

# Phosphorylation at serine 331 is required for Aurora B activation

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**A**urora B kinase activity is required for successful cell division. In this paper, we show that Aurora B is phosphorylated at serine 331 (Ser331) during mitosis and that phosphorylated Aurora B localizes to kinetochores in prometaphase cells. Chk1 kinase is essential for Ser331 phosphorylation during unperturbed prometaphase or during spindle disruption by taxol but not nocodazole. Phosphorylation at Ser331 is required for optimal phosphorylation of INCENP at TSS residues, for Survivin association with the chromosomal passenger complex, and for complete Aurora B activation, but it is

dispensable for Aurora B localization to centromeres, for autophosphorylation at threonine 232, and for association with INCENP. Overexpression of Aurora B<sup>S331A</sup>, in which Ser331 is mutated to alanine, results in spontaneous chromosome missegregation, cell multinucleation, unstable binding of BubR1 to kinetochores, and impaired mitotic delay in the presence of taxol. We propose that Chk1 phosphorylates Aurora B at Ser331 to fully induce Aurora B kinase activity. These results indicate that phosphorylation at Ser331 is an essential mechanism for Aurora B activation.

## Introduction

The conserved kinase Aurora B is an important regulator of mitotic cell division (Carmena et al., 2009). Aurora B forms the catalytic core of the chromosomal passenger complex (CPC), which includes the regulatory proteins INCENP, Survivin, and Borealin (Carmena et al., 2009). The CPC associates with the centromere from prophase until metaphase and transfers to the spindle midzone in anaphase and the midbody in late cytokinesis, and Aurora B localization requires all three regulatory subunits of the CPC (Carvalho et al., 2003; Honda et al., 2003; Jeyaprasath et al., 2007). Furthermore, Survivin directly interacts with Aurora B and the N terminus of INCENP and mediates targeting of the CPC to chromosomes (Wheatley et al., 2001; Vader et al., 2006; Kelly et al., 2010).

Aurora B has a well-established function in promoting release of misattached kinetochore microtubules until bipolar attachment is achieved (Tanaka et al., 2002; Liu et al., 2009). Furthermore, Aurora B is required for cleavage furrow ingression (Fuller et al., 2008), and impaired Aurora B activity correlates with chromosome misalignment in metaphase, missegregated chromosomes during anaphase, and failure of cytokinesis (Adams et al., 2001; Ditchfield et al., 2003).

Aurora B is also involved in the spindle checkpoint, a surveillance mechanism that delays anaphase until all chromosomes are correctly bioriented; however, its precise role is a matter of active investigation (Nezi and Musacchio, 2009). In budding yeast, Ipl1/Aurora is required for spindle checkpoint function in response to a lack of tension across attached kinetochores (Biggins and Murray, 2001; Cheeseman et al., 2002). In higher eukaryotic cells, catalytic activity of Aurora B is required for recruitment of checkpoint protein BubR1 to kinetochores and sustained mitotic arrest in the absence of tension (Ditchfield et al., 2003; Lampson and Kapoor, 2004). Furthermore, recent studies in yeast (Pinsky et al., 2009; Vanoosthuyse and Hardwick, 2009) and vertebrate cells (Maldonado and Kapoor, 2011; Santaguida et al., 2011; Saurin et al., 2011) have shown that potent inhibition of Aurora B activity weakens the mitotic arrest in the presence of many unattached kinetochores.

Aurora B is associated with INCENP throughout the cell cycle; however, Aurora B kinase activity peaks in mitosis (Bolton et al., 2002). Activation of Aurora B occurs through a two-step mechanism: in the first step, Aurora B binds to the

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Abbreviation used in this paper: CPC, chromosomal passenger complex.

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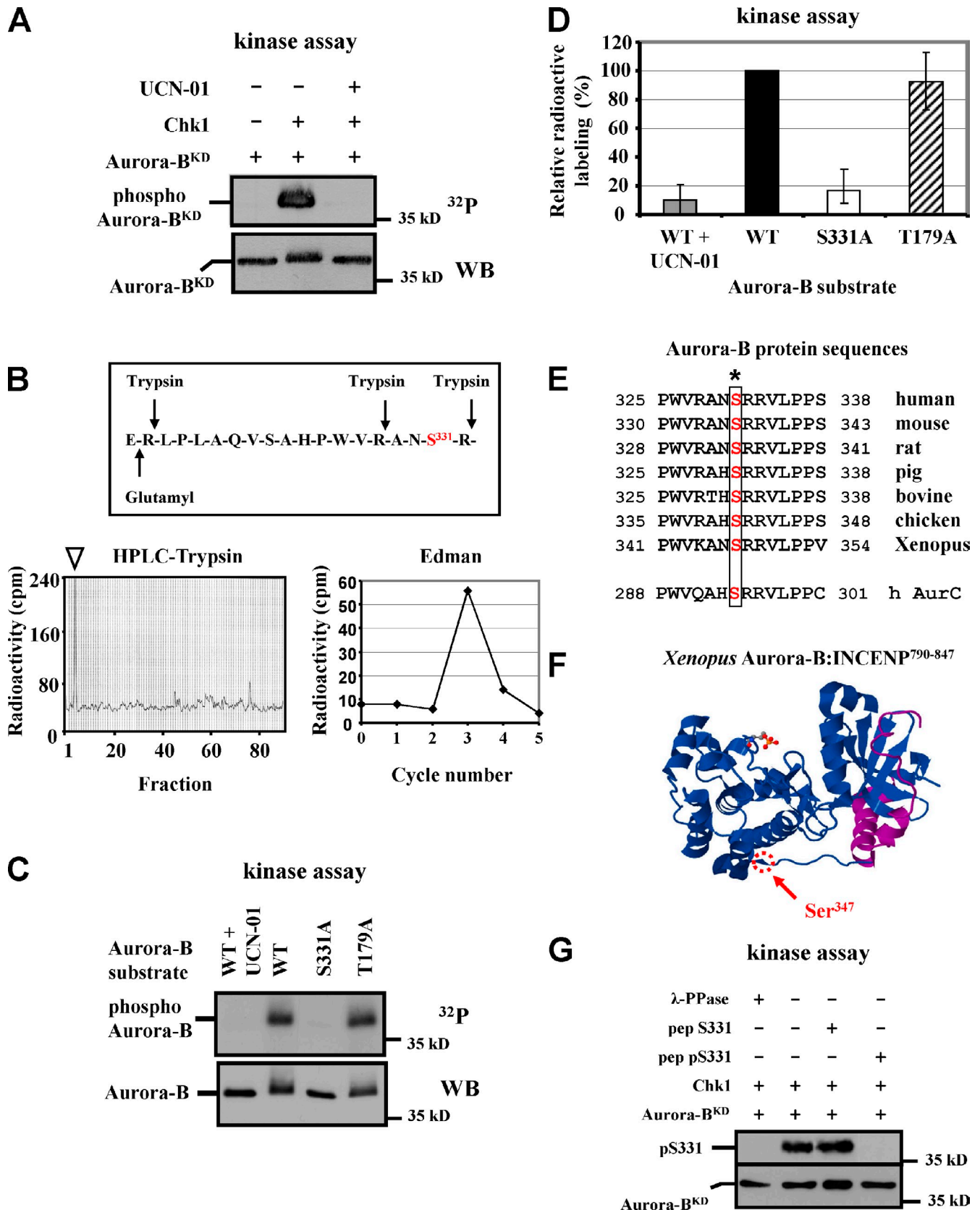


Figure 1. Chk1 phosphorylates Aurora B at Ser331. (A) Chk1 in vitro kinase assay. (top) Autoradiography analysis (<sup>32</sup>P) of phosphorylated (phospho) Aurora B<sup>KD</sup>. (bottom) Western blot (WB) analysis of total Aurora B<sup>KD</sup>. (B) Mapping the Chk1 phosphorylation site. (top) Amino acids inclusive and surrounding Ser331 (S<sup>331</sup>) showing N terminal trypsin and glutamyl endopeptidase cleavage sites. (bottom left) HPLC analysis of radiolabeled Aurora B<sup>KD</sup> after digestion with trypsin. The main radioactive fraction is indicated by an arrowhead. (bottom right) Edman degradation analysis of the phosphopeptide from the main radioactive fraction. The graph shows radioactivity released from the membrane. (C) Chk1 in vitro kinase assay using purified proteins Aurora B<sup>WT</sup>

C-terminal IN box sequence of INCENP spanning amino acids 822–900 (human numbering) and is autophosphorylated at threonine 232 (Thr232) within its activation loop (Honda et al., 2003; Yasui et al., 2004). This step represents an intermediate state of Aurora B activation in which the Aurora B C-terminal tail stabilizes an open conformation of the catalytic cleft (Sessa et al., 2005). In the second step, Aurora B phosphorylates INCENP in trans at two adjacent serine residues of the conserved TSS sequence (Bishop and Schumacher, 2002; Honda et al., 2003). Phosphorylation of the TSS motif and release of the Aurora B C-terminal tail through an undescribed mechanism generate the fully active kinase (Sessa et al., 2005). TD-60, microtubules, and priming phosphorylation of Aurora B substrates by Plk1 and haspin catalyze the first step of Aurora B activation in vitro (Rosasco-Nitcher et al., 2008). Furthermore, local clustering at chromosomes stimulates Aurora B autoactivation, and Mps1 kinase phosphorylates Borealin to enhance Aurora B activity by an unknown mechanism (Kelly et al., 2007; Jelluma et al., 2008).

Chk1 kinase is a well-established component in the DNA damage and DNA replication checkpoints (Smith et al., 2010). Furthermore, Chk1 protects cells against spontaneous chromosome missegregation and is required for sustained mitotic arrest in the presence of taxol, a drug that dampens microtubule dynamics and primarily interferes with kinetochore tension but not when microtubules are completely depolymerized by nocodazole (Zachos et al., 2007; Peddibhotla et al., 2009). Spindle checkpoint failure in Chk1-deficient cells is associated with impaired localization of BubR1 to kinetochores and reduced Aurora B kinase activity. However, the mechanism of Aurora B regulation by Chk1 has not been previously described (Zachos et al., 2007).

In the present study, we identify the conserved residue serine 331 (Ser331) of human Aurora B as the Chk1 phosphorylation site in vitro and, using a phosphospecific antibody raised against this site, we show that Chk1 is required for Ser331 phosphorylation during unperturbed prometaphase or spindle disruption by taxol but not during prophase, cytokinesis, or treatment of cells with nocodazole. Furthermore, using cells overexpressing wild-type or nonphosphorylatable S331A mutant Aurora B, we propose that Ser331 phosphorylation is required for optimal phosphorylation of INCENP at the TSS motif, Survivin association with the CPC, and complete Aurora B activation but not for Aurora B localization to centromeres, phosphorylation at Thr232, or association with INCENP. In addition, overexpression of S331A Aurora B results in spontaneous chromosome missegregation, cell multinucleation, defective accumulation of BubR1 at kinetochores, and impaired mitotic delay in response to taxol. On the basis of these findings, we propose that Ser331 phosphorylation by Chk1 is an essential mechanism for Aurora B activation.

## Results

### Chk1 phosphorylates Aurora B at Ser331 in vitro

Chk1 phosphorylated kinase-dead human Aurora B (Aurora B<sup>KD</sup>) in vitro, and this phosphorylation was abolished in the presence of the selective Chk1 inhibitor UCN-01 (Fig. 1 A). To map the Chk1 phosphoacceptor sites, Aurora B<sup>KD</sup> was radiolabeled by Chk1 and digested with Trypsin, and the resulting peptides were resolved by HPLC (Fig. 1 B). The phosphopeptide from the main radioactive fraction was subjected to Edman degradation, and radioactivity was released after three cycles (Fig. 1 B). This process was repeated after digestion of radiolabeled Aurora B<sup>KD</sup> with glutamyl endopeptidase and resulted in a main radioactive fraction that released radioactivity after 17 cycles (Fig. S1, A and B). Ser331 is the only residue on human Aurora B consistent with both phosphorylation patterns. Therefore, our results suggest Ser331 is the main Chk1 phosphorylation site in vitro (Fig. 1 B).

To verify this, bacterially expressed wild-type Aurora B (Aurora B<sup>WT</sup>), Aurora B<sup>S331A</sup> harboring a nonphosphorylatable mutation of Ser331 to alanine (S331A), or Aurora B<sup>T179A</sup>, in which threonine 179 was changed to alanine (negative control) was used as a substrate in Chk1 in vitro kinase assays. Mutation of Ser331 to alanine or inhibition of Chk1 activity by UCN-01 markedly reduced substrate phosphorylation compared with Aurora B<sup>WT</sup> (Fig. 1, C and D). In comparison, phosphorylation of Aurora B<sup>T179A</sup> by Chk1 was similar to Aurora B<sup>WT</sup> (Fig. 1, C and D). These results show that Chk1 phosphorylates human Aurora B at Ser331 in vitro.

Multiple sequence alignment demonstrated that Ser331 is conserved in Aurora B proteins from different species and also in human Aurora C, an Aurora parologue that exhibits structural properties similar to Aurora B and can perform related mitotic functions in the absence of Aurora B (Fig. 1 E; Han et al., 2007; Slattery et al., 2009). Ser331 is located at the foot of the Aurora B C-terminal tail, which interacts with the IN box of INCENP in the partially active complex (Fig. 1 F; Sessa et al., 2005).

To further investigate Ser331 phosphorylation, an antiphospho-Ser331 (anti-pS331) antiserum was raised against the human protein sequence. As shown in Fig. 1 G, this antiserum recognized Aurora B<sup>KD</sup> phosphorylated by Chk1 in vitro, and immunoreactivity was abolished after substrate treatment with  $\lambda$  protein phosphatase (Fig. 1 G,  $\lambda$ -PPase) or incubation of the antiserum with the phosphorylated (Fig. 1 G, pep pS331) but not the unphosphorylated (Fig. 1 G, pep S331) synthetic peptides.

(wild type [WT]), Aurora B<sup>S331A</sup> (S331A), or Aurora B<sup>T179A</sup> (T179A) as substrates. (top) Autoradiography analysis. (bottom) Western blot analysis of the reactions. (D) Densitometric analysis of Chk1 in vitro kinase assay from C. Radioactive labeling of the wild-type protein was plotted as 100%. Error bars show the standard deviation from the mean from three independent experiments. (E) Alignment of Aurora B and human Aurora C (h AurC) protein sequences. Ser331 is marked by asterisk. (F) View of *Xenopus* Aurora B–INCENP<sup>790–847</sup> (blue and magenta ribbons, respectively) in complex with hesperadine (ball and stick model). Ser<sup>347</sup> of *Xenopus* Aurora B (equivalent of human Ser331) is circled and indicated by an arrow. (G) Chk1 in vitro kinase assay. Western blot analysis of phosphorylated (pS331) and total Aurora B<sup>KD</sup> using the anti-pS331 antiserum.  $\lambda$ -PPase,  $\lambda$  protein phosphatase; pep S331, unphosphorylated Ser331 synthetic peptide; pep pS331, phospho-S331 synthetic peptide.

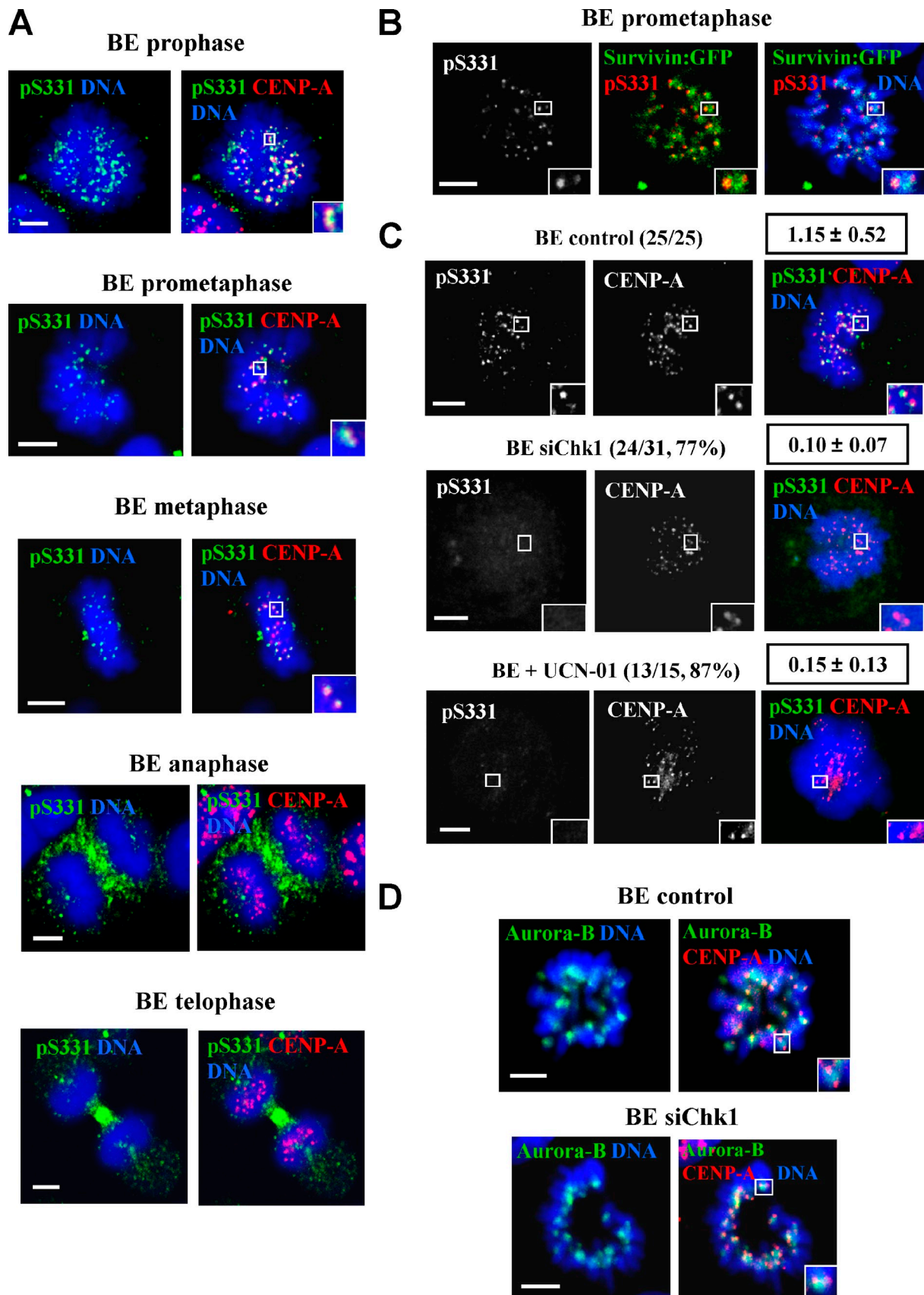


Figure 2. **Chk1 is required for Ser331 phosphorylation during unperturbed prometaphase.** (A) Ser331 phosphorylation (pS331) in BE cells during unperturbed mitosis. (B) Localization of pS331 and Survivin-GFP. (C) BE cells were transfected with negative siRNA (control) and Chk1 siRNA (siChk1) or treated with UCN-01 for 16 h. The frequency of cells exhibiting the respective phenotype and mean pS331/CENP-A fluorescence intensity values (boxed numbers) are shown. (D) Localization of total Aurora B in BE cells transfected as in C. Insets show magnified kinetochores. Bars, 5  $\mu$ m.

Table I. pS331 fluorescence intensity values at prometaphase kinetochores

Treatment	BE control		BE siChk1		P-values
	Fluorescence intensity (pS331/CENP-A)	n (kinetochore pairs, cells)	Fluorescence intensity (pS331/CENP-A)	n (kinetochore pairs, cells)	
Unperturbed	1.15 ± 0.52	196, 12	0.10 ± 0.07	80, 12	P < 0.01
Taxol	0.87 ± 0.43	188, 12	0.08 ± 0.06	174, 12	P < 0.01
Nocodazole	0.88 ± 0.45	162, 12	0.92 ± 0.44	193, 12	P = 0.76

Mean fluorescence intensity values are normalized to CENP-A staining, and *n* represents the number of kinetochore pairs quantified followed by the number of cells analyzed. The p-values were calculated using the Student's *t* test.

### Aurora B is phosphorylated at Ser331 in mitosis

Confocal microscopy analysis of BE human colon carcinoma cells during unperturbed prometaphase showed that pS331 Aurora B was juxtaposed to CENP-A, near kinetochores (Fig. 2 A), whereas total Aurora B localized along the extended centromere as previously described (Fig. 2 D; Cooke et al., 1987). Importantly, pS331 staining was only detectable in the presence of the protein phosphatase inhibitor microcystin, thus suggesting that pS331 is sensitive to phosphatase activity (Gorbsky and Ricketts, 1993). Furthermore, pS331 localized to kinetochores during prophase and metaphase, to the midzone in anaphase and the midbody in telophase and late cytokinesis (Fig. 2 A). In addition, BE cells transiently expressing Survivin fused to GFP (Survivin-GFP) exhibited pS331 juxtaposed to Survivin-GFP, near kinetochores (Fig. 2 B). Depletion of Aurora B expression by transient transfection of BE cells with Aurora B siRNA (Fig. S1 C, siAurora B) impaired pS331 staining at prometaphase kinetochores (Fig. S1 D). In some cells, pS331 staining was also detectable at centrosomes; however, this signal persisted after depletion of Aurora B by siRNA and was likely spurious (Fig. S1 E; Posch et al., 2010). Furthermore, pS331 kinetochore staining was reduced after incubation of the anti-pS331 antiserum with the phosphorylated peptide pS331 compared with the unphosphorylated peptide S331 synthetic peptides (Fig. S1 F). These results show that pS331 localizes to kinetochores in unperturbed prometaphase, the midzone in anaphase, and the midbody in telophase and cytokinesis.

### Chk1 is required for Ser331 phosphorylation during unperturbed prometaphase

To investigate the role of Chk1 for Ser331 phosphorylation, BE cells transiently transfected with negative siRNA (control), Chk1 siRNA (siChk1), or treated with UCN-01 were analyzed by confocal microscopy (Figs. 2 C and S2 A). Depletion of Chk1 or inhibition of Chk1 kinase activity by UCN-01 diminished pS331 staining at prometaphase kinetochores as indicated by reduced pS331/CENP-A fluorescence intensity compared with controls (Fig. 2 C and Tables I and S1). Significantly, impaired pS331 staining after Chk1 depletion was not caused by reduced levels of Aurora B expression (Fig. S2 B). Furthermore, total Aurora B localized to centromeres in all control (22/22) and Chk1-depleted (20/20) cells examined in prometaphase (Fig. 2 D). These results show that Chk1 activity is required for optimal phosphorylation of Aurora B at Ser331 during

unperturbed prometaphase. Remarkably, depletion of Chk1 did not detectably reduce pS331 staining compared with controls in prophase or cytokinesis (Fig. S2, C and D).

### Chk1 is required for Ser331 phosphorylation in the presence of taxol

Treatment with taxol or nocodazole activates the spindle checkpoint in checkpoint-proficient cells (Zachos et al., 2007). In the presence of taxol, Chk1-depleted BE cells exhibited impaired pS331 staining at prometaphase kinetochores compared with controls (Fig. 3 A and Table I). In contrast, after treatment with nocodazole, Chk1-depleted and control BE cells exhibited similar levels of pS331 at kinetochores (Fig. 3 B and Table I). Importantly, total Aurora B localized to centromeres in the presence of taxol or nocodazole in all control (17/17) and Chk1-depleted (20/20) cells examined in prometaphase (Fig. 3 C). Collectively, these results show that Chk1 is required for Ser331 phosphorylation in the presence of taxol but not nocodazole.

### Generation of CHO<sup>WT</sup> and CHO<sup>S331A</sup> cell lines

To investigate the significance of Ser331 phosphorylation for Aurora B functions, CHO cells expressing 6xMyc-tagged Aurora B<sup>WT</sup> (CHO<sup>WT</sup>) or Aurora B<sup>S331A</sup> (CHO<sup>S331A</sup>) under control of a tetracycline-induced promoter were generated. Addition of tetracycline for 8–24 h stimulated accumulation of 6xMyc–Aurora B<sup>WT</sup> and 6xMyc–Aurora B<sup>S331A</sup> at approximate levels 10-fold higher than the endogenous protein (Figs. 3 D and S2 E). This level of expression was selected to disrupt endogenous Aurora B functions while maintaining correct localization of wild-type 6xMyc–Aurora B to centromeres (Ditchfield et al., 2003). After induction with tetracycline, 6xMyc–Aurora B<sup>WT</sup> and 6xMyc–Aurora B<sup>S331A</sup> proteins localized to centromeres during unperturbed prometaphase in all (30/30) cells examined (Fig. 3 E). Similar results were obtained in the presence of taxol or nocodazole (Fig. 3 F) in all (30/30) cells examined. Please note that CENP-B localizes to sister kinetochores in CHO cells (Cooke et al., 1990; Wordeman and Mitchison, 1995). Furthermore, CHO cells expressing wild-type, but not the S331A mutant, Aurora B exhibited pS331 staining (Figs. 4 A and S2 F), and pS331 was juxtaposed to CENP-A–GFP in prometaphase (Fig. 4 A).

### Phosphorylation at Ser331 is required for optimal Aurora B kinase activity

To investigate the significance of Ser331 phosphorylation for Aurora B catalytic activity, CHO<sup>WT</sup> or CHO<sup>S331A</sup> cells induced with tetracycline were treated with taxol or nocodazole, and

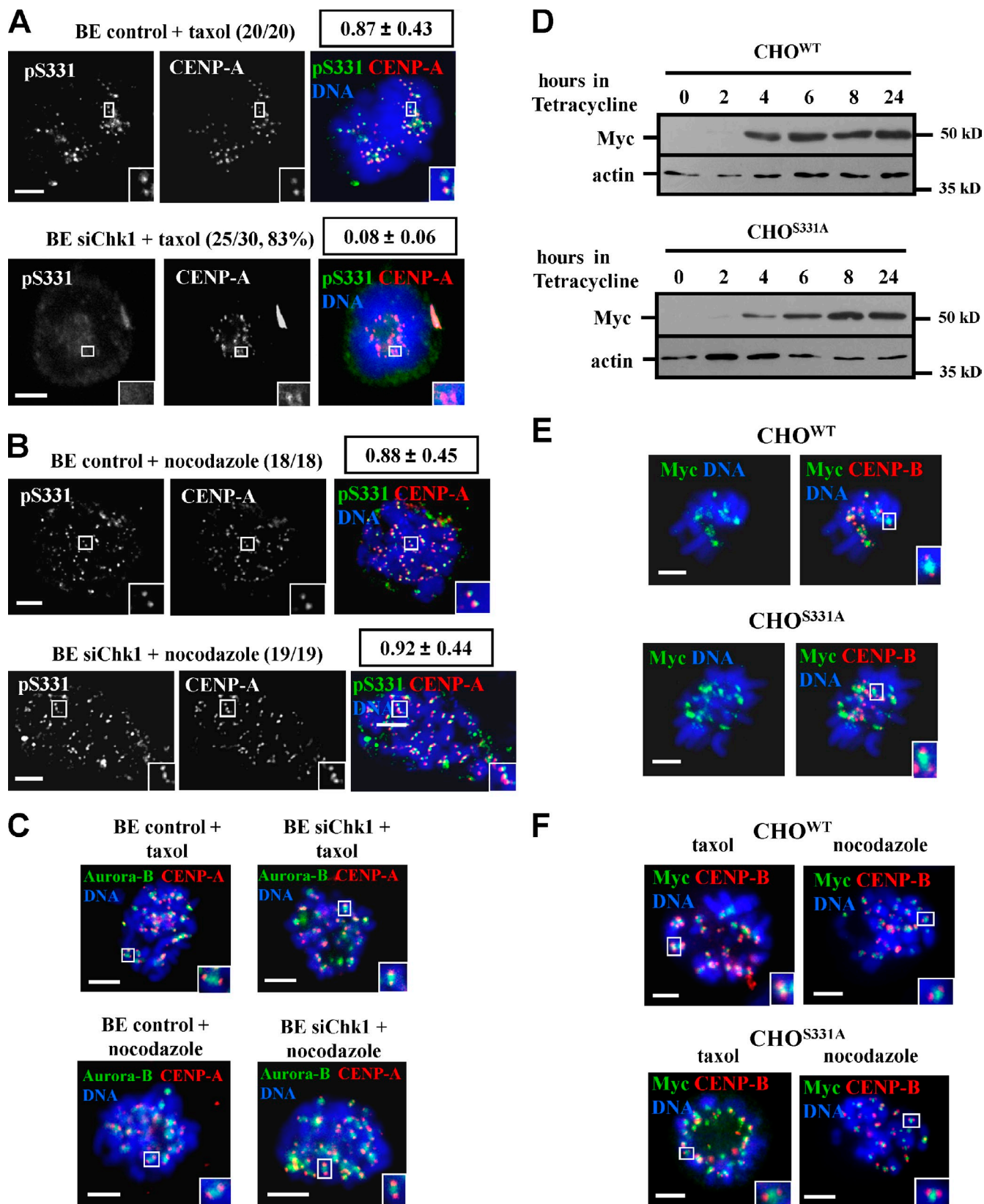
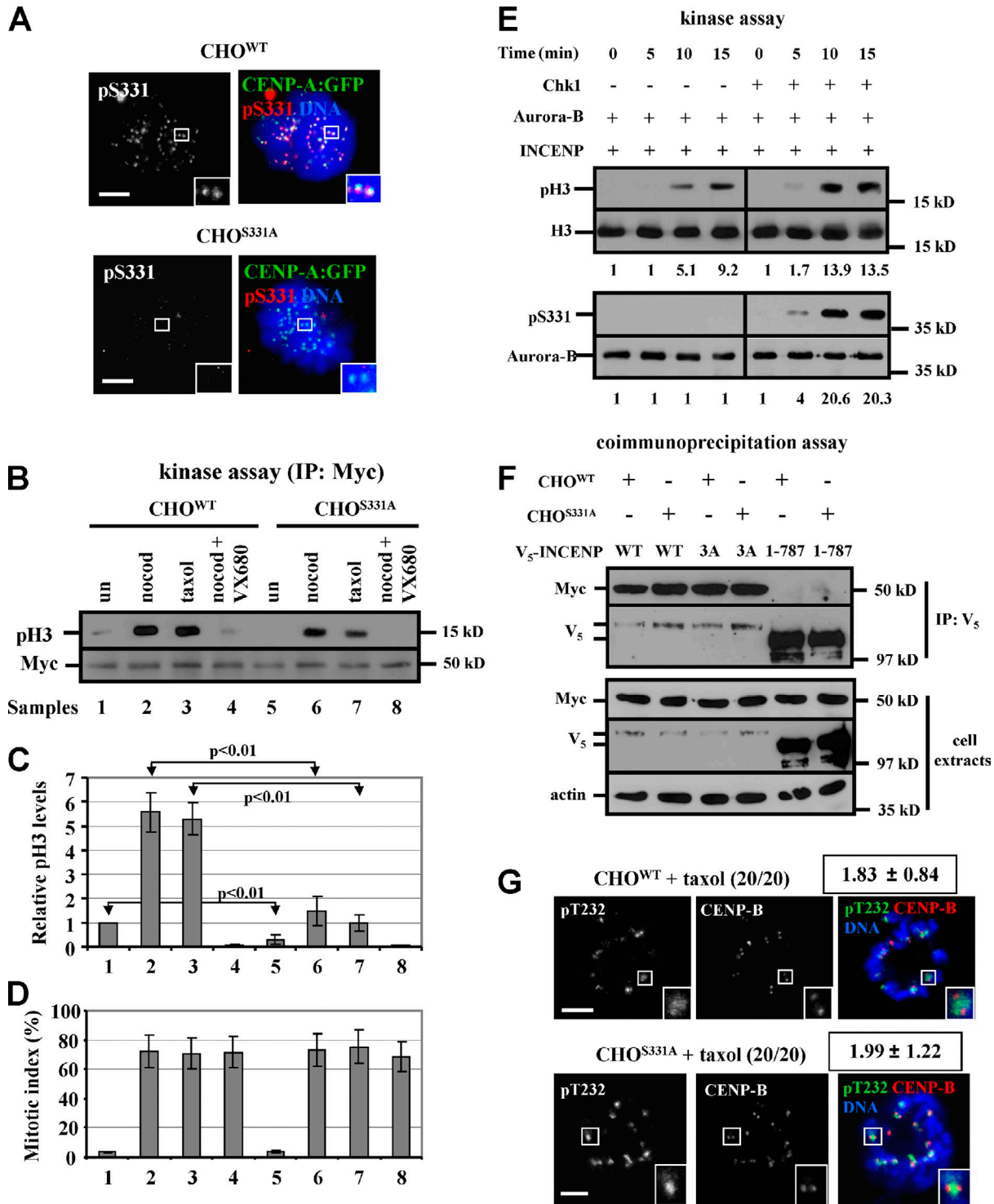


Figure 3. Chk1 is required for Ser331 phosphorylation in the presence of taxol. (A and B) BE cells transfected with negative siRNA (control) or Chk1 siRNA (siChk1) were treated with taxol (A) or nocodazole (B) for 4 h. The frequency of cells exhibiting the respective phenotype and mean pS331/CENP-A fluorescence intensity values (boxed numbers) are shown. (C) Localization of total Aurora B. BE cells were transfected as in A and treated with taxol or nocodazole for 4 h. (D) Western blot analysis of 6×Myc–Aurora B (Myc) and actin in CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells after induction with tetracycline. (E and F) Localization of 6×Myc–Aurora B proteins. Tetracycline-induced CHO<sup>WT</sup> or CHO<sup>S331A</sup> cells were untreated (E) or treated with taxol or nocodazole (F) for 4 h. Insets show magnified kinetochores. Bars, 5 μm.



**Figure 4. Phosphorylation of Ser331 is required for complete Aurora B activation.** (A) Phosphorylation of Ser331 (pS331) during unperturbed prometaphase. CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells expressing CENP-A-GFP were induced with tetracycline. (B) Immunoprecipitation kinase assay. CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells induced with tetracycline were untreated (un) or treated with nocodazole (nocod) or taxol for 8 h in the absence or presence of VX-680. (top) Western blot analysis of Myc-associated phosphorylation of Ser10 of histone H3 (pH3). (bottom) Western blot of immunoprecipitated (IP) Myc. (C) Densitometric analysis of pH3 levels from B. pH3 levels in sample 1 were arbitrarily set to 1. Error bars show the standard deviation from the mean from three independent experiments. The p-values were calculated using the Student's *t* test. (D) Mitotic index of each sample from B at the time of harvesting (untreated or shake-off cells). (E) In vitro kinase assay. Complexes of purified Chk1, Aurora B, and GST-INCENP<sup>826-919</sup> were incubated with histone H3, and pH3 (top) or pS331 activities (bottom) were determined. Values show the relative levels of pH3 or pS331, and levels at 0 min were arbitrarily set to 1. Western blot analysis of total H3 and Aurora B is also shown. Black lines indicate that intervening lanes have been spliced out. (F) Coimmunoprecipitation assay. (top) Western blot analysis of immunoprecipitated (IP) 6xMyc-Aurora B (Myc) and V<sub>5</sub>-INCENP (V<sub>5</sub>) after induction of CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells with tetracycline. (bottom) Western blot analysis of total Myc, V<sub>5</sub>, and actin. (G) Phosphorylation of Thr232 (pT232). CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells were induced with tetracycline and treated with taxol for 4 h. The frequency of cells exhibiting the respective phenotype and mean pT232/CENP-B fluorescence intensity values (boxed numbers) are shown. Insets show magnified kinetochores. WT, wild type. Bars, 5 μm.

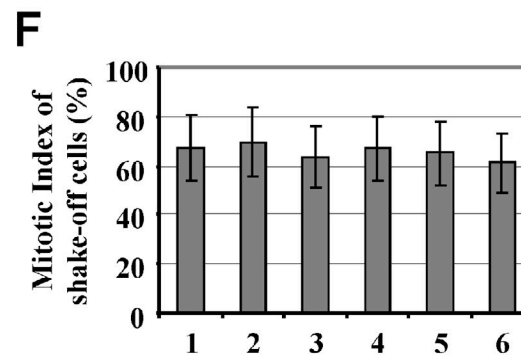
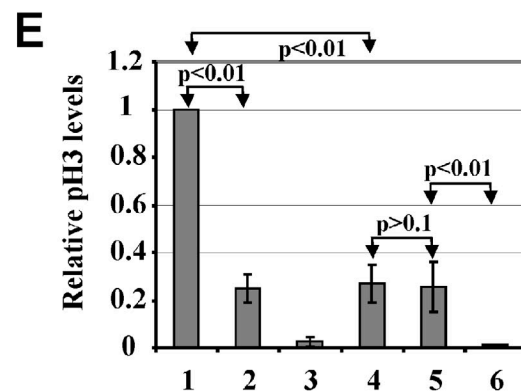
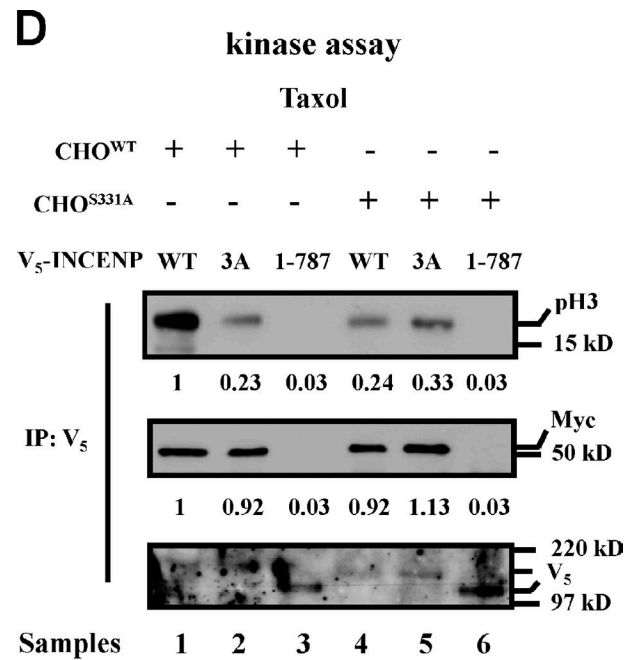
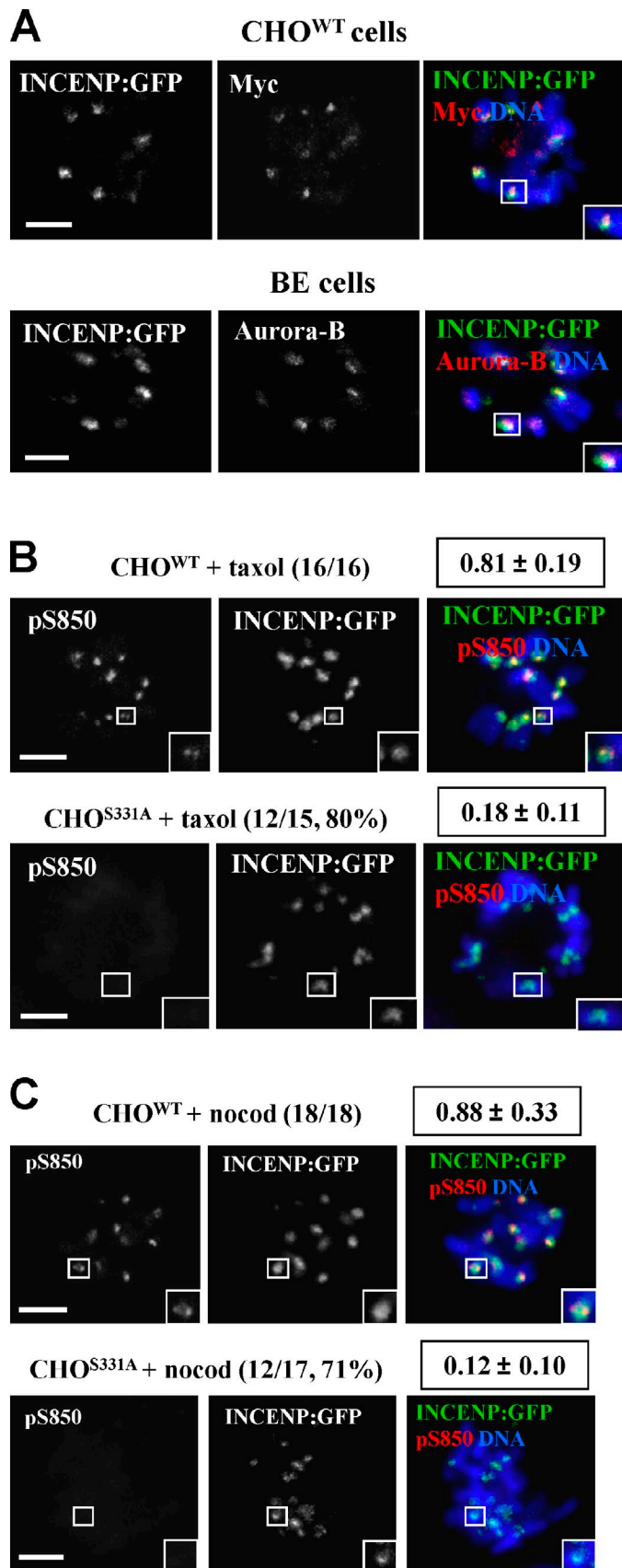


Figure 5. Phosphorylation of Ser331 is required for TSS phosphorylation. (A) Localization of INCENP-GFP. (top) CHO<sup>WT</sup> cells expressing INCENP-GFP were induced with tetracycline. (bottom) BE cells expressing INCENP-GFP. (B and C) Phosphorylation of INCENP at Ser850 (pS850). CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells expressing INCENP-GFP were induced with tetracycline and treated with taxol (B) or nocodazole (nocod, C) for 4 h. The frequency of cells



Table II. **pS850 fluorescence intensity values at prometaphase kinetochores**

Treatment	CHO <sup>WT</sup> cells		CHO <sup>S331A</sup> cells		P-values
	Fluorescence intensity (pS850/GFP)	n (kinetochore pairs, cells)	Fluorescence intensity (pS850/GFP)	n (kinetochore pairs, cells)	
Taxol	0.81 ± 0.19	89, 10	0.18 ± 0.11	81, 10	P < 0.01
Nocodazole	0.88 ± 0.33	88, 11	0.12 ± 0.10	74, 10	P < 0.01

Mean fluorescence intensity values are normalized to GFP staining, and *n* represents the number of kinetochore pairs quantified followed by the number of cells analyzed. The p-values were calculated using the Student's *t* test.

mitotic cells were selectively isolated by shake off or left untreated and harvested without shake off. Cell extracts were analyzed for Myc-associated kinase activity against histone H3 serine 10, a physiological substrate of Aurora B, by immunoprecipitation kinase assays (Adams et al., 2001). As shown in Fig. 4 B and quantified in Fig. 4 C, treatment with taxol or nocodazole induced H3 serine 10 phosphorylation (pH3) compared with untreated cells. However, pH3 activity was decreased in CHO<sup>S331A</sup> compared with CHO<sup>WT</sup> cells (Fig. 4, B and C) despite similar mitotic indices in the respective samples (untreated or shake-off cells) in both cell lines (Fig. 4 D). In comparison, VX-680 almost completely inhibited pH3 activity (Fig. 4, B and C).

Furthermore, we analyzed the ability of Chk1 to enhance Aurora B catalytic activity, indicated by pH3, in vitro (Zachos et al., 2007). Chk1 stimulated pH3 activity in the presence or absence of GST-INCENP<sup>826-919</sup>, and this coincided with Aurora B phosphorylation at Ser331 (Figs. 4 E and S3 A). Collectively, these results suggest that Ser331 phosphorylation is required for optimal Aurora B activation. Mutation of Ser331 to aspartate did not restore Aurora B kinase activity in immunoprecipitation kinase assays, thus suggesting that this mutation doesn't effectively mimic Ser331 phosphorylation in this case (unpublished data).

### Ser331 phosphorylation is not essential for Aurora B binding to INCENP or Thr232 phosphorylation

Binding to INCENP and phosphorylation at Thr232 stimulate Aurora B activity. To investigate the significance of Ser331 phosphorylation for Aurora B association with INCENP, CHO<sup>WT</sup>, or CHO<sup>S331A</sup>, cells transiently expressing V<sub>5</sub>-tagged human INCENP were induced with tetracycline, V<sub>5</sub>-INCENP was precipitated from cell extracts using an antibody against V<sub>5</sub>, and associated 6xMyc-Aurora B was detected by Western blotting against Myc. As shown in Fig. 4 F, similar levels of wild-type or S331A Aurora B precipitated with wild-type V<sub>5</sub>-INCENP<sup>WT</sup> or mutant V<sub>5</sub>-INCENP<sup>3A</sup>, in which the TSS motif was changed to AAA (Honda et al., 2003). In comparison, 6xMyc-Aurora B proteins did not associate with truncated V<sub>5</sub>-INCENP<sup>1-787</sup> lacking the

C-terminal segment for binding to Aurora B (Fig. 4 F). Furthermore, similar levels of wild-type or S331A Aurora B precipitated with V<sub>5</sub>-INCENP<sup>WT</sup> after treatment of cells with taxol or nocodazole (Fig. S3 B). These results suggest that phosphorylation at Ser331 is not required for Aurora B association with INCENP.

Furthermore, CHO cells expressing wild-type or S331A Aurora B exhibited similar levels of phospho-Thr232 (pT232) Aurora B at centromeres in the presence of taxol (Fig. 4 G and Table S1) or nocodazole (Fig. S3 C and Table S1) as determined by pT232/CENP-B fluorescence intensity levels. Collectively, these results suggest that phosphorylation at Ser331 is not required for Aurora B binding to INCENP or phosphorylation at Thr232.

### Ser331 phosphorylation is required for INCENP phosphorylation at TSS residues

Phosphorylation of the TSS motif is required for full Aurora B activation. To investigate phosphorylation at TSS residues, CHO<sup>WT</sup>, CHO<sup>S331A</sup>, or BE cells transiently expressing *Xenopus laevis* INCENP fused to GFP (INCENP-GFP) were examined for phosphorylation at Ser850 (pS850) inside the *Xenopus* TSS motif by confocal microscopy (Knowlton et al., 2006). INCENP-GFP colocalized with 6xMyc-Aurora B in CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells and also with endogenous Aurora B in BE cells (Fig. 5 A and not depicted).

After induction with tetracycline and treatment with taxol or nocodazole, CHO<sup>WT</sup> prometaphase cells exhibited pS850 staining, and pS850 partially colocalized with INCENP-GFP near kinetochores (Fig. 5, B and C). In contrast, CHO<sup>S331A</sup> cells exhibited diminished pS850 staining compared with controls as shown by pS850/INCENP-GFP fluorescence intensity (Fig. 5, B and C; and Table II).

Furthermore, BE cells expressing INCENP-GFP and treated with UCN-01 exhibited reduced pS850 staining compared with controls in the presence of taxol (Fig. S3 D and Table S1). Remarkably, in the presence of nocodazole, both control and UCN-01-treated BE cells exhibited similar levels of pS850 staining (Fig. S3 E and Table S1), and this is consistent with Chk1 being required for Ser331 phosphorylation in

exhibiting the respective phenotype and mean pS850/INCENP-GFP fluorescence intensity values (boxed numbers) are shown. (D) Immunoprecipitation kinase assay. CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells expressing V<sub>5</sub>-INCENP were induced with tetracycline and treated with taxol for 8 h. (top) Western blot analysis of V<sub>5</sub>-associated phosphorylation of Ser10 of histone H3 (pH3). (middle and bottom) Western blot analysis of immunoprecipitated Myc and V<sub>5</sub>. Values show the relative pH3 and Myc levels with levels at sample 1 arbitrarily set to 1. (E) Densitometric analysis of pH3 levels from D. The p-values were calculated using the Student's *t* test. (F) Mitotic index of each sample from D at the time of harvesting. Error bars show the standard deviation from the mean from three independent experiments. Insets show magnified kinetochores. WT, wild type. Bars, 5 μm.

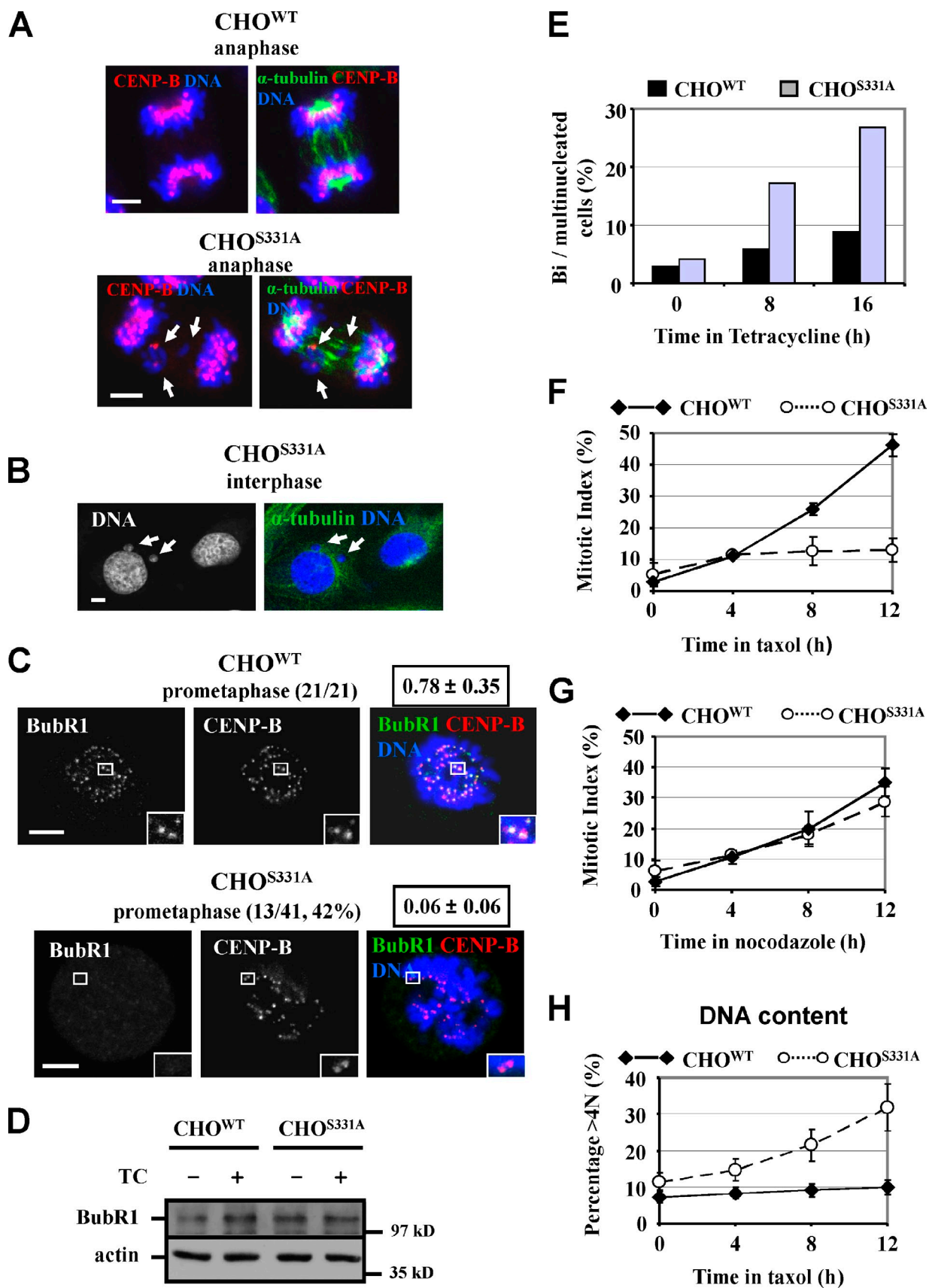


Figure 6. Expression of S331A Aurora B correlates with chromosome missegregation and impaired accumulation of BubR1 at kinetochores. (A–E) CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells were induced with tetracycline. (A) Missegregated chromosomes are indicated by arrows. (B) Micronuclei are indicated by arrows. A single image plane is shown. (C) Localization of BubR1. The frequency of cells exhibiting the respective phenotype and mean BubR1/CENPB fluorescence

the presence of taxol but not nocodazole. Collectively, these results suggest that phosphorylation at Ser331 is required for optimal phosphorylation of INCENP at TSS residues.

#### **Phosphorylations at Ser331 and TSS stimulate complete Aurora B activation**

To further investigate the mechanism of Aurora B activation, CHO<sup>WT</sup> or CHO<sup>S331A</sup> cells transiently expressing V<sub>5</sub>-INCENP were treated with tetracycline and taxol, mitotic cells were selectively isolated by shake off, and V<sub>5</sub>-associated pH3 activity was determined by immunoprecipitation kinase assay. As shown in Fig. 5 D and quantified in Fig. 5 E, cells expressing 6×Myc–Aurora B<sup>WT</sup>/V<sub>5</sub>-INCENP<sup>3A</sup> or 6×Myc–Aurora B<sup>S331A</sup>/V<sub>5</sub>-INCENP<sup>WT</sup> exhibited reduced levels of pH3 activity compared with those expressing the wild-type complex despite similar mitotic indices in shake-off cells in all samples (Fig. 5 F). Significantly, mutation of both Ser331 and TSS to alanine did not further reduce pH3 activity (Fig. 5, D and E), and this is consistent with Ser331 being required for TSS phosphorylation. In comparison, V<sub>5</sub>-associated pH3 activity was almost undetectable in cells expressing truncated V<sub>5</sub>-INCENP<sup>1-787</sup> (Fig. 5, D and E). These results show that phosphorylations at Ser331 and TSS are required for complete Aurora B activation.

#### **Expression of S331A Aurora B correlates with spontaneous chromosome missegregation, impaired accumulation of BubR1 at kinetochores, and cell multinucleation**

Reduced Aurora B activity is associated with defects in chromosome alignment, segregation, and cytokinesis. Microscopic examination of CHO<sup>S331A</sup> cells after induction with tetracycline revealed that 20/50 (40%) of metaphases exhibited misaligned chromatin (Fig. S4 A), and 23/50 (46%) of anaphases showed one or few missegregated chromosomes (Fig. 6 A) compared with only 5/50 (10%) and 4/50 (8%), respectively, in CHO<sup>WT</sup> cells. Missegregated chromosomes can give rise to micronuclei (Gisselsson, 2008). After induction with tetracycline for 16 h, 26/136 (19%) of CHO<sup>S331A</sup> cells in interphase exhibited one or few micronuclei compared with 4/121 (3%) of CHO<sup>WT</sup> cells (Fig. 6 B).

Furthermore, CHO<sup>S331A</sup> cells induced with tetracycline exhibited impaired accumulation of BubR1 at prometaphase kinetochores as shown by reduced BubR1/CENP-B fluorescence intensity compared with controls (Fig. 6 C and Table S1). However, BubR1 levels per se were not diminished by overexpression of Aurora B transgenes (Fig. 6 D).

Incomplete cytokinesis can cause bi- or multinucleation (Xu et al., 2010). In the presence of tetracycline, CHO<sup>S331A</sup> cells exhibit a rapid increase in the bi/multinucleation index compared with CHO<sup>WT</sup> cells (Figs. 6 E and S4 B). Collectively, these

results suggest that phosphorylation of Aurora B at Ser331 is required for optimal chromosome alignment and segregation, accumulation of BubR1 at kinetochores, and successful cytokinesis in the absence of spindle poisons.

#### **Phosphorylation of Ser331 is required for sustained mitotic delay and BubR1 maintenance to kinetochores in the presence of taxol**

Furthermore, treatment with tetracycline and taxol induced accumulation of CHO<sup>WT</sup> cells in mitosis as determined by microscopic examination of condensed chromatin (Fig. 6 F). In contrast, although the proportion of mitotic cells in CHO<sup>S331A</sup> cultures treated with taxol initially increased, this increase was not sustained, and the mitotic index was significantly reduced after 8–12 h compared with CHO<sup>WT</sup> cells (Fig. 6 F). Importantly, CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells treated with tetracycline and nocodazole accumulated in mitosis with similar kinetics (Fig. 6 G). Also, after prolonged treatment with taxol, ~30% of CHO<sup>S331A</sup> cells accumulated DNA content greater than 4N as determined by flow cytometry (Fig. 6 H).

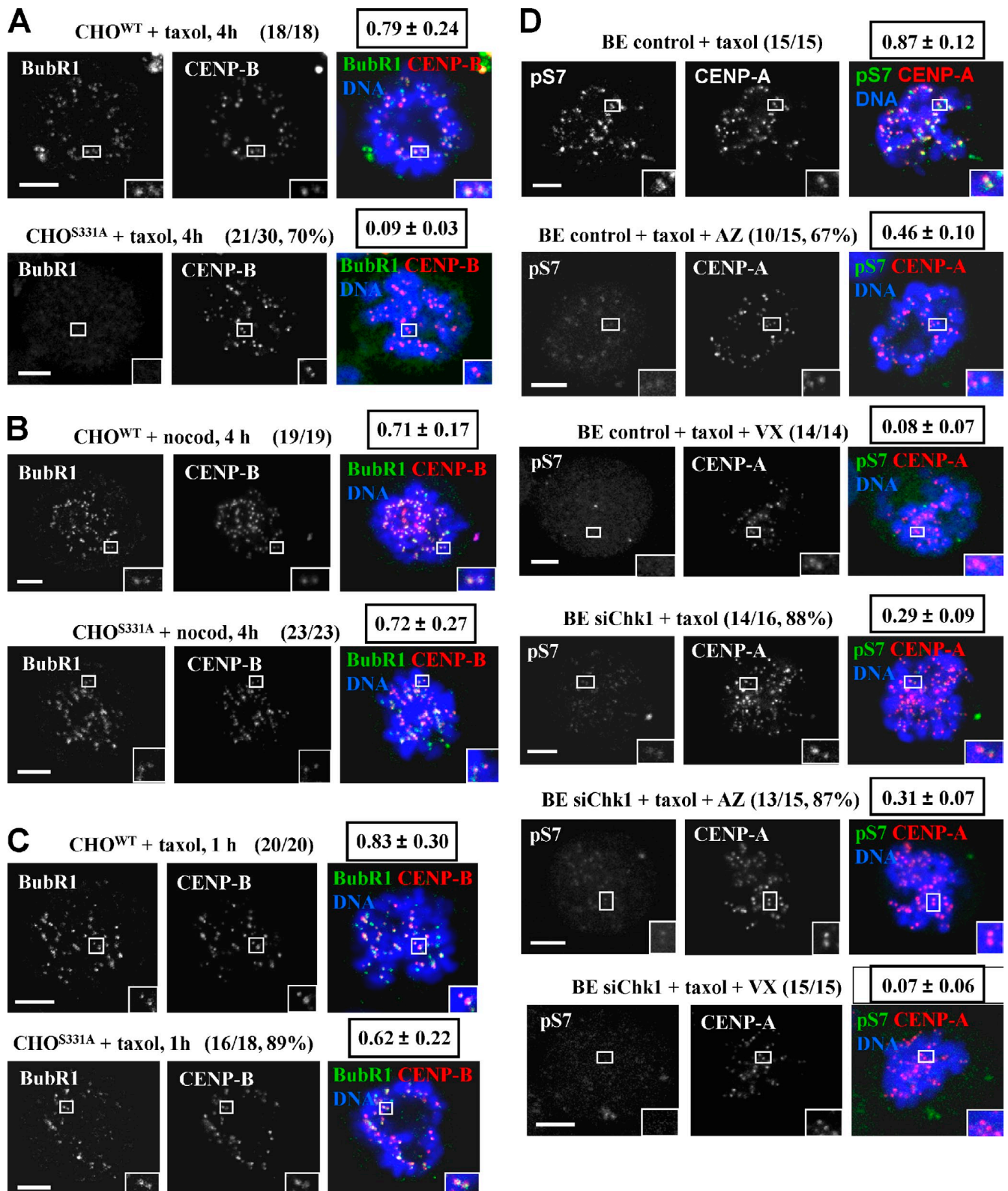
After treatment with taxol for 4 h, CHO<sup>S331A</sup> cells exhibit reduced staining of BubR1 at kinetochores by approximately ninefold compared with CHO<sup>WT</sup> cells as determined by BubR1/CENP-B fluorescence intensity (Fig. 7 A and Table S1). In contrast, after treatment with nocodazole for 4 h, CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells exhibit similar levels of BubR1 staining at kinetochores (Fig. 7 B and Table S1), therefore showing that kinetochores in CHO<sup>S331A</sup> cells are capable of BubR1 binding. Importantly, after treatment with taxol for 1 h, BubR1 was readily detectable at kinetochores in CHO<sup>S331A</sup> cells, and BubR1/CENP-B fluorescence intensity was reduced by only 1.3-fold compared with CHO<sup>WT</sup> cells ( $P = 0.036$ ; Fig. 7 C and Table S1). These results show that BubR1 is recruited to kinetochores in CHO<sup>S331A</sup> cells in the presence of taxol and that it is BubR1 maintenance at kinetochores rather than its initial recruitment that is affected in CHO<sup>S331A</sup> cells. Collectively, these results suggest that phosphorylation of Aurora B at Ser331 is required for sustained mitotic delay and BubR1 maintenance at kinetochores in the presence of taxol.

#### **Inhibition of Mps1 does not further reduce Aurora B activity in Chk1-depleted cells**

Mps1 enhances Aurora B activity. Depletion of Chk1 by siRNA or inhibition of Mps1 activity by AZ3146 in BE cells treated with taxol diminished Aurora B kinase activity by approximately threefold ( $P < 0.001$ ) and 1.9-fold ( $P < 0.001$ ), respectively, compared with controls, as indicated by lower levels of phosphorylated serine 7 (pS7) of CENP-A (Fig. 7 D and Table S1; Zeitlin et al., 2001; Hewitt et al., 2010). Significantly, treatment of Chk1-depleted cells with AZ3146 did not further

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intensity values (boxed numbers) are shown. Insets show magnified kinetochores. (D) Western blot analysis of BubR1 and actin. (E) Bi/multinucleation indices of interphase cells. A minimum of 300 cells per sample from one experiment was analyzed. TC, tetracycline. (F and G) Mitotic index analysis of CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells after induction with tetracycline and treatment with taxol (F) or nocodazole (G). (H) Polyploidy in CHO<sup>S331A</sup> cells. CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells were treated with tetracycline and taxol, and DNA content greater than 4N was determined. Error bars show the standard deviation from the mean from three experiments. Bars, 5  $\mu$ m.



**Figure 7. Cells expressing S331A Aurora B exhibit unstable binding of BubR1 to kinetochores in response to taxol.** (A–C) Localization of BubR1. CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells were induced with tetracycline and treated with taxol (A and C) or nocodazole (nocod, B) for the indicated times. The frequency of cells exhibiting the respective phenotype and mean BubR1/CENP-B fluorescence intensity values (boxed numbers) are shown. (D) Phosphorylation of Ser7 of CENP-A (pS7). BE cells transfected with negative siRNA (control) or Chk1 siRNA (siChk1) were treated with taxol for 4 h in the absence or presence of AZ3146 (AZ) or VX-680 (VX). The frequency of cells exhibiting the respective phenotype and mean pS7/CENP-A fluorescence intensity values (boxed numbers) are shown. Insets show magnified kinetochores. Bars, 5  $\mu$ m.

reduce Aurora B activity (Fig. 7 D). In comparison, VX-680 further diminished Aurora B activity in Chk1-depleted cells by approximately fourfold ( $P < 0.001$ ; Fig. 7 D). These results show that combining inhibition of Mps1 with Chk1 depletion does not exhibit an additive effect on Aurora B activity.

#### **Phosphorylation of Ser331 is required for optimal Survivin association with the CPC**

The phenotype described for CHO<sup>S331A</sup> cells is similar to the phenotype reported after depletion of Survivin in human cells (Carvalho et al., 2003). As shown in Fig. 8 (A and B), Survivin associated with 6×Myc–Aurora B was reduced in CHO<sup>S331A</sup> compared with CHO<sup>WT</sup> cells after treatment with tetracycline and taxol. However, Survivin-GFP localized to centromeres in all CHO<sup>WT</sup> (20/20) and CHO<sup>S331A</sup> (25/25) cells transiently expressing Survivin-GFP in the presence of taxol (Fig. 8 C). These results suggest that Ser331 phosphorylation is required for optimal association of Survivin with the CPC but not for Survivin localization to centromeres. Furthermore, depletion of Survivin by siRNA (Fig. S4 C, siSurvivin) reduced accumulation of wild-type or S331A 6×Myc–Aurora B to centromeres compared with controls (Figs. 8 D and S4 D).

## **Discussion**

Complete Aurora B activation occurs through a two-step mechanism: in the first step, Aurora B binds to INCENP and is partially activated, and in the second step, Aurora B phosphorylates INCENP at the TSS motif and becomes fully active (Bishop and Schumacher, 2002). In the present study, we show that human Aurora B is phosphorylated at the conserved residue Ser331 during unperturbed mitosis or treatment of cells with spindle poisons. Aurora B phosphorylated at Ser331 localizes to kinetochores in prometaphase, the midzone in anaphase, and the midbody in telophase and cytokinesis. Chk1 is required for Ser331 phosphorylation during unperturbed prometaphase or treatment of cells with taxol, a drug that stabilizes microtubules and primarily interferes with tension at kinetochores; however, Chk1 is dispensable for Ser331 phosphorylation during prophase, cytokinesis, or complete microtubule depolymerization by nocodazole. To our knowledge, this is the first non-T-loop phosphorylation reported for Aurora B, and Chk1 is the first kinase known to phosphorylate Aurora B (Yasui et al., 2004).

To investigate the significance of Ser331 phosphorylation, we generated CHO cells expressing wild-type or S331A Aurora B, in which Ser331 was mutated to alanine, under control of a tetracycline-induced promoter. Using the aforementioned cell lines, we proposed that phosphorylation at Ser331 is required for efficient phosphorylation of INCENP at TSS residues and complete Aurora B activation (Honda et al., 2003). However, it is not essential for Aurora B localization to centromeres, autophosphorylation at Thr232, or association with INCENP (Yasui et al., 2004; Rosasco-Nitcher et al., 2008).

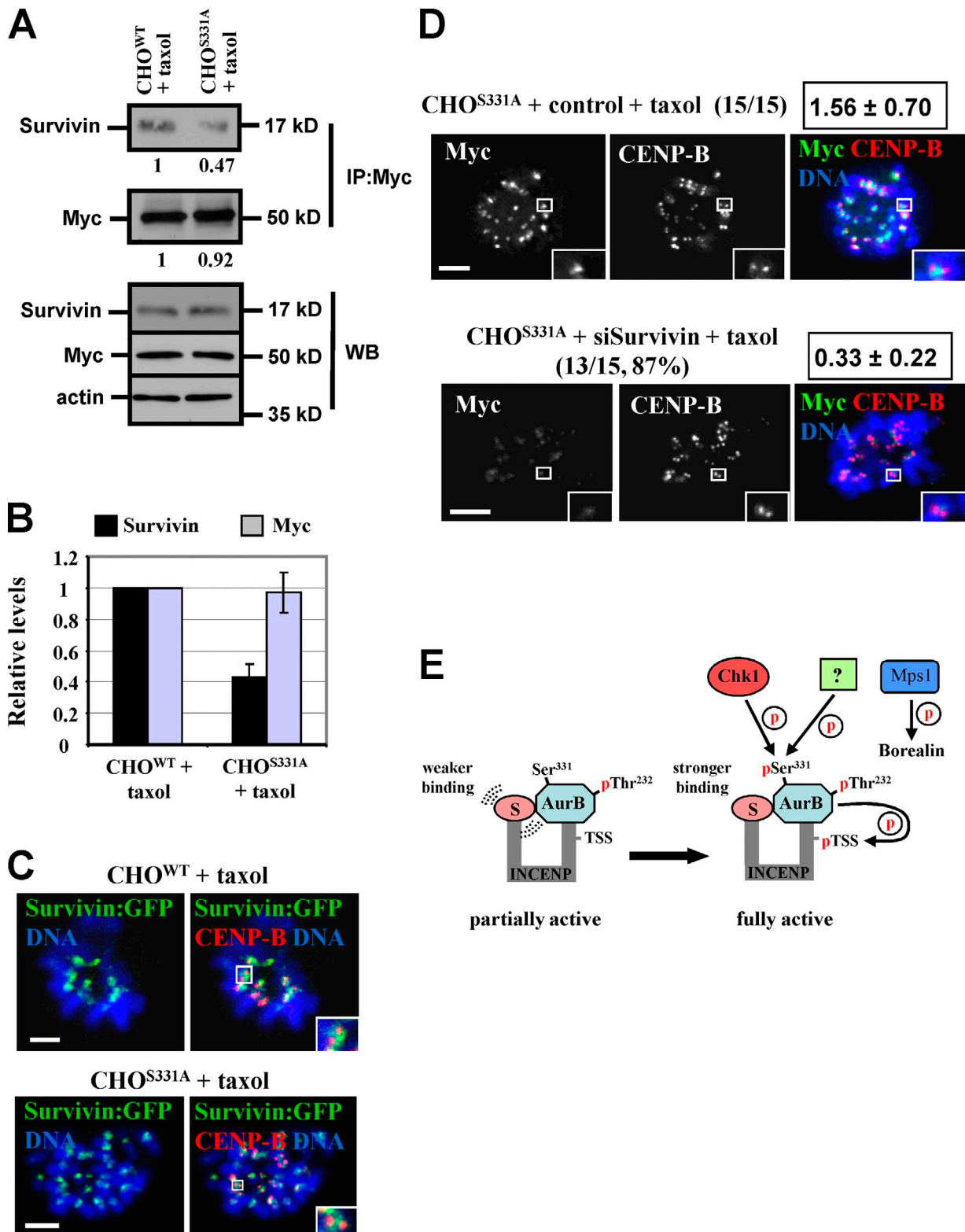
In agreement with previous findings, reduced Aurora B activity in CHO<sup>S331A</sup> cells correlates with chromosome missegregation and impaired accumulation of BubR1 at kinetochores during unperturbed mitosis (Kaitna et al., 2000; Ditchfield

et al., 2003). Furthermore, CHO<sup>S331A</sup> cells exhibit multinucleation and phenocopy DT40 cells expressing TAA mutant INCENP, which is consistent with Ser331 being required for TSS phosphorylation (Xu et al., 2010). In addition, overexpression of Aurora B<sup>S331A</sup> impaired mitotic delay and BubR1 maintenance to kinetochores during treatment of cells with taxol. However, it did not diminish mitotic accumulation induced by nocodazole. It is possible that low Aurora B activity in cells expressing Aurora B<sup>S331A</sup> is sufficient for spindle checkpoint response when microtubules are absent, whereas phosphorylation at Ser331 and higher levels of kinase activity are required for a robust response against taxol (Xu et al., 2009; Santaguida et al., 2011).

Combining inhibition of Mps1 with Chk1 depletion did not exhibit an additive effect on Aurora B activity, thus suggesting that Mps1 and Chk1 are involved in the same step of Aurora B activation. Mps1 phosphorylates Borealin to enhance Aurora B activity (Jelluma et al., 2008; Sliedrecht et al., 2010). One possibility is that phosphorylation of Borealin by Mps1 facilitates Ser331 or TSS phosphorylation, for example by stabilizing interactions between CPC proteins (Vader et al., 2006). Clarifying how Borealin contributes to stimulation of Aurora B activity is required to test this idea.

CHO<sup>S331A</sup> exhibits lower levels of Survivin associated with the CPC compared with CHO<sup>WT</sup> cells. Because Survivin binds to the CPC as a monomer, we propose that Survivin association with the partially active complex is weaker compared with the fully active complex (Jeyapakash et al., 2007). Furthermore, depletion of Survivin reduced accumulation of wild-type and S331A Aurora B to centromeres, consistent with a role for Survivin in mediating targeting of the CPC to chromosomes (Vader et al., 2006; Kelly et al., 2010). Our preliminary results suggest that depletion of Survivin in CHO<sup>S331A</sup> cells further impairs the checkpoint response to taxol by reducing localization of Aurora B<sup>S331A</sup> to centromeres, thus further diminishing Aurora B activity at centromeres and kinetochores.

On the basis of those findings, we propose the following model for Aurora B activation (Fig. 8 E). Aurora B associated with INCENP and Survivin is autophosphorylated at Thr232 and partially active. During unperturbed prometaphase or treatment of cells with taxol, Chk1 phosphorylates Aurora B at Ser331, and this phosphorylation is required for phosphorylation of INCENP at the TSS motif and complete kinase activation. In contrast, cells in prophase, anaphase, and cytokinesis or treated with nocodazole, can phosphorylate Ser331 through an unidentified kinase (Fig. 8 E, question mark) and achieve full Aurora B activity independently of Chk1. Furthermore, Survivin binding to the CPC is stronger in the fully active compared with the partially active complex, and Mps1 contributes to full Aurora B activation, presumably by phosphorylating Borealin (for simplicity not depicted in the complex). Our model raises several important questions regarding the significance of Ser331 phosphorylation for Aurora B protein conformation, the role of this phosphorylation in modulating Survivin association with the CPC, and the potential role of protein phosphatases and kinases in regulating phosphorylation of Ser331.



**Figure 8. Expression of S331A Aurora B correlates with reduced association of Survivin with the CPC.** (A) Coimmunoprecipitation assay. CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells were induced with tetracycline and treated with taxol for 4 h. (top and middle) Western blot analysis of immunoprecipitated (IP) Survivin and 6xMyc-Aurora B (Myc). Values show the relative levels of Survivin and Myc with levels at CHO<sup>WT</sup> cells set to 1. (bottom) Western blot (WB) analysis of total Survivin, Myc, and actin. (B) Densitometric analysis of immunoprecipitated Survivin and Myc levels from A. Error bars show the standard deviation from the mean from three independent experiments. (C) Localization of Survivin-GFP. CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells expressing Survivin-GFP were treated as in A. (D) Localization of 6xMyc-Aurora B (Myc). CHO<sup>S331A</sup> cells were transfected with control or Survivin siRNA (siSurvivin), induced with tetracycline, and treated with taxol for 4 h. The frequency of cells exhibiting the respective phenotype and mean Myc/CENP-B fluorescence intensity values (boxed numbers) are shown. (E) Model for Aurora B (AurB) activation (see Discussion for details). P, phosphorylation; S, Survivin; question mark shows an unidentified kinase. Insets show magnified kinetochores. Bars, 5  $\mu$ m.

Serine 331 is at the foot of the Aurora B C-terminal tail inside a sequence essential for Aurora B activation (Scrittore et al., 2005). Phosphorylation of Ser331 may trigger release of the Aurora B C-terminal tail, phosphorylation of the TSS sequence, and complete Aurora B activation. Alternatively, phosphorylation of Aurora B at Ser331 may change INCENP conformation through an allosteric mechanism to make TSS residues more accessible to Aurora B, and the release of the C-terminal tail may be a consequence of TSS phosphorylation (Sessa et al., 2005). Crystallographic analysis of an Aurora B–INCENP complex harboring phosphorylated Ser331 is required to distinguish between these possibilities.

Aurora B phosphorylates Survivin at threonine 117 (Thr117), and mutation of Thr117 to alanine prevented immunoprecipitation of Survivin with INCENP (Wheatley et al., 2004, 2007). It is possible that Survivin is inefficiently phosphorylated at Thr117 in CHO<sup>S331A</sup> compared with wild-type cells. Alternatively, phosphorylation at Ser331 may regulate Survivin association with the CPC indirectly, through changes in conformation of Aurora B–INCENP. Increased binding of Survivin to the fully active complex may facilitate interactions with Aurora B substrates and enhance Aurora B catalytic activity (Bolton et al., 2002).

Ser331 appears constitutively phosphorylated under all conditions examined. This is in agreement with a recent study showing that Aurora B activity does not significantly change during mitosis, based on phosphorylation of an artificial fluorescence resonance energy transfer probe substrate targeted to CENP-B (Liu et al., 2009). Furthermore, Aurora B phosphorylated at Ser331 localizes to kinetochores, where it is presumably near its substrates. This is consistent with findings that active Aurora B efficiently promotes microtubule detachment if tethered within the kinetochore but not the centromere (Liu et al., 2009) and also with a recent study showing a small population of pT232 Aurora B at kinetochores (Posch et al., 2010). Interestingly, pS331 and pT232 proteins are only detectable at kinetochores under conditions that inhibit protein phosphatase activity (Gorbsky and Ricketts, 1993; Posch et al., 2010). Protein phosphatases are known to oppose Aurora B–mediated signaling (Emanuele et al., 2008; Meadows et al., 2011), and Sds22, a conserved regulator of Protein Phosphatase 1, was recently shown to regulate Aurora B activity (Posch et al., 2010). Identifying phosphatases that reverse Ser331 phosphorylation and reduce Aurora B kinase activity will provide new insight into mechanisms that silence the spindle checkpoint (Meadows et al., 2011; Rosenberg et al., 2011).

In the absence of Chk1, other kinases mediate Ser331 phosphorylation during prophase, cytokinesis, or treatment of cells with nocodazole. Phosphorylation of Ser331 by as yet unidentified kinases may accelerate phosphorylated group formation when increased counteracting protein phosphatase activity has to be overcome, for example, during assembly of the outer kinetochore in prophase, or when Aurora B regulates large scale events, such as formation of the cleavage furrow in cytokinesis (Emanuele et al., 2008; Fuller et al., 2008). Identifying the full panel of Ser331 kinases will improve our understanding of how CPC functions are controlled in mitosis.

## Materials and methods

### Antibodies and peptides

Anti-pS331 antiserum was generated in rabbits by immunization against the phosphorylated peptide pS331 (H2N-CPWVVRANS[PO3H2]RRVLPPS-CONH2) of human Aurora B (Eurogentec). The unphosphorylated peptide S331 (H2N-CPWVVRANSRRVLPPS-CONH2) was used in competition experiments as appropriate.

Monoclonal antibodies against Chk1 (G-4), Myc (9E10), and Survivin (D-8) and polyclonal antibodies against CENP-B and pH3 were obtained from Santa Cruz Biotechnology, Inc. Monoclonal antibodies against  $\alpha$ -tubulin (DM1A),  $\gamma$ -tubulin (GTU-88), and actin (AC-40) were obtained from Sigma-Aldrich. Monoclonal antibody to CENP-A (3–19) and polyclonal antibodies against human Aurora B (ab2254) and murine BubR1 (ab28193) were obtained from Abcam. Monoclonal (AIM-1) antibody to Aurora B was purchased from BD, polyclonal antibody against V<sub>5</sub> (ab3792) was purchased from Millipore, polyclonal phospho–CENP-A (Ser7) antibody was purchased from Cell Signaling Technology, and polyclonal antibody against histone H3 (pan) was obtained from Millipore. Monoclonal antibody against pT232 (pAB2.1) was a gift from M. Inagaki (Aichi Cancer Center Research Institute, Nagoya, Japan), and rabbit polyclonal antiserum against phosphorylated Ser850 of *Xenopus* INCENP was a gift from T. Stukenberg (University of Virginia, Charlottesville, VA).

### Purified proteins

Recombinant proteins histone H1, histone H3, Chk1, GST-INCENP<sup>826–916</sup>, kinase-active Aurora B, and Aurora B<sup>KD</sup> (D200A) were obtained from Millipore. Human Aurora B<sup>WT</sup>, Aurora B<sup>S331A</sup>, and Aurora B<sup>T179A</sup> proteins were expressed in BL21 (DE3) cells (Agilent Technologies), purified using the 6xHis purification kit (B-PER; Thermo Fisher Scientific), and used as substrates in kinase reactions.

### Mutagenesis, cloning, and generation of cell lines

Human Aurora B cDNA was obtained by reverse transcription PCR by using total RNA from human fibroblasts. The PCR product was introduced as a EcoRI–NotI fragment into the pET-28a(+) vector (EMD) and completely sequenced. Point mutations T1052G (changing Ser331 to alanine) and A535G (changing Thr179 to alanine) were generated by site-directed mutagenesis using the site-directed mutagenesis kit (QuikChange; Agilent Technologies).

To produce inducible CHO<sup>WT</sup> and CHO<sup>S331A</sup> cell lines, wild-type or T1052G Aurora B cDNAs were subcloned as EcoRI–NotI fragments into the 6xMyc-pcDNA3 vector (a gift from D. Kardassis, University of Crete, Iraklion, Greece), excised with BamHI–NotI, and introduced into the pcDNA5/FRT/TO plasmid (Invitrogen). These vectors were then transfected into CHO cells (T-REx; Invitrogen) stably expressing the tetracycline repressor together with the pTK-Hyg selection vector (Agilent Technologies) conferring resistance to hygromycin (Invitrogen).

To generate the V<sub>5</sub>-INCENP<sup>WT</sup> and V<sub>5</sub>-INCENP<sup>3A</sup> expression plasmids, human INCENP cDNA was excised with BamHI–XhoI from RH100/pBSK INCENP or RH100/pBSK INCENP (TSS-AAA) plasmids, respectively (gifts from E. Nigg, Biozentrum, University of Basel, Basel, Switzerland), and cloned into the pcDNA3.1/V<sub>5</sub>-His vector (Invitrogen). Truncated V<sub>5</sub>-INCENP<sup>1–787</sup> was amplified by PCR from the RH100/pBSK INCENP plasmid and cloned as a BamHI–XhoI fragment into the pcDNA3.1/V<sub>5</sub>-His vector.

Plasmid pCS2+ GFP INCENP coding GFP fused to *Xenopus* INCENP was obtained from T. Stukenberg (Knowlton et al., 2006). Plasmid CENP-A–GFP coding for human CENP-A fused to GFP in the pBos vector was obtained from K. Sullivan (Centre for Chromosome Biology, University of Ireland, Galway, Ireland; Kanda et al., 1998), and human Survivin–GFP plasmid was obtained from W. Earnshaw (Wellcome Centre for Cell Biology, University of Edinburgh, Edinburgh, Scotland, UK; Wheatley et al., 2001).

### Cell culture, treatments, and RNA interference

Human colon carcinoma BE cells were grown in DME (Invitrogen) containing 10% fetal bovine serum and CHO cells (T-REx CHO) in Ham's F12 (Invitrogen) supplemented with 10% fetal bovine serum. All cells were cultured at 37°C and 5% CO<sub>2</sub>.

Cells were treated with 1  $\mu$ M nocodazole (Sigma-Aldrich), 1  $\mu$ M taxol (Sigma-Aldrich), 3  $\mu$ M VX-680 (Kava Technology, Inc.), or 2  $\mu$ M AZ3146 (Axon) as appropriate. To induce expression of Aurora B transgenes, CHO<sup>WT</sup> or CHO<sup>S331A</sup> cells were treated with 17 or 30 ng/ml tetracycline (Sigma-Aldrich), respectively, for 16 h before analysis or further treatment with drugs.

Negative siRNA or siRNA duplexes designed to repress human Chk1 (Thermo Fisher Scientific), human Aurora B (Thermo Fisher Scientific), or rat Survivin (QIAGEN) was transfected into BE or CHO cells 48 h (siChk1), 24 h (Aurora B siRNA), or 72 h (Survivin siRNA) before analysis or treatment with drugs using Lipofectamine 2000 (Invitrogen). For transient expression of V<sub>5</sub>-INCENP or GFP proteins, plasmids were transfected into cells 24 h before analysis or treatment with drugs using Lipofectamine 2000.

### Immunoprecipitations and kinase assays

For Myc immunoprecipitations shown in Fig. 8 A, cells were incubated in ice-cold E1A buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 1 mM PMSF, 1 mM NaF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 30 µg/ml RNase, 10 mM sodium β-glycerophosphate, 1 mM sodium vanadate, and 0.1 µM microcystin) for 30 min (Harlow et al., 1986). Approximately 1 mg cell lysate was incubated with 1 µg anti-Myc (9E10) antibody for 2 h followed by addition of 20 µl protein A/G PLUS-agarose beads (Invitrogen) for 16 h at 4°C. Samples were spun down, washed twice with E1A buffer, and analyzed by SDS-PAGE.

For the Myc immunoprecipitations shown in Fig. 4 B, cells were sonicated 3× for 10 s in ice-cold radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 30 µg/ml RNase, 20 mM sodium β-glycerophosphate, and 0.3 mM sodium vanadate). 0.3–1 mg cell lysate was incubated with 0.5 µg anti-Myc antibody for 1 h followed by addition of 20 µl protein A/G PLUS-agarose beads for 16 h at 4°C. Samples were spun down and washed twice with radioimmunoprecipitation buffer, once with wash buffer (50 mM Tris-HCl, pH 8.0, 0.4 M NaCl, 1% NP-40, 0.5% deoxycholate, 1 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml aprotinin), and twice with Tris buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1 mM PMSF) essentially as previously described (Honda et al., 2003).

For V<sub>5</sub> immunoprecipitations, cells were sonicated 3× for 10 s in ice-cold immunoprecipitation buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 0.1 mM PMSF, 10 mM sodium β-glycerophosphate, 0.1 mM Na<sub>2</sub>VO<sub>4</sub>, 1 mM NaF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin). 0.3–1 mg cell lysate was incubated with 0.5 µg anti-V<sub>5</sub> antibody for 1 h followed by addition of 20 µl protein A/G PLUS-agarose beads for 16 h at 4°C. Samples were spun down, washed twice with immunoprecipitation buffer, and analyzed by SDS-PAGE (Figs. 4 F and S3 B) or included in kinase reactions (Fig. 5 D).

For Figs. 4 B and 5 D, immunoprecipitated proteins on agarose beads were included into a 20-µl reaction containing 1 µg histone H3, 50 mM Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM DTT, 5 mM NaF, 5 mM sodium β-glycerophosphate, 50 µM sodium vanadate, and 0.1 mM ATP (Honda et al., 2003). For Figs. 4 E and S3 A, 0.2 µg recombinant kinase-active Aurora B was included into a 20-µl reaction containing 1 µg histone H3, 50 mM Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM DTT, 5 mM NaF, 5 mM sodium β-glycerophosphate, 50 µM sodium vanadate, and 10 µM ATP. Where appropriate, 0.5 µg recombinant Chk1 and/or 0.2 µg GST-INCENP<sup>826-916</sup> was included in the kinase reaction. Unless otherwise stated, reactions were incubated for 20 min at 30°C, stopped by addition of 2 µl 10× gel sample buffer, and analyzed by SDS-PAGE and Western blotting using a polyclonal antibody against pH3.

For in vitro Chk1 kinase assays in Fig. 1, 0.5 µg recombinant Chk1 was incubated with 1 µg protein substrate in 20 µl kinase buffer (20 mM MOPS, pH 7.2, 5 mM EGTA, 10 mM MgCl<sub>2</sub>, 25 mM sodium β-glycerophosphate, 1 mM sodium vanadate, 1 mM DTT, 100 µM ATP, and 1 µCi γ-ATP) for 20 min at 30°C before analysis by SDS-PAGE. Radioactive labeling of Chk1 substrates was determined by autoradiography and densitometric analysis of the bands (Zachos et al., 2007). Where appropriate, 145 ng UCN-01 (Sigma-Aldrich) was included in the kinase reaction.

### Indirect immunofluorescence microscopy

For pS331 staining, cells were rinsed twice in PHEM buffer (60 mM Pipes, 25 mM Hepes, pH 7.0, 10 mM EGTA, and 4 mM MgSO<sub>4</sub>) extracted in PHEM supplemented with 0.5% CHAPS and 100 nM microcystin (Sigma-Aldrich) for 5 min at room temperature, fixed with cold methanol for 5 min at -20°C, washed twice with PBS, and immunostained (Gorbsky and Ricketts, 1993). For all other fluorescence microscopy applications, cells were fixed in 4% paraformaldehyde in cytoskeleton buffer (1.1 M Na<sub>2</sub>HPO<sub>4</sub>, 0.4 M KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 5 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM EGTA, 5 mM Pipes, and 5 mM glucose, pH 6.1) for 5 min at 37°C, permeabilized in 0.5% Triton X-100 in cytoskeleton buffer at room temperature, and immunostained as appropriate (Zachos et al., 2007).

FITC- or rhodamine-TRITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) were used, DNA was stained with 10 µM TO-PRO-3 iodide (642/661; Invitrogen), and cells were mounted in VECTASHIELD medium (Vector Laboratories). Images were collected using a laser-scanning spectral confocal microscope (TCS SP2; Leica), LCS Lite software (Leica), and a 63× Apochromat 1.40 NA oil objective except for Figs. 3 B, 6 (A and B), S1, S2, and S4 A, in which a 40× Apochromat 1.25 NA oil objective was used. A low fluorescence immersion oil (11513859; Leica) was used, and imaging was performed at room temperature. Unless otherwise stated, mean projections of image stacks were obtained using the LCS Lite software.

To analyze fluorescence intensities, background readings were subtracted, and fluorescence intensities were quantified using LCS Lite. The pS331 values were normalized against the CENP-A signal, and the pS850 was normalized against the GFP signal (also see Table S1). For Table I, four cells per experiment from three independent experiments were analyzed for each treatment. For Table II, three to four cells per experiment from three independent experiments were analyzed for each treatment. For Table S1, a minimum of three cells per experiment from at least two independent experiments were analyzed for each treatment.

### Mitotic and bi/multinucleation indices

Cells were fixed in 4% paraformaldehyde in cytoskeleton buffer, permeabilized in 0.5% Triton X-100, stained, and examined for condensed chromatin or bi/multinucleation by fluorescence microscopy. For Fig. 6 E, a minimum of 300 cells per sample was analyzed. For Fig. 6 (F and G), a minimum of 100 cells per sample was analyzed.

### DNA content

Cells were fixed in 70% ethanol-PBS at 4°C, stained with 20 µg/ml propidium iodide in PBS (Sigma-Aldrich) and analyzed for DNA content by use of a flow cytometer (FACScan; BD).

### Western blotting and densitometry

Cells were lysed in ice-cold whole-cell extract buffer (20 mM Hepes, 5 mM EDTA, 10 mM EGTA, 0.4 M KCl, 0.4% Triton X-100, 10% glycerol, 5 mM NaF, 50 ng/ml okadaic acid, 1 mM DTT, 5 µg/ml leupeptin, 50 µg/ml PMSF, 1 mM benzamide, 5 µg/ml aprotinin, and 1 mM Na<sub>2</sub>VO<sub>4</sub>) for 30 min on ice. Lysates were cleared by centrifugation at 15,000 g for 10 min. Densitometric analysis was performed using ImageJ (National Institutes of Health).

### HPLC-Edman degradation

Radiolabeled Aurora B<sup>KD</sup> was excised from an SDS-PAGE gel and digested with trypsin or glutamyl endopeptidase (ICN Pharmaceuticals, Inc.). Samples were acidified and loaded onto a C-18 column (Vydac) for HPLC essentially as previously described (Sanchez et al., 1997). Fractions were collected at 1-min intervals and counted for radioactivity, and selected fractions were immobilized on membrane discs for N-terminal sequencing (Sullivan and Wong, 1991).

### Protein Data Bank image

The image of *Xenopus* Aurora B-INCENP<sup>790-847</sup> in complex with hesperadine (Sessa et al., 2005) was downloaded from the Protein Data Bank using Jmol (accession no. 2BFY).

### Online supplemental material

Fig. S1 shows HPLC-Edman analysis of radiolabeled Aurora B<sup>KD</sup> after digestion with glutamyl endopeptidase, Aurora B expression levels after treatment of BE cells with Aurora B siRNA, and specificity of the anti-pS331 antiserum by immunofluorescence. Fig. S2 shows Chk1 and Aurora B expression levels after treatment of BE cells with Chk1 siRNA and Ser331 phosphorylation in prophase and cytokinesis as well as Aurora B expression levels and Ser331 phosphorylation in CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells. Fig. S3 shows Chk1 in vitro kinase assays, immunoprecipitation of 6×Myc-Aurora B with V<sub>5</sub>-INCENP, Aurora B phosphorylation at Thr232, and INCENP phosphorylation at Ser850. Fig. S4 shows examples of CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells in metaphase and interphase, expression of Survivin, and localization of 6×Myc-Aurora B after treatment of CHO<sup>WT</sup> cells with Survivin siRNA. Table S1 shows fluorescence intensity values at prometaphase kinetochores after various treatments. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201104023/DC1>.

We thank J. Papamatheakis and T. Stukenberg for comments on the manuscript and C. Ward (University of Glasgow, Glasgow, Scotland, UK) for technical help with HPLC-Edman. We also thank W. Earnshaw, M. Inagaki, P. Jallepalli,



D. Kardassis, E. Nigg, T. Stukenberg, K. Sullivan, and S. Taylor for generously sharing reagents.

This work was funded by the Association for International Cancer Research (to E. Petsalaki, T. Akoumianaki, and G. Zachos).

Submitted: 6 April 2011

Accepted: 23 September 2011

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