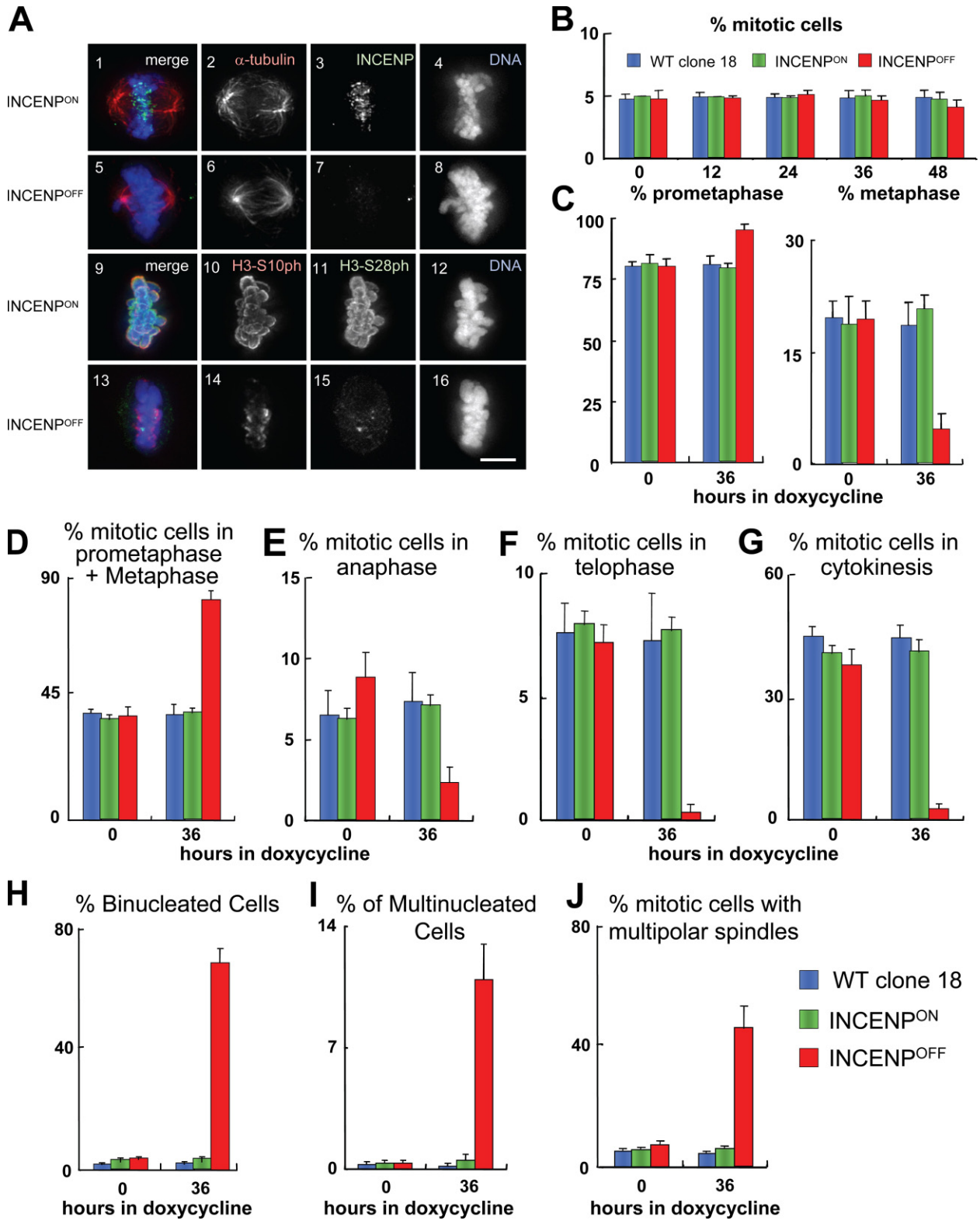

Figure 2. **Phenotypic analysis of conditional INCENP knockout cell line.** (A) Immunofluorescence images showing INCENP^{ON} and INCENP^{OFF} cells stained with specific antibodies recognizing endogenous INCENP (green; panels 3 and 7), α-tubulin (red; panels 2 and 6), and DAPI for DNA (blue; panels 4 and 8). After incubation with doxycycline for 26 h, endogenous INCENP was hardly detected (INCENP^{OFF}; panel 7). Panels 9–16 show immunofluorescence images of H3S10ph (green; panels 10 and 14) and H3S28ph (red; panels 11 and 15) plus DAPI for DNA (blue; panels 12 and16) in INCENP^{ON} (panels 9–12) and INCENP^{OFF} (panels 13–16) cells. Merged images are shown in panels 1, 5, 9, and 13. INCENP^{ON} and INCENP^{OFF} cells were harvested and fixed at the same time. Images were acquired using the same microscope settings for all experiments. Bar, 5 µm. (B) Mitotic indices of wild-type, INCENP^{ON}, and INCENP^{OFF} cells. (C) Scoring of mitotic cells in late prometaphase and metaphase where cells are bioriented and two spindle poles are on opposite sides. (D–G) Percentage of mitotic cells in prometaphase or metaphase (D), anaphase (E), telophase (F), or cytokinesis (G) is shown. (H–J) Percentage of binucleated cells (H), multinucleated cells (I), and mitotic cells with multipolar spindles (J) is shown. Error bars indicate SD.

with aurora B, Borealin, and Survivin (Fig. S1, D–F). During anaphase, TrAP-INCENP^{WT} accumulated in the cleavage furrow and midbody. TrAP-INCENP^{WT} was also competent in CPC formation. INCENP^{OFF} cells expressing TrAP-INCENP^{WT} grown in doxycycline for 28 h were treated with nocodazole from hours 16–28 to enrich for mitotic cells. Subsequent streptavidin pull-downs confirmed that TrAP-INCENP^{WT} was associated with endogenous aurora B, Borealin, and Survivin in vivo (Fig. 4, A and B, lane 1).

Because exogenous TrAP-INCENP^{WT} could functionally replace endogenous INCENP in DT40 cells, this permitted us to study the physiological effects of INCENP structural mutants against a background where endogenous INCENP is essentially absent.

Isolation of an INCENP point mutant that fails to bind aurora B

Only four residues in the IN box (spanning residues 755–812 of chicken INCENP) are fully conserved: Glu756, Trp766, Phe802, and Lys811 (Fig. 3 A). Of these, structural and in vitro experiments examining interactions between an IN box fragment and aurora B had suggested that Trp766 and Phe802 (chicken numbering) might be important for activation and binding of the kinase, respectively (Sessa et al., 2005). Thus, our initial site-directed mutagenesis experiments focused on these two residues. Additional functional characterization of the C-terminal TSS motif will be published elsewhere (unpublished data).

We selected INCENP$^{ON/OFF}$-conditional cell lines stably expressing TrAP-INCENP^{W766G} (kinase-binding deficient) and TrAP-INCENP^{F802A} (kinase activation deficient) mutants that expressed levels of mutant INCENP protein similar to those in wild-type clone 18 DT40 cells (Fig. 3, F and G). In an INCENP^{ON} background, these clones grew with kinetics indistinguishable from wild-type clone 18 DT40 cells (Fig. 3, C and D), and the endogenous passenger proteins showed their typical dynamic localization. However, after the addition of doxycycline, both mutant cell lines died with kinetics resembling the INCENP^{OFF} cultures (Fig. 3, C and D).

Strikingly, although TrAP-INCENP^{W766G} failed to bind aurora B, this protein formed a CPC subcomplex with Survivin and Borealin (Fig. 4, A and B, lane 2). TrAP-INCENP^{F802A} bound to aurora B and formed an intact CPC with Survivin and Borealin (Fig. 4, A and B, lane 3). This is in contrast to predictions from an earlier structural analysis (Sessa et al., 2005).

A CPC subcomplex lacking aurora B fails to transfer to the spindle midzone

We used clones expressing TrAP-INCENP^{W766G} (kinase-binding deficient) to test the hypothesis that Survivin and Borealin target the CPC to its diverse locations during mitosis (Gassmann et al., 2004; Klein et al., 2006; Vader et al., 2006a) by examining the behavior of a CPC subcomplex lacking aurora B.

As expected, TrAP-INCENP^{W766G} colocalized with Survivin and Borealin throughout mitosis in INCENP^{OFF} cells (Fig. S2, A and B). This INCENP–Survivin–Borealin subcomplex localized to centromeres in early mitosis, as confirmed by costaining with CENP-A (Fig. 4 F and Fig. S3 B). In contrast, aurora B failed to localize normally in these cells and was instead dispersed throughout the cytoplasm (Fig. 4 E and Fig. S3 A).

Aurora B kinase activity was significantly decreased in INCENP^{OFF}/TrAP-INCENP^{W766G} cells. As measured by quantitative immunoblotting, levels of H3S10ph fell essentially to those seen in INCENP^{OFF} cells (Fig. 5, A, B, and E). A similar decline in the levels of H3S10ph and H3S28ph was observed by indirect immunofluorescence (Fig. S3 F). These data support the accepted notion that INCENP–aurora B complex formation is required for full kinase activation (Adams et al., 2000; Bishop and Schumacher, 2002; Bolton et al., 2002; Honda et al., 2003).

In INCENP^{OFF}/TrAP-INCENP^{W766G} cells, Survivin and Borealin levels fell significantly by 12 h in doxycycline (Fig. 3, F–H). However, this paralleled the overall INCENP levels, which were overexpressed in INCENP^{ON} cells but fell to near wild-type levels after doxycycline addition in INCENP^{OFF}/TrAP-INCENP^{W766G} cells. Thus, INCENP overexpression is accompanied by an increase in Survivin and Borealin levels. This suggests that INCENP levels may determine the stable levels of all three subcomplex members. Interestingly, aurora B levels fell no more than twofold at 24 h after abolition of the association of the kinase with INCENP (Fig. 3, E and F).

These experiments offered the first opportunity to examine the behavior of a CPC subcomplex lacking aurora B. Strikingly, although the INCENP–Survivin–Borealin subcomplex did target to centromeres, it failed to efficiently transfer to the central spindle at anaphase onset, instead remaining associated with the chromosome arms as sister chromatids separated and moved polewards (Fig. 6 B; Fig. S2, A and B; and Fig. S3, A and B). This suggests that either full CPC formation or aurora B activation is required for transfer of the complex to the spindle midzone during mitotic exit.

INCENP^{OFF}/TrAP-INCENP^{W766G} cells (kinase-binding deficient) accumulate in prometaphase/metaphase and show a pronounced decline in the later stages of mitosis (Fig. S4, B–E). The prometaphase/metaphase accumulation suggests that at least some degree of spindle checkpoint activity is present. Because these cultures exhibit a normal mitotic index of ~5% (Fig. S4 A), we hypothesized that the cells must undergo an abnormal mitotic exit. This was supported by the observation of dramatic increases in the percentage of binucleated (two) and multinucleated (more than two) cells over time in doxycycline (Fig. S4, G and H), which is indicative of mitotic exit without cytokinesis. We also observed an accumulation of mitotic cells with more than two spindle poles (Fig. S4 F).

Together, data obtained with this aurora B–nonbinding mutant indicate that formation of the CPC holocomplex is not required for mitotic entry, spindle checkpoint activation, or sister chromatid separation. However, holocomplex formation and/or aurora B activation are required for CPC transfer to the anaphase spindle midzone and for completion of cytokinesis.

A CPC holocomplex fails to transfer to the spindle midzone if aurora B is not fully activated

The aforementioned experiments suggest two possible hypotheses for the failure of the CPC subcomplex to transfer to

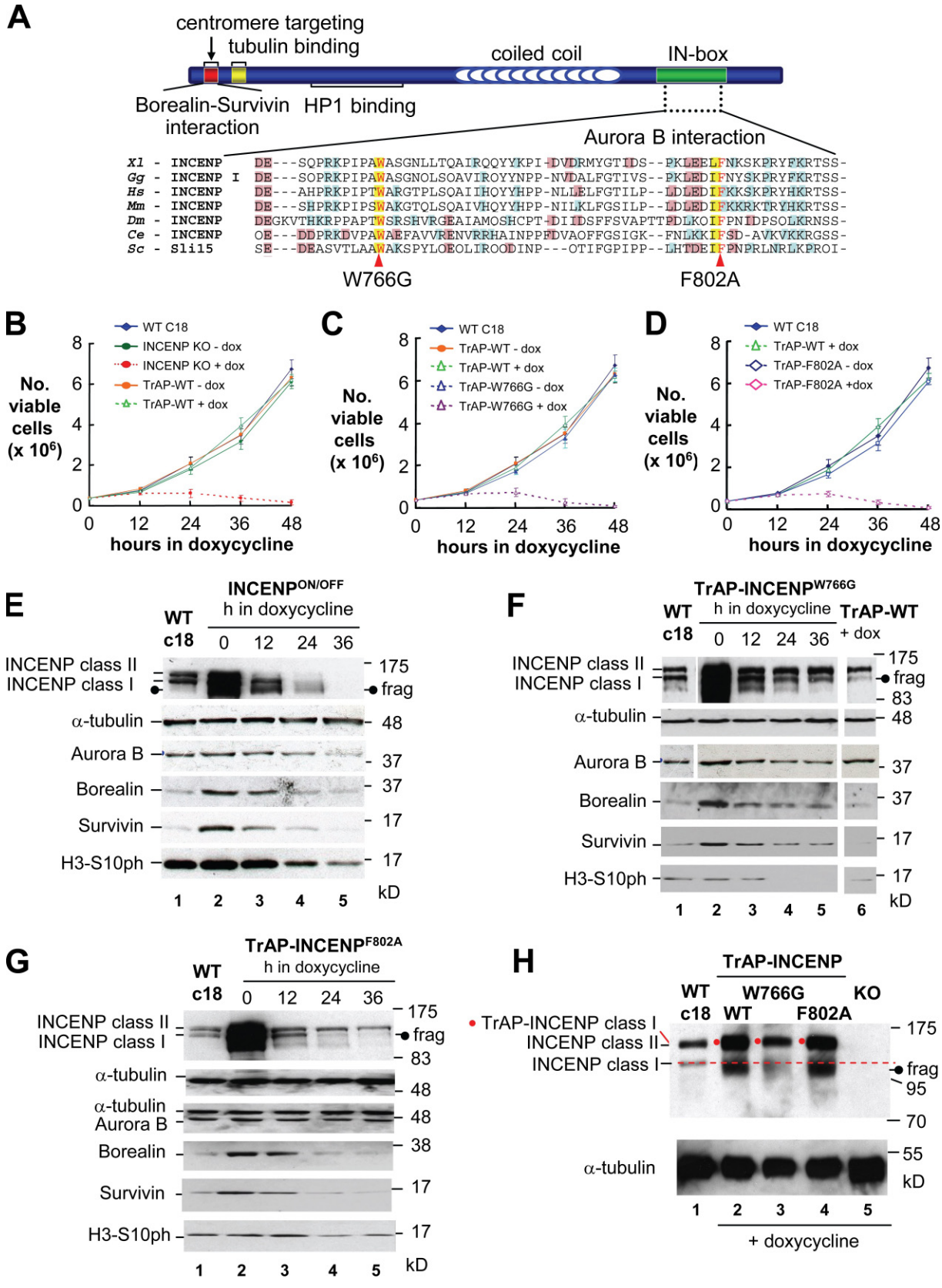

Figure 3. **Generation of INCENP structural mutants to dissect INCENP–aurora B interactions.** (A) Schematic representation of the main domains of INCENP with a sequence alignment of the INCENP IN box in several model organisms. Red triangles indicate key residues mutated in this study. Xl, *Xenopus laevis*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Sc, *Saccharomyces cerevisiae*. (B–D) Growth curves of DT40 wild-type (WT) and INCENP$^{ON/OFF}$ cells expressing various forms of INCENP in the presence and absence of doxycycline. The expressed INCENP was TrAP-INCENP^{WT} (B), TrAP-INCENP^{WT} plus TrAP-INCENP^{W766G} (C), and TrAP-INCENP^{WT} plus TrAP-INCENP^{F802A} (D). Growth of

the spindle midzone: (1) the CPC might fail to transfer unless all four subunits are associated with one another, or (2) CPC transfer to the spindle midzone might require a threshold of aurora B activation.

To distinguish between these two hypotheses, we examined CPC behavior in INCENP^{OFF} cells expressing TrAP-INCENP^{F802A} (kinase activation deficient). As shown in Fig. 4, and contrary to predictions based on in vitro binding of INCENP fragments to aurora B (Sessa et al., 2005), TrAP-INCENP^{F802A} could scaffold the formation of a complete CPC holocomplex containing aurora B (Fig. 4, A and B, lane 3). Thus, as expected, TrAP-INCENP^{F802A} colocalized with aurora B, Survivin, and Borealin throughout mitosis (Fig. 4 G; Fig. 6 C; Fig. S2, C and D; and Fig. S3 C).

Phe802 in the INCENP IN box was predicted from the crystal structure to be involved in setting the opening angle of the kinase catalytic cleft and therefore to be important for aurora B activation (Sessa et al., 2005). Indeed, the activity of aurora B against histone H3 serines 10 and 28 was significantly decreased in INCENP^{OFF} cells expressing INCENP^{F802A} (Fig. 5, A, B, and E; and Fig. S3 G). As a control, levels of H3T3ph, a measure of haspin kinase activity, were essentially unaltered in these cells, which is consistent with haspin being upstream of aurora B (Rosasco-Nitcher et al., 2008). Importantly, aurora B protein levels were unaffected by the replacement of INCENP^{WT} with INCENP^{F802A}, and Survivin and Borealin levels paralleled those of INCENP (Fig. 3 G).

In view of the importance of this residue for aurora B activation, it was no surprise that INCENP^{OFF} cells expressing TrAP-INCENP^{F802A} rapidly lost viability after shutoff of the endogenous INCENP allele (Fig. 3 D). Phenotypically, these cells resembled INCENP^{OFF} cells with an increase in prometaphases/metaphases coupled to significant decreases in cells in the latter stages of mitosis (Fig. S4, J–N). These cells also exited mitosis abnormally, with increases in bi- and multinucleated cells, indicating a failure to complete cytokinesis (Fig. S5, P and Q).

The CPC containing TrAP-INCENP^{F802A} localized normally to centromeres in early mitosis (Fig. 4 H; Fig. 6 C; and Fig. S3, C and D) but failed to transfer to the spindle midzone in anaphase (Fig. 6 C; and Fig. S2, C and D). Thus, both holocomplex formation and aurora B activation are required for CPC transfer to the spindle midzone.

Aurora B activity is required for CPC holocomplex transfer to the spindle midzone

The aforementioned experiments suggested that aurora B activation by INCENP is required for CPC transfer to the spindle midzone. As an independent test of this hypothesis, we examined the effects of direct aurora B inhibition with ZM447439 (Ditchfield et al., 2003) on CPC transfer to the anaphase spindle.

As expected, aurora B activity was efficiently inhibited by addition of ZM447439 to cultures of either wild-type clone 18 cells or INCENP^{OFF} cells expressing TrAP-INCENP^{WT} (Fig. 5, A and B; and see Fig. 8 G). Indeed, quantitative immunoblotting of H3S10ph levels showed an effect even more profound than that seen with INCENP^{OFF} cells (Fig. 5 E), suggesting that basal aurora B activity is present in INCENP^{OFF} cultures.

The original report of ZM447439 described a prometaphase arrest with chromosomes distributed around the periphery of the mitotic spindle (Ditchfield et al., 2003). Indeed, after a brief (3 h) exposure of wild-type clone 18 or control INCENP^{OFF}/TrAP-INCENP^{WT} cells to ZM447439, the predominant phenotype was as previously described (Ditchfield et al., 2003). However, a few cells did progress to anaphase. Strikingly, these cells phenocopied INCENP^{OFF} cells expressing TrAP-INCENP^{W766G} and TrAP-INCENP^{F802A}; the CPC localized normally to centromeres in early mitosis, but then failed to transfer to the anaphase spindle midzone, remaining instead associated with the anaphase chromatids (Fig. 6, D and E; and Fig. S5, A and B). Thus, aurora B activity is critical for the CPC to transfer to the spindle midzone.

The CPC is not required for formation of a spindle midzone

One possible explanation for the failure of CPC components to transfer to the spindle midzone at anaphase after the inhibition of aurora B activity could be that the CPC is required for the assembly of a functional kinetochore or central spindle. Perturbation of kinetochore assembly results in spindle abnormalities, as the two spindle poles are not correctly linked to one another through the paired sister kinetochores (Cheeseman et al., 2004). However, inner and outer kinetochores appeared to be normal in cells depleted of INCENP, as judged by the localization of CENP-A, -E, -H, -O, -T, and Hec1/Ndc80 (Fig. 7 and Fig. 8 B).

We previously observed that the bundling of central spindle microtubules was often deficient in HeLa cells expressing dominant INCENP mutants (Mackay et al., 1998). Therefore, to test the hypothesis that normal CPC function is required for the assembly of a functional central spindle, we examined the behavior of CENP-E and PRC1 in the aforementioned cell lines as well as in wild-type clone 18 cells after aurora B inhibition by ZM447439. However, both CENP-E and PRC1 localization throughout mitosis is independent of CPC complex formation and function in DT40 cells. Both proteins localize normally in INCENP^{OFF} cells, which lack the CPC (Fig. 8 B

cultures expressing TrAP-INCENP^{WT} was indistinguishable from wild-type cells. All cultures expressing only mutant INCENP died. (E–G) Immunoblots show levels of chromosomal passenger proteins in INCENP$^{ON/OFF}$ cells (E) or INCENP$^{ON/OFF}$ cells expressing TrAP-INCENP^{W766G} (F) or TrAP-INCENP^{F802A} (G). INCENP^{OFF} cells expressing TrAP-INCENP^{WT} are shown as controls. (E) Black circles indicate a proteolytic fragment of INCENP. At time 0, INCENP, which was driven by the tTA expressed from the KIF4A promoter, is ~20× overexpressed, and the levels of the other passengers are correspondingly increased. All levels decrease to those shown in wild-type clone 18 cells after the addition of doxycycline provided that some form of INCENP was present. Levels of H3S10ph are shown as a measure of aurora B activity and α-tubulin as a loading control. White lines indicate that intervening lanes have been spliced out. (H) Immunoblots shows higher resolutions of INCENP and its fragment. TrAP-INCENP class I comigrate with INCENP class II. The red line indicates that the proteolytic fragments seen in all cells expressing TrAP-INCENP constructs are not INCENP class I. α-Tubulin was used as a loading control. (F–H) Black circles indicate the position of INCENP degradation fragments. Error bars indicate SD.

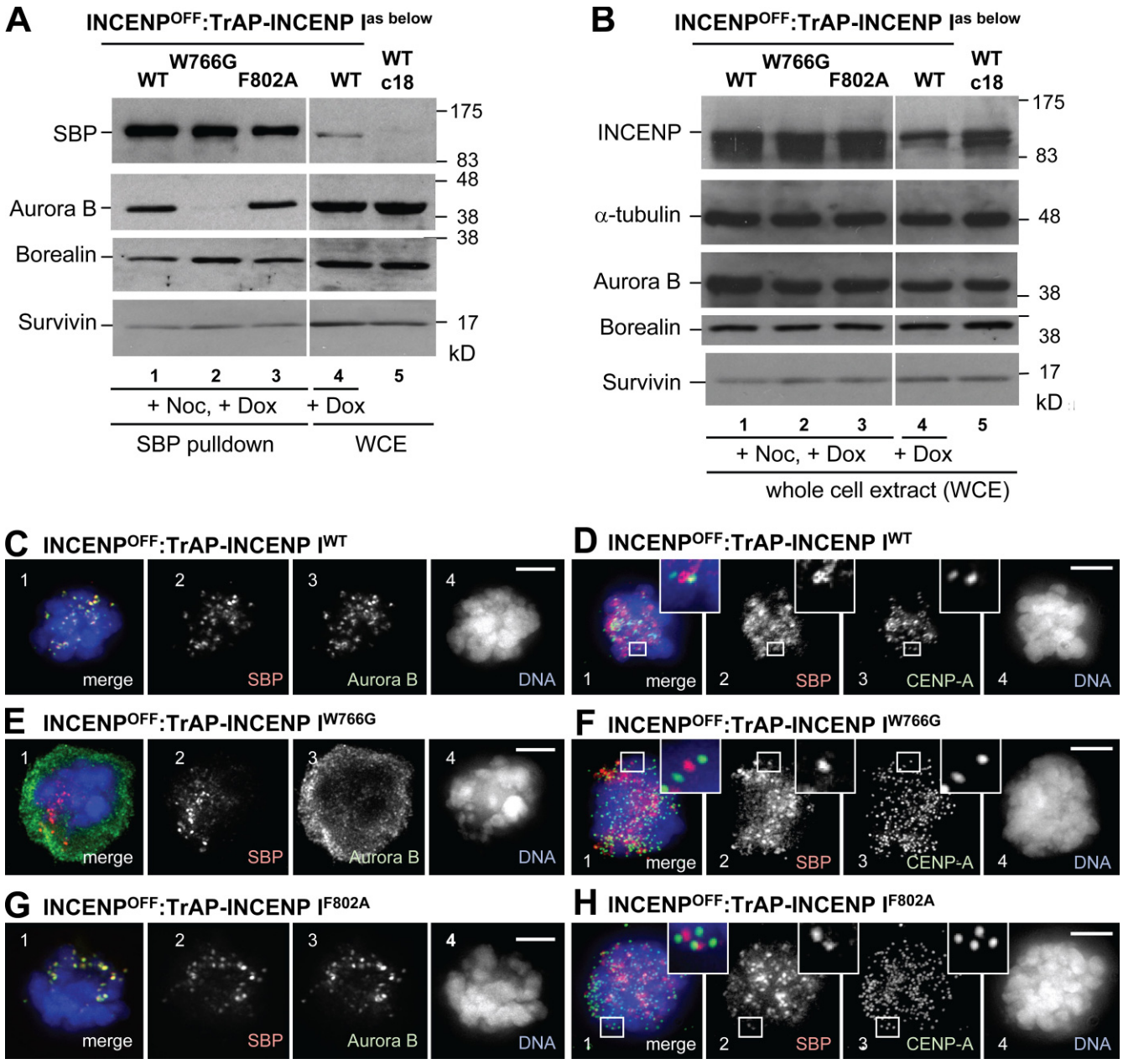

Figure 4. **In vivo analysis of CPC formation.** (A and B) INCENP$^{ON/OFF}$ cells stably expressing TrAP-tagged INCENP mutants were grown in doxycycline (Dox) to shut off expression of wild-type (WT) INCENP plus nocodazole (Noc; or not) to enrich for mitotic cells. (A) Immunoblots of streptavidin pull-downs with anti-SBP to reveal the proteins associated with INCENP. (B) Immunoblots of whole cell lysates with antibodies to INCENP (monoclonal antibody 3D3; Cooke et al., 1987) and the other passenger proteins. Equal numbers of cells were loaded per lane. α-Tubulin is used as a loading control. White lines indicate that intervening lanes have been spliced out. (C–H) Localization of exogenous TrAP-INCENP (red; panel 2) plus endogenous aurora B (green; C, E, G, and I [panel 3]) or CENP-A (green; D, F, H, and J [panel 3]) in INCENP^{OFF} cells expressing TrAP-INCENP^{WT} (C and D), TrAP-INCENP^{W766G} (E and F), or TrAP-INCENP^{F802A} (G and H). These images are of the nocodazole-treated cells used for the SBP pull-down experiment in A and B. (E) In all cases, the TrAP-INCENP localizes to centromeres, but only in INCENP^{OFF}/TrAP-INCENP^{W766G} cells is aurora B localization diffuse. Images were acquired using the same microscope settings for all experiments. Insets show magnified views of boxed regions. Bars, 5 μm.

and not depicted), as well as in INCENP^{OFF} cells expressing the INCENP mutants or after inhibition of aurora B with ZM447439 (Fig. 8, C–G).

Our experiments confirm that CENP-E and the CPC transfer to the spindle midzone by independent mechanisms. In the presence of ZM447439, INCENP transfer to the anaphase spindle midzone was defective in the same cells where CENP-E transfer appeared normal (Fig. 8 F and Fig. S5 C).

Differing levels of aurora B activity fine tune spindle checkpoint activity

The aforementioned cell lines allowed us to examine the relationship between levels of aurora B activity and the spindle checkpoint response. Consistent with a previous study (Ditchfield et al., 2003), aurora B activity is not essential for a normal checkpoint response to nocodazole or to high dose taxol treatment (Fig. 9, A, D, and E; Yue et al., 2008). Interestingly, it is

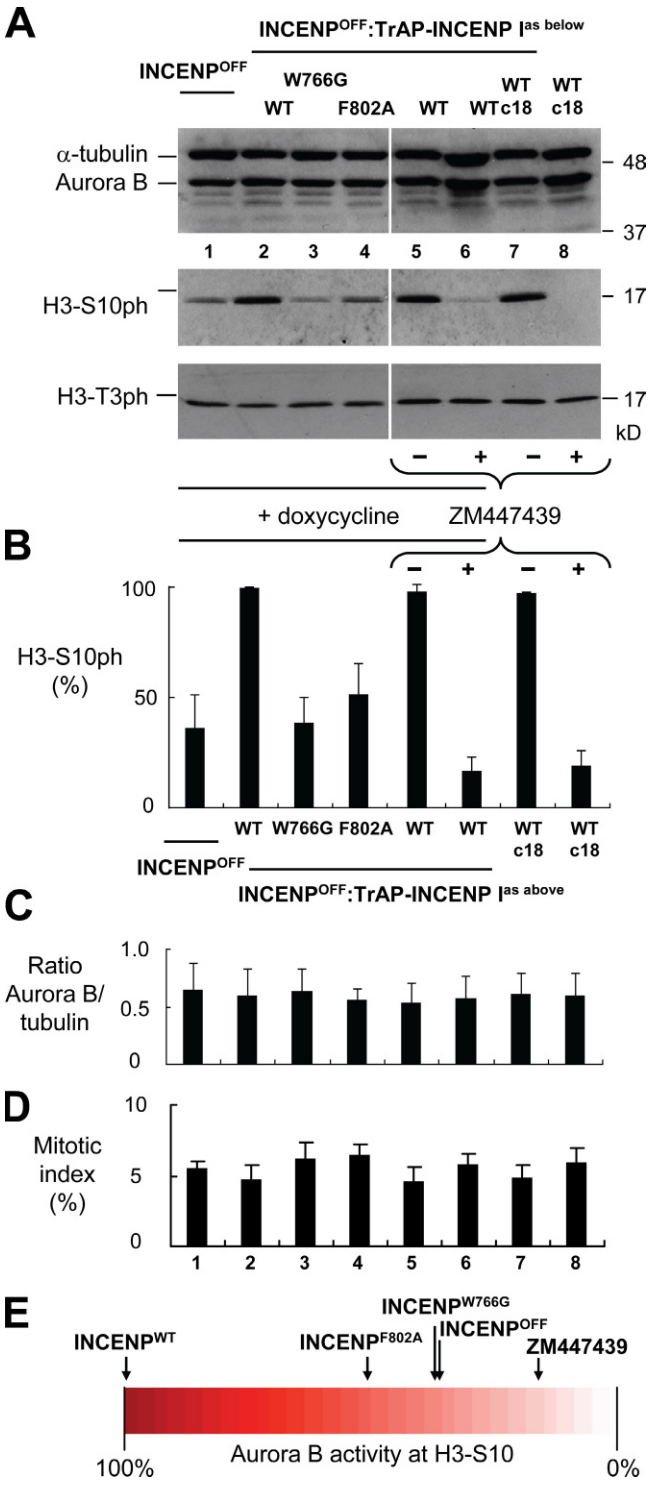

Figure 5. **Quantitation of aurora B kinase activity in INCENP mutants.** (A) Estimation of aurora B activity by immunoblotting. Asynchronous cells were harvested after treatment with doxycycline for 28 h or with 2 µM ZM447439 for 5 h (Fig. S1 C), and lysates were subjected to immunoblotting with the indicated antibodies. α-Tubulin and haspin kinase substrate H3T3ph are shown as controls. White lines indicate that intervening lanes have been spliced out. (B) Measurement of H3S10ph levels in the same samples using Odyssey. (C) Ratio of aurora B protein levels versus the loading control α-tubulin for each sample as measured using Odyssey. (D) Mitotic index of each cell line at the time of harvesting. (E) Diagram showing the relative levels of aurora B activity based on the level of H3S10ph measured using the Odyssey assay. WT, wild type. Error bars indicate SD.

required for a response to low dose taxol, and indeed, the response appears to depend on the level of aurora B activity (Fig. 9, B and D). Wild-type DT40 cells exposed to 10 nM taxol in the presence of ZM447439 mount a very weak checkpoint response. INCENP^{OFF} and INCENP^{OFF}/INCENP^{W766G} cells (kinase-binding defective), which exhibit only very low levels of aurora B activity, respond weakly to 10 nM taxol, as seen previously after Survivin RNAi or conditional knockout (Carvalho et al., 2003; Lens et al., 2003; Yue et al., 2008). Remarkably, INCENP^{OFF}/INCENP^{F802A} cells (kinase activation defective) in which the kinase is only slightly more active mount a robust checkpoint response to 10 nM taxol. Thus, a level of aurora B activity that is insufficient to promote transfer of the CPC to the anaphase spindle midzone is sufficient for a robust spindle checkpoint response.

Discussion

CPC localization during mitosis is intimately linked with its mitotic functions (Tanaka et al., 2002; Liu et al., 2009). Interactions involving the N terminus of INCENP are critical for CPC localization to centromeres and the regulation of kinetochore–microtubule attachments (Mackay and Earnshaw, 1993; Ainsztein et al., 1998; Klein et al., 2006; Vader et al., 2006a; Jeyaprakash et al., 2007). In contrast, the role of association of the C-terminal IN box with aurora B (Adams et al., 2000; Kaitna et al., 2000) in controlling CPC behavior and function is less studied.

In this study, we have expressed two INCENP mutants against the null background of an INCENP-conditional knockout created by promoter hijack of the *incenp* gene in DT40 cells (Samejima et al., 2008). The INCENP^{W766G} mutant is unable to bind aurora B kinase, whereas the INCENP^{F802A} mutant does bind the kinase but cannot activate it fully. This mutation corresponds to *Xenopus laevis* INCENP^{F837A}, which was shown to be involved in determining the conformation of the catalytic cleft in aurora B (Sessa et al., 2005). Together with the use of specific aurora B inhibitor ZM447439 (Ditchfield et al., 2003; Girdler et al., 2008), these mutants have enabled us for the first time to create cells with a graded series of levels of aurora B activity (assayed by quantitation of phosphorylation of H3S10).

In cells expressing wild-type INCENP treated with ZM447439, aurora B H3S10 kinase activity falls to 15–20% of maximum. This residual activity may reflect the phosphorylation of H3S10 by other kinases. In INCENP^{OFF} cells or INCENP^{OFF} cells expressing INCENP^{W766G}, H3S10 kinase activity is roughly 30–40% of maximum. This 15–20% increase could reflect the basal level of aurora B activity without interaction with INCENP, or it could conceivably reflect the activation of aurora B by extremely low levels of the paralogue INCENP-like (although the latter is unlikely given its extremely low expression levels in DT40 cells). Regardless, our experiments reveal that these cells rapidly die, so this residual level of kinase activity is unable to support life. In INCENP^{OFF} cells expressing INCENP^{F802A}, the aurora B activity is higher, roughly 50% of maximal. Thus, in this experimental system, INCENP can act analogous to a rheostat for aurora B, with differing interactions adjusting the level of kinase activity (Fig. 10).

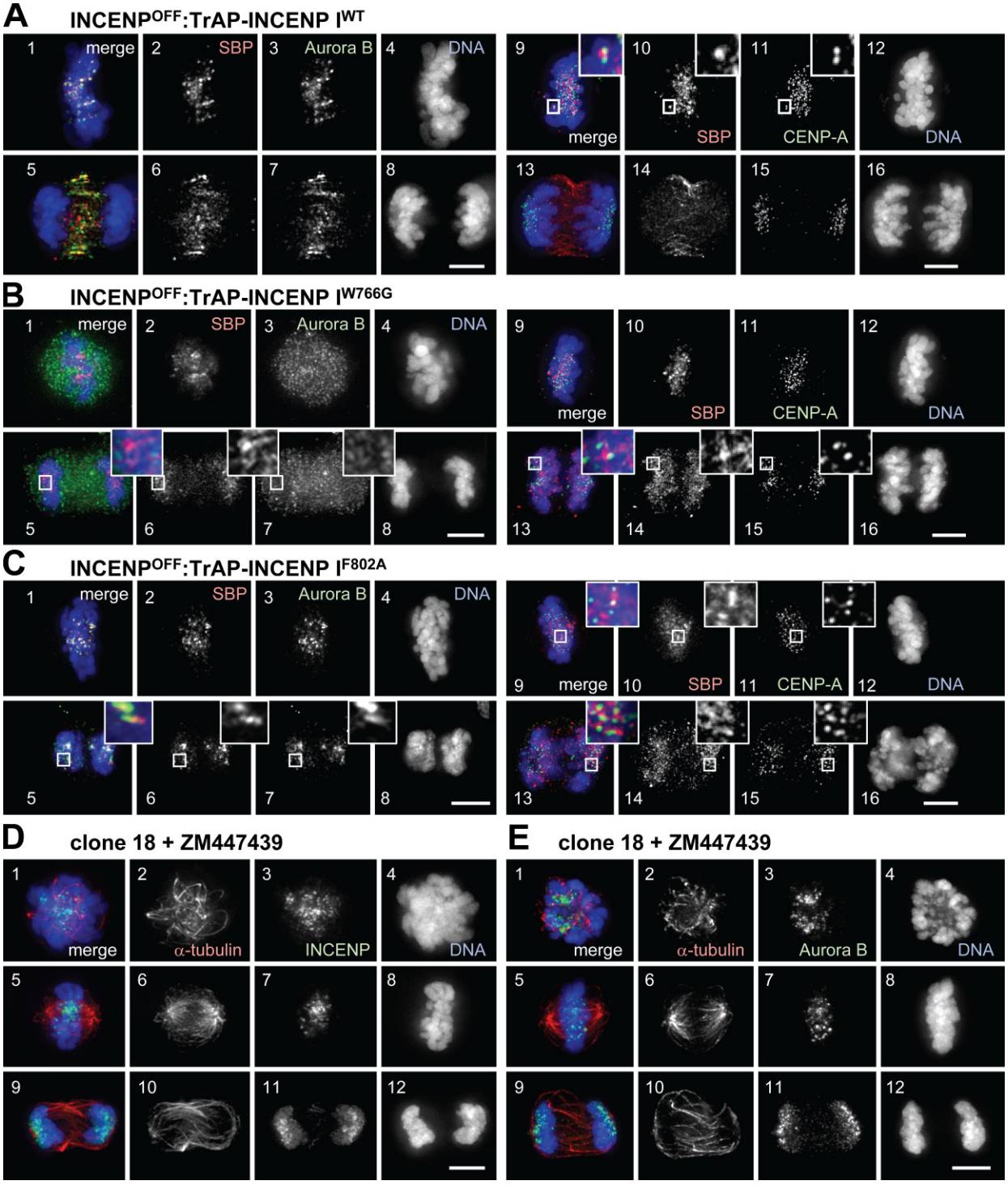

Figure 6. **Active aurora B is required for aurora B and INCENP to transfer to the spindle midzone.** (A–C) Localization of exogenous TrAP-INCENP (red; panels 2, 6, 10, and 14) plus endogenous aurora B (green; panels 3 and 7) or CENP-A (green; panels 11 and 15) and DNA (blue; panels 4, 8, 12, and 16) in INCENP^{OFF} cells expressing TrAP-INCENP^{WT} (A), TrAP-INCENP^{W766G} (B), or TrAP-INCENP^{F802A} (C). In all cases, TrAP-INCENP localizes to centromeres at metaphase, but in INCENP^{OFF} cells expressing TrAP-INCENP^{W766G} or TrAP-INCENP^{F802A} (B and C), it fails to transfer to the spindle midzone at anaphase. Aurora B colocalizes with INCENP except for cells expressing TrAP-INCENP^{W766G}, where it is diffuse. (D and E) INCENP and aurora B localize to centromeres in early mitosis but fail to transfer to the spindle at anaphase in wild-type clone 18 cells grown in the aurora B inhibitor ZM447439. Cells were stained for INCENP or aurora B (green; panels 3, 7, and 11) plus α-tubulin (red; panels 2, 6, and 10) and DAPI for DNA (blue; panels 4, 8, and 12). Insets show magnified views of boxed regions. Bars, 5 µm.

Interestingly, our results indicate that these differing levels of kinase activity are functionally significant. For example, we demonstrate that relatively robust levels of kinase activity are required for the CPC to transfer from the chromosomes to the spindle midzone at anaphase. However, even a tripartite CPC lacking aurora B can target to centromeres in

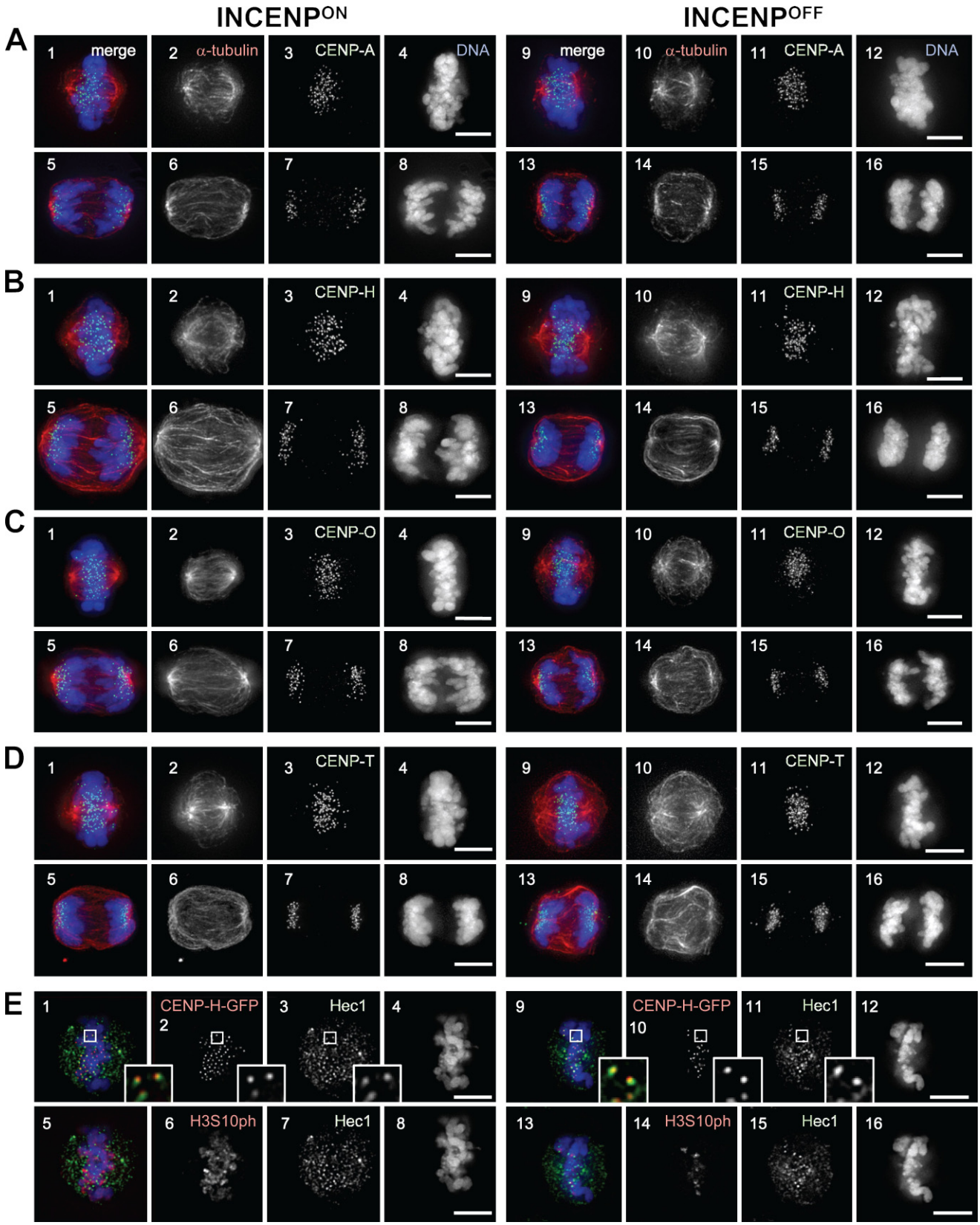

Figure 7. **Kinetochore assembly is normal in cells lacking a functional CPC.** (A–E) Kinetochore proteins CENP-A (A), CENP-H (B), CENP-O (C), CENP-T (D), and Hec1 (E) localize normally during metaphase and anaphase in INCENP^{ON} cells (panels 1–8) and INCENP^{OFF} cells (panels 9–16). CENP proteins are shown in green, α-tubulin or CENP-H–GFP in red, and DAPI (DNA) in blue. Insets show magnified views of boxed regions. Bars, 5 μm.

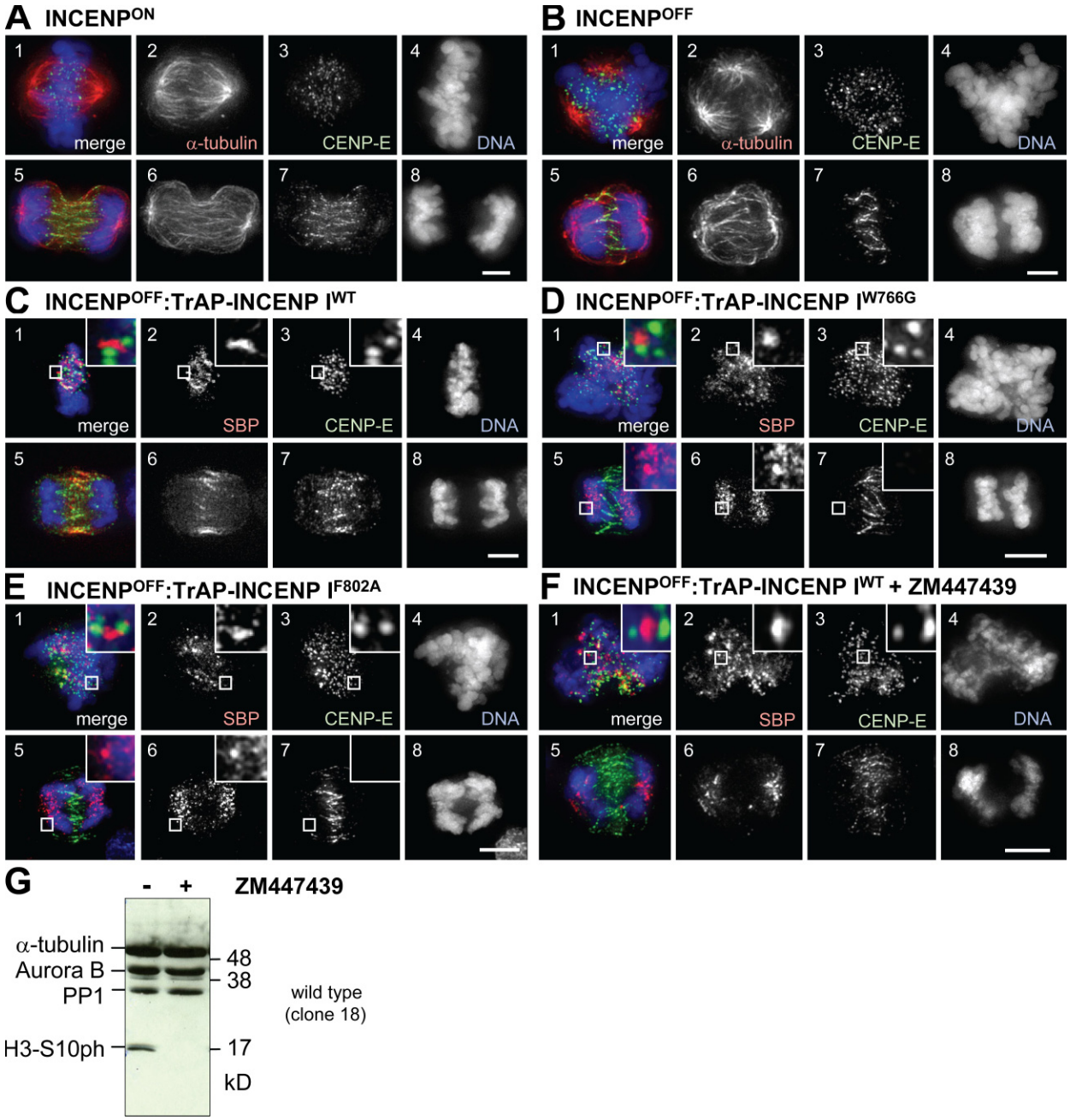

Figure 8. **The CPC and CENP-E exhibit distinct central spindle transfer mechanisms during anaphase.** (A–F) CENP-E transfers to the central spindle at anaphase even when the CPC remains stuck on the chromatin. CENP-E (green; panels 3 and 7) and α-tubulin or TrAP-INCENP (red; panels 2 and 6) plus DAPI for DNA (blue; panels 4 and 8) are shown for INCENP^{ON} cells (A), INCENP^{OFF} cells (B), or INCENP^{OFF} cells expressing TrAP-INCENP^{WT} (C), TrAP-INCENP^{W766G} (D), TrAP-INCENP^{F802A} (E), or TrAP-INCENP^{WT} in the presence of ZM447439 (F). CENP-E localizes to the outer kinetochore in all cells (insets) and transfers to the central spindle at anaphase, even in cells expressing TrAP-INCENP^{W766G} or TrAP-INCENP^{F802A} in which the CPC remains trapped on the chromatin. Insets show magnified views of boxed regions. Bars, 5 µm. (G) The level of H3S10ph is reduced significantly in the presence of ZM447439 as a result of aurora B inhibition. Levels of aurora B and PP1 remained the same. α-Tubulin was used as a loading control.

early mitosis, but that complex remains on the chromosomes during anaphase. This was seen in cells expressing the INCENP^{W766G} mutant, where INCENP, Borealin, and Survivin form a complex, and aurora B localization is diffuse (Fig. 10 A). Even increasing the kinase activity level to ~50% and assembling the full quadripartite CPC was not sufficient to trigger transfer to the anaphase spindle (seen in cells expressing the INCENP^{F802A} mutant).

These results with INCENP mutants were also confirmed with ZM447439. Despite confirming earlier reports that ZM447439 interferes with chromosome alignment on the spindle (Ditchfield et al., 2003), we were able to observe a small number of drug-treated cells in anaphase. Strikingly, in all instances, the CPC components remained on the chromosomes.

The best example of the functional consequences of fine regulation of the aurora B activity by INCENP comes when

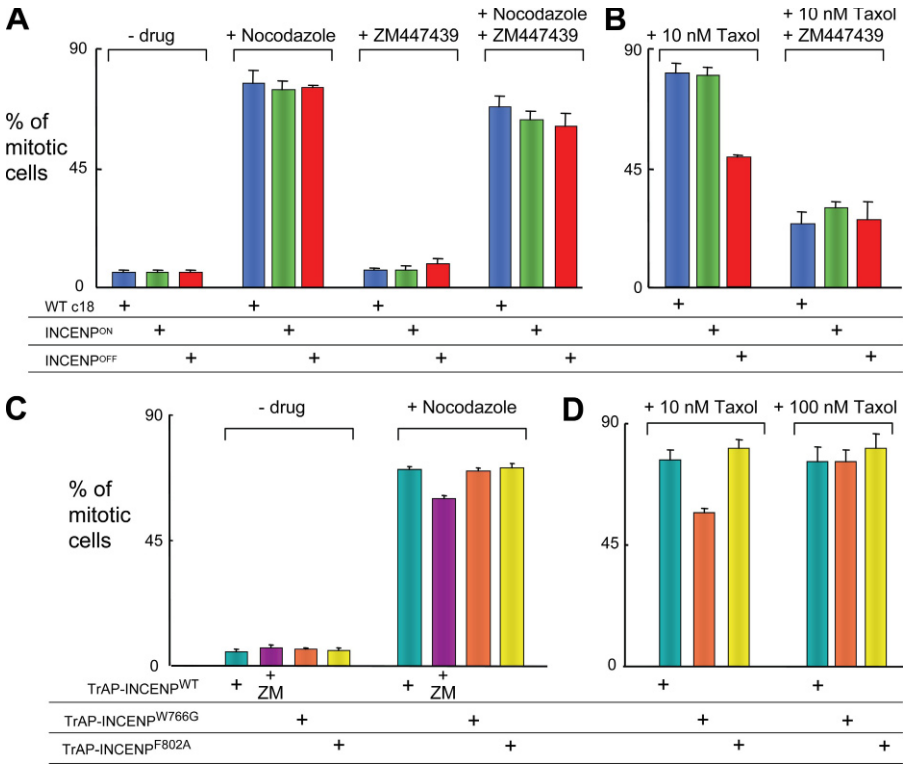

Figure 9. **Effect of INCENP mutations on the spindle checkpoint.** (A) INCENP^{OFF} cells exhibit a robust spindle checkpoint response to nocodazole treatment that is slightly reduced if aurora B is inhibited with ZM447438 (P = 0.01). (B) INCENP^{OFF} cells exhibit an impaired spindle checkpoint response to 10 nM taxol treatment that is significantly worsened by the addition of ZM447439 (P = 0.005). (C) INCENP^{OFF} cells expressing TrAP-INCENP^{WT}, TrAP-INCENP^{W766G}, and TrAP-INCENP^{F802A} all exhibit a robust spindle checkpoint response to nocodazole treatment that is reduced for TrAP-INCENP^{WT} if ZM447438 (ZM) is added (P = 0.003). (D) INCENP^{OFF} cells expressing TrAP-INCENP^{WT} or TrAP-INCENP^{F802A} exhibit a robust spindle checkpoint response to 10 nM taxol treatment. INCENP^{OFF} cells expressing TrAP-INCENP^{W766G} exhibit an impaired response to 10 nM taxol (P = 0.0014). All three cell lines exhibit a normal checkpoint response if treated with 100 nM taxol. Three independent experiments were performed. WT, wild type. Error bars indicate SD.

considering the spindle checkpoint. In DT40 cells, as reported in previous studies (Ditchfield et al., 2003; Yue et al., 2008), the CPC is not required for a robust spindle checkpoint response when microtubules are disassembled, e.g., with nocodazole. However, the response to taxol is complex. Low dose taxol suppresses microtubule dynamics, but some degree of intrakinetochore stretch remains (Maresca and Salmon, 2009). Our results show that 15–20% aurora B activity is insufficient to give a robust response to low dose taxol (10 nM). Indeed, only a marginally stronger checkpoint response was observed in cells with 30–40% kinase activity. Remarkably, increasing the level of aurora B activity only slightly further, to ~50% maximal, was sufficient

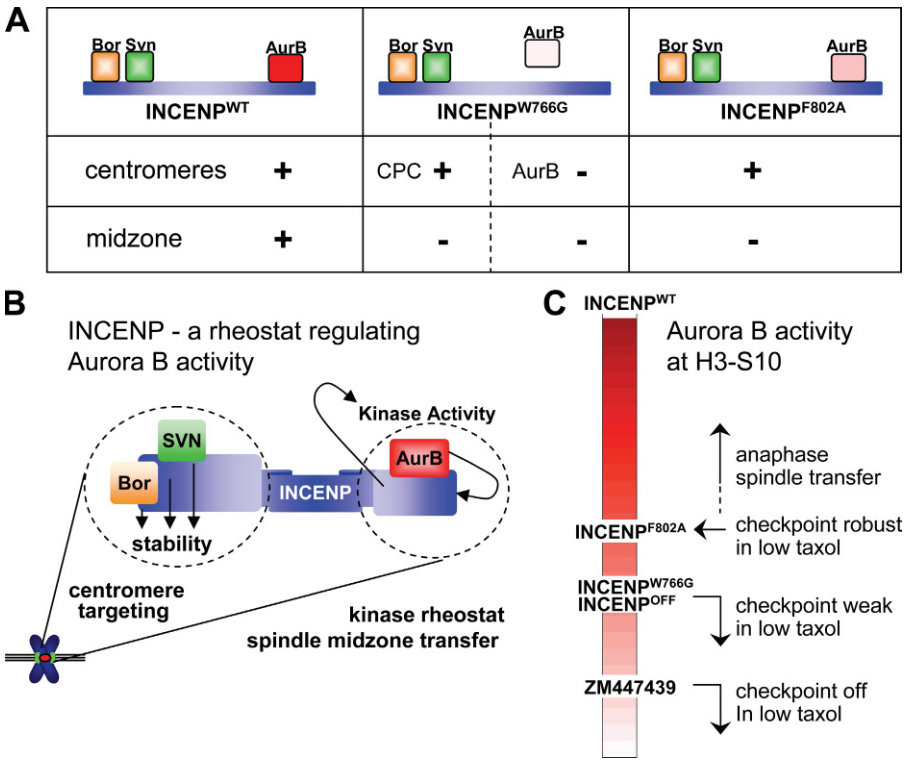

Figure 10. **INCENP acts like a rheostat to adjust levels of aurora B activity and determine CPC behavior.** (A) Status of the CPC, levels of aurora B (AurB) activity (shown as a degree of red shading), and localization of the complex during metaphase and anaphase in cells expressing the different INCENP mutants. Levels of aurora B activity are based on those measured in Fig. 5 B. (B) Nonenzymatic subunits of CPC regulate aurora B localization and activity. An INCENP–Borealin (Bor)–Survivin (Svn) subcomplex is sufficient for centromere targeting and is required for the stability of the individual components. The INCENP C terminus acts as a rheostat, controlling the level of aurora B kinase activity. (C) Functional consequences of the differing levels of aurora B kinase activity set by interactions with the INCENP C terminus. If aurora B is inhibited with ZM447439, the kinase is inactive, and the spindle checkpoint response to low dose taxol is defective. In INCENP^{OFF} cells and INCENP^{OFF} cells expressing INCENP^{W766G}, aurora B activity is ~15–20% higher, but the spindle checkpoint response to low dose taxol remains defective. In INCENP^{OFF} cells expressing INCENP^{F802A}, aurora B activity is a further ~10% higher. These cells now exhibit a normal checkpoint response to low dose taxol. In all of these cells, aurora B activity is insufficient to promote CPC transfer from centromeres to the anaphase spindle midzone.

to give a robust response against low dose taxol. It is important to note that in all cases, a wild-type response was observed against 100 nM taxol, which highly suppresses microtubule dynamics and introduces bundling of microtubules but would still be scored as an intermediate level of drug in one recent study (Yang et al., 2009).

This kinase rheostat in which the checkpoint response to taxol varied depending on the level of aurora B kinase activity may explain some of the controversy in recent studies looking at the role of the CPC in the spindle checkpoint response (Nezi and Musacchio, 2009; Yang et al., 2009). We postulate that effects seen at 10 nM taxol are unlikely to be the result of significant differences in the stability of the microtubules or in spindle tension but could instead reflect subtle differences in the ability of aurora B to correct kinetochore misattachments. A final explanation of this response may require the development of in vitro assays to look at the correction of microtubule attachments.

The question of why the transfer of the CPC from the chromosomes to the spindle midzone requires significantly higher levels of kinase activity also merits further study. Key substrates could be in the centromere, where CENP-A phosphorylation has been implicated in the control of cytokinesis (Zeitlin et al., 2001). However, we have shown in this study that even in the absence of any CPC (INCENP^{OFF} cells), kinetochores assemble CENP-A, -H, -O, -T, -E, and Hec1/Ndc80 and appear to function normally, as determined by the ability of sister chromatids to migrate poleward at anaphase. This disagrees with previous reports that CPC activity is required for assembly of the outer kinetochore (Emanuele et al., 2008; Yang et al., 2008).

Although the CPC does influence the bundling of midzone microtubules (Mackay et al., 1998; Fuller et al., 2008), a structurally distinct spindle midzone can form in the absence of the CPC, as shown by the movements of CENP-E (Fuller et al., 2008). Thus, CENP-E must use a central spindle–targeting mechanism either distinct from or upstream from that of the CPC.

It could be that successful cytokinesis requires subtle aspects of CPC localization that we are unable to detect in our present system. Alternatively, critical substrates, which may include vimentin (Goto et al., 2003; Slawson et al., 2008), Mklp1 (Guse et al., 2005; Zhu et al., 2005; Neef et al., 2006), MgcRacGAP/Cyk4 (Minoshima et al., 2003), and myosin II regulatory light chain (Murata-Hori et al., 2000), might simply require a higher level of kinase activity to be phosphorylated beyond a critical threshold. This could either be because of weaker binding to the kinase or because of more localized counteracting protein phosphatase activity.

Transfer of the budding yeast CPC from centromeres to the overlapping microtubules of the central spindle requires CDC14 phosphatase (Pereira and Schiebel, 2003). Interestingly, Cdc14 phosphatase is required for central spindle formation in *Caenorhabditis elegans* (Gruneberg et al., 2002). The role of CDC14 isoforms in vertebrate cells is less clear (Trinkle-Mulcahy and Lamond, 2006; Bassermann et al., 2008; Clifford et al., 2008; Queralt and Uhlmann, 2008) and merits further study.

These experiments reveal a new complexity to CPC regulation in mitosis. Whether wild-type INCENP is able to activate aurora B to differing extents at different cellular locations in vivo remains to be determined. It is possible that interactions of INCENP with Cdk1 or Plk1 (Goto et al., 2006) could contribute to a rheostat, fine-tuning aurora B activity in vivo.

Materials and methods

Cell culture

DT40 cells were grown in suspension in RPMI 1640 medium supplemented with 10% FBS, 1% chicken serum, and 100 U/ml penicillin streptomycin and 300 mg/ml L-glutamine (Invitrogen) and maintained in 5% CO_2 at 39°C at no more than 10^6 cells/ml (70–90 ml cultures). Doxycycline at a final concentration of 10–500 ng/ml was added to the culture medium to repress transcription of the promoter-hijacked endogenous INCENP locus (Samejima et al., 2008).

Gene targeting

The *incenp1* gene is located at chromosome *5*, position 18,267,63–18,291,339.

Library screening

A DT40 genomic library (Lambda FixII; Agilent Technologies) was screened using a 1.2-kb (NdeI–AflII) fragment from the cloned INCENP cDNA (8.1; Mackay et al., 1993). After the third round of screening, one clone (λ18), which contained the ATG of *incenp1*, was used for the construction of the targeting vectors.

Promoter hijack vector

The promoter hijack–targeting vector was assembled in modified pTre-tight plasmid (Samejima et al., 2008). The 5′ arm (18,259,114–18,261,543; 2,430 bp) and the 3′ arm (18,267,671–18,27,1112; 3,442 bp) obtained from phage DNA were transferred into the SpeI and PvuII–XbaI sites within the modified pTre-tight (LoxP puro), respectively (Samejima et al., 2008). The resultant targeting vector was linearized using PvuI and transfected into wild-type DT40 clone 18 cells. Targeting events were verified with a 5′ probe (18,258,574–18,259,074; 501 bp) amplified from phage DNA.

After the first targeting, the resistance cassette was excised by transient transfection of a plasmid encoding Cre recombinase. The heterozygote cells were transfected with a *kif4a*-tTA2 construct (Samejima et al., 2008) before second allele targeting.

Disruption vector

The disruption vector was based on a pBK-CMV plasmid containing a 4,528-bp *incenp* genomic DNA-covering region 18,265,044–18,269,571. A puromycin resistance cassette (Sugawara et al., 1997) was inserted into the SalI site 81 bp downstream from the ATG in the *incenp1* locus in the opposite orientation from the *incenp* open reading frame. The resultant targeting vector was linearized using KpnI. A 5′ probe (18,264,709–18,265,043; 334 bp) was obtained by BglII–SpeI digest of a plasmid containing *incenp* genomic DNA.

Biochemical analysis, immunoblotting, and antibodies

Whole cell lysates were prepared, and the equivalent to 10^6 cells was loaded onto a polyacrylamide gel. SDS-PAGE and immunoblotting were performed following standard procedures. Anti–α-tubulin antibody (B512; Sigma-Aldrich), anti-H3S10ph (Millipore), and anti-H3S28ph (Abcam) were used. Rabbit polyclonal (WCE1186), mouse monoclonal anti-INCENP (3D3), and mouse monoclonal anti-SBP antibodies were previously described (Cooke et al., 1987; Earnshaw and Cooke, 1991; Hudson et al., 2008). Rabbit polyclonal antibodies against chicken kinetochore proteins were previously described (Fukagawa et al., 2001; Hori et al., 2003, 2008; Okada et al., 2006). For the Survivin antibody, a 6× His-Survivin full-length fusion protein was purified over nickel sepharose beads, resolved by SDS-PAGE, and used to immunize four rabbits (Diagnostics Scotland), all of which produced antibodies recognizing a band running just below the 16.5-kD marker on immunoblots of DT40 whole cell lysates. Serum from rabbit 1576 was affinity purified against the 6× His-Survivin fusion protein and used for further experiments. Antibodies to chicken Borealin and chicken aurora B were raised against *Escherichia coli*–expressed

GST fusion proteins (GST-Borealin$^{1–301}$, rabbit R2098/R2099; GST–aurora B$^{1–201}$, rabbit R2100/R2101). Polyclonal sera were subsequently affinity purified against the GST fusion proteins.

SBP pull-down in DT40 cells

INCENP$^{ON/OFF}$ cells expressing exogenous INCENP (TrAP-INCENP^{WT}, TrAP-INCENP^{W766G}, TrAP-INCENP^{F802A}, and TrAP-INCENP^{TS414AS815A}) constructs were incubated with doxycycline for 16 h to shut off expression of the endogenous promoter-hijacked INCENP locus. At t = 16 h, nocodazole was added for a further 10 h to increase the mitotic index to 40–70%. To prepare lysates, 50–80 × 10^6 DT40 cells were harvested, washed twice with PBS, and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 1% NP-40, 0.5% deoxycholate, 1 mM PMSF, 20 mM β-mercaptoethanol, and 0.3 mM Na vanadate; Honda et al., 2003) supplemented with the protease inhibitors 1 mM PMSF (Sigma-Aldrich) and 1 µg/ml CLAP (chymostatin, leupeptin, antipain, pepstatin A; Sigma-Aldrich). After thorough sonication, cellular debris was removed by centrifugation at 20,000 *g* for 20 min at 4°C. Cell lysates were incubated with ImmunoPure-immobilized streptavidin beads (Thermo Fisher Scientific) at 4°C for 1.5 h. Beads were washed twice in lysis buffer, once in wash buffer (50 mM Tris-HCl, 0.2% NP-40, 0.4 M NaCl, and 0.2 mM PMSF), and the final wash was performed in Tris buffer (50 mM Tris-HCl, 0.4 M NaCl, and 0.1 mM PMSF). The beads were finally boiled in Laemmli sample buffer. All samples were subjected to SDS-PAGE and analyzed by immunoblotting.

Indirect immunofluorescence microscopy

Cells were incubated at 39°C on polylysine-coated slides (Polysine; VWR International) for 15 min before fixation in 4% PFA in cytoskeleton buffer (137 mM NaCl, 5 mM KCl, 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM EGTA, 5 mM Pipes, and 5.5 mM glucose; Yue et al., 2008) and permeabilization in 0.15% Triton X-100 in CB buffer. Both steps were performed at 37°C. After blocking in 1% BSA in PBS, cells were probed with the aforementioned antibodies, and slides were mounted using Vectashield containing DAPI (Vector Laboratories). For immunofluoresence, 3D datasets were acquired at room temperature using a cooled charge-coupled device camera (CH350; Photometrics) on a widefield microscope (Deltavision Spectris; Applied Precision) with 100×/1.4 NA Plan Apo lens. 3D datasets were acquired and subsequently deconvolved using a constrained iterative algorithm implemented in SoftWoRx software (Applied Precision).

Time-lapse imaging

INCENP$^{ON/OFF}$ cells stably expressing histone H2BRFP were treated with 9 µM (Cdk inhibitor) RO3306 for 6 h to allow enrichment of cells in G2. Data acquisition started immediately after release from the drug. Cells were seeded on concanavalin A–treated coverslips, assembled into Rose chamber, and maintained at 39°C with Leibovitz's L-15 medium (Invitrogen) supplemented with 10% FBS and 1% chicken serum. Differential interference contrast and RFP 3D image datasets were collected every 2 min using MetaMorph software (MDS Analytical Technologies) on a fluorescence microscope (Eclipse TE2000-E; Nikon) with a 100×/1.2 NA objective equipped with a high speed camera (CoolSnapHQ; Photometrics). Entry into mitosis and anaphase onset of individual cells were scored based on the chromosome morphology.

Site-directed mutagenesis

INCENP point mutants were generated by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis kit; Agilent Technologies) using the plasmid pTrAP-GgINCENP class I and transferred into the knockout cells by electroporation. The following primer sequences were used: W766G (forward), 5′-CCATCCCTGCCGGGGCCAGTGGG-3′ and (reverse) 5′-CCCACTGGCCCCGGCAGGGATGG-3′; F802A (forward), 5′-CCCCAAGTTGGAGGACATCGCTTACAAAAGCAAACCACGC-3′ and (reverse) 5′-GCGTGGTTTGCTTTTGTAAGCGATGTCCTCCAACTTGGGG-3′. Stable transformants were selected by 400 µg/ml Zeocin. Stable knockout lines homogeneously expressing the TrAP-tagged fusion protein at levels comparable with the endogenous INCENP in wild-type cells were isolated and kept at 39°C.

Quantitative analysis

Growth curves were generated by seeding the various cell lines at 2 × 10^5 cells/ml at 39°C (unless indicated otherwise) and counting the cell number every 12 h until 48 h. To avoid the effects of overgrowth, cells were diluted to 4 × 10^5 cells/ml whenever the number exceeded 10^6 cells/ml. The cell number at each time point was multiplied by the appropriate dilution factor to get a true count.

To determine the multinucleation or mitotic index, a total of 500 cells were scored at each time point for each individual experiment ($n > 3$). The multinucleation index was calculated by dividing the number of multinucleated interphase cells by the total number (500) of interphase cells. The mitotic index was calculated by dividing the number of mitotic cells by the number of interphase and mitotic cells.

To assess the distribution of mitotic phases, cells were subject to immunofluorescence using appropriate antibodies (e.g., aurora B, α-tubulin, and γ-tubulin). A total of at least 200 mitotic cells were scored at each time point. The frequency of multipolar spindles was determined in an independent experiment by dividing the number of mitotic cells with more than two spindle poles by the total number of 200 mitotic cells (cells were either stained with aurora B, α-tubulin plus DAPI, or aurora B, γ-tubulin plus DAPI). Each quantitation was performed independently at least three times.

Quantitative immunoblotting

Membranes were first incubated with primary antibodies recognizing aurora B, α-tubulin, H3S10ph, and subsequently with IRDye-labeled secondary antibodies (LI-COR Biosciences). The fluorescence intensities were subsequently determined using a charge-coupled device scanner (Odyssey; LI-COR Biosciences) according to the manufacturer's instructions.

Quantitative real-time RT-PCR

Total RNA was extracted from DT40 cells using TRizol (Invitrogen) following the manufacturer's instructions. Oligo-dT–primed cDNA derived from the total RNA and primers specific for chicken *incenp* (3′ untranslated region) or *incenp-like* (exon 19) were used to determine the expression of *incenp-like* relative to *incenp* in clone 18 cells. Primers used for *incenp* are 5′-GGGCAGTGACATTTTTCAAGGAA-3′ and 5′-TGCAGCCATGAATGCTCACC-3′. Primers used for *incenp-like* are 5′-TAATCAAGCCATTCTGCACCAA-3′ and 5′-GGTGGGGAATTCCAAACTGC-3′.

Spindle checkpoint assay

Exponentially growing cells were treated with 10 or 100 nM taxol/0.5 µg/ml nocodazole/2 µM ZM447439 for 9 h. Cells were plated on polylysine-coated slides, fixed with 4% PFA, and stained by DAPI. A total of 200 cells were scored from each sample to determine mitotic index. Experiments were performed at least three times.

Online supplemental material

Fig. S1 shows that INCENP class I is sufficient to keep DT40 cells alive. Fig. S2 shows distribution of Survivin and Borealin in cells expressing only mutant INCENP. Fig. S3 shows distribution of aurora B and CENP-A and levels of histone H3 phosphorylation in INCENP^{OFF} cells expressing various INCENP mutants. Fig. S4 shows analysis of mitotic parameters in INCENP^{OFF} cells expressing INCENP^{W766G} and INCENP^{F802A}. Fig. S5 shows the effect of aurora B kinase inhibitor ZM447439 on CPC and CENP-E behavior in INCENP^{OFF}/TrAP-INCENP^{WT} cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200906053/DC1.

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