Chk1 and Mps1 jointly regulate correction of merotelic kinetochore attachments

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Summary
If uncorrected, merotelic kinetochore attachments can induce mis-segregated chromosomes in anaphase. We show that checkpoint kinase 1 (Chk1) protects vertebrate cells against merotelic attachments and lagging chromosomes and is required for correction of merotelic attachments during a prolonged metaphase. Decreased Chk1 activity leads to hyper-stable kinetochore microtubules, unstable binding of MCAK, Kif2b and Mps1 to centromeres or kinetochores and reduced phosphorylation of Hec1 by Aurora-B. Phosphorylation of Aurora-B at serine 331 (Ser331) by Chk1 is high in prometaphase and decreases significantly in metaphase cells. We propose that Ser331 phosphorylation is required for optimal localization of MCAK, Kif2b and Mps1 to centromeres or kinetochores and for Hec1 phosphorylation. Furthermore, inhibition of Mps1 activity diminishes initial recruitment of MCAK and Kif2b to centromeres or kinetochores, impairs Hec1 phosphorylation and exacerbates merotelic attachments in Chk1-deficient cells. We propose that Chk1 and Mps1 jointly regulate Aurora-B, MCAK, Kif2b and Hec1 to correct merotelic attachments. These results suggest a role for Chk1 and Mps1 in error correction.

Key words: Chk1, Aurora-B, Mps1, Merotelic, Mitosis

Introduction
Accurate chromosome segregation during mitosis requires that sister kinetochores attach to microtubules emanating from opposite spindle poles [bipolar attachment or bi-orientation (Cimini, 2008; Tanaka, 2013)]. Merotelic attachments in which a single kinetochore binds to microtubules coming from both poles occur spontaneously in early mitosis and are not detected by the mitotic spindle checkpoint, the mechanism that prevents anaphase onset until all sister kinetochores bi-orient (Cimini, 2008; Tanaka, 2013). If uncorrected before anaphase, merotelic attachments will lead to lagging chromosomes, typically consisting of a single sister chromatid, and can result in chromosome mis-segregation and aneuploidy (Cimini et al., 2001; Cimini et al., 2003).

The chromosomal passenger complex (CPC), comprising Aurora-B kinase, INCENP, Survivin and Borealin, plays a central role in correction of kinetochore mis-attachments (Tanaka, 2013; van der Waal et al., 2012a). Inhibition of Aurora-B activity increases the frequency of merotelic and syntelic attachments, in which sister kinetochores are bound to microtubules coming from the same spindle pole, and leads to hyper-stable kinetochore microtubules (kMTs; (Hauf et al., 2003; Cimini et al., 2006; Knowlton et al., 2006). It has been proposed that Aurora-B promotes detachment of incorrectly attached microtubules by phosphorylating kinetochore substrates to promote turnover of kMTs; however, the molecular pathways involved are a matter of active investigation (Cheeseman et al., 2002; Cimini et al., 2006; Pinsky et al., 2006; Akiyoshi et al., 2010).

The microtubule-depolymerising kinesins MCAK and Kif2b destabilize kMTs to correct mis-attachments (Kline-Smith et al., 2004; Manning et al., 2007; Bakhoum et al., 2009). MCAK localizes to several mitotic structures including spindle poles, centromeres in the absence of tension or kinetochores in the presence of tension (Andrews et al., 2004; Manning et al., 2007). MCAK promotes turnover of kMTs in metaphase cells and depletion of MCAK correlates with chromosome mis-segregation (Kline-Smith et al., 2004; Bakhoum et al., 2009). Furthermore, Aurora-B phosphorylates MCAK to recruit it to centromeres and inhibits its microtubule depolymerization activity (Andrews et al., 2004; Lan et al., 2004; Knowlton et al., 2006; Tanenbaum et al., 2011).

Kif2b localizes to spindle poles, microtubules and kinetochores where it regulates kMT dynamics during prometaphase and Aurora-B promotes localization of Kif2b to kinetochores by an undescribed mechanism (Manning et al., 2007; Bakhoum et al., 2009). However, in metaphase, Kif2b is replaced by the astrin, SKAP and Ska protein complexes at kinetochores to promote kMT stability and chromosome alignment (Manning et al., 2010; Schmidt et al., 2010; Chan et al., 2012).

Furthermore, Aurora-B phosphorylates the kinetochore protein Hec1 on several N-terminal residues including serine 55 (Ser55) and serine 44 (Ser44) to promote detachment of kMTs; however, the molecular pathways involved are a matter of active investigation (DeLuca et al., 2006; DeLuca et al., 2011). Hec1 mediates kMT attachments and expression of non phosphorylatable Hec1 increases merotelic attachments and anaphase lagging chromosomes (Cheeseman et al., 2006; DeLuca et al., 2006). In addition, mitotic Hec1 phosphorylation is high in prometaphase and decreases significantly in metaphase cells (DeLuca et al., 2011).

Checkpoint kinase 1 (Chk1) is a well established component in the DNA damage and DNA replication pathways (Smith et al.,
Chk1 is also required for optimal chromosome segregation and for spindle checkpoint signalling during unperturbed mitosis or treatment of cells with taxol (Zachos et al., 2007; Peddibhotla et al., 2009). Chk1 phosphorylates Aurora-B at Ser331 to induce Aurora-B kinase activity; however, a role for Chk1 in error correction has not been previously reported (Petsalaki et al., 2011).

Mps1 kinase is required for mitotic arrest in the presence of unattached kinetochores, proper chromosome alignment and segregation (Abrieu et al., 2001; Stucke et al., 2002; Jelluma et al., 2008a; Hewitt et al., 2010; Santaguida et al., 2010). Recent studies have shown that Mps1 phosphorylates Borealin to enhance Aurora-B kinase activity (Jelluma et al., 2008b; Saurin et al., 2011); however, other studies did not detect changes in Aurora-B activity upon Mps1 inhibition. Instead, Aurora-B activity was required for optimal localization of Mps1 to kinetochores (Hewitt et al., 2010; Maciejowski et al., 2010; Santaguida et al., 2010). However, a role for Mps1 in preventing merotelic attachments has not been previously described.

In the present study, using human cells depleted of Chk1 by small interfering RNA (siRNA) and DT40 avian B-lymphoma cells in which Chk1 was genetically ablated by gene targeting (Zachos et al., 2003), we show that Chk1 protects against anaphases with merotelic attachments and lagging chromosomes and is required for correction of merotelic attachments in metaphase-delayed cells. Reduced Chk1 activity leads to hyper-stable kMTs and Chk1 is required for stable binding of MCAK, Kif2b and Mps1 to centromeres or kinetochores and for mitotic phosphorylation of Hec1 on Ser55 and Ser44. Furthermore, Aurora-B phosphorylated at Ser331 localizes at kinetochores and this phosphorylation is high in prometaphase and decreases significantly in metaphase cells. Using cells overexpressing wild-type or non-phosphorylatable Ser331 to alanine (S331A) mutant Aurora-B (Petsalaki et al., 2011), we propose that Ser331 phosphorylation is required for optimal MCAK, Kif2b and Mps1 localization to centromeres or kinetochores and for Hec1 phosphorylation. Furthermore, inhibition of Mps1 activity or Mps1 depletion diminish initial recruitment of MCAK and Kif2b to centromeres or kinetochores, impair mitotic Hec1 phosphorylation and exacerbate merotelic attachments and lagging chromosomes in Chk1-deficient cells. On the basis of those findings, we propose that Chk1 and Mps1 jointly regulate Aurora-B, MCAK, Kif2b and Hec1 functions to promote correction of merotelic attachments.

**Results**

**Chk1-deficient cells exhibit high levels of anaphases with merotelic attachments and lagging chromosomes**

To investigate a role for Chk1 in preventing anaphases with merotelic attachments, human colon carcinoma BE cells transiently transfected with negative siRNA (control), Chk1 siRNA (siChk1) or treated with the selective Chk1 inhibitor UCN-01 were analyzed by confocal microscopy. Depletion of Chk1 or inhibition of Chk1 activity by UCN-01 increased the frequency of anaphases with merotelic attachments (13.3% and 13.4%, respectively) compared to controls (3.9%; Fig. 1A,B).

**Fig. 1.** Chk1 is required for correction of merotelic attachments in metaphase-delayed cells. (A,B) BE cells were transfected with negative siRNA (control) or Chk1 siRNA (siChk1) or were treated with UCN-01 for 3 hours. (A) Examples of anaphases. A single sister centromere is connected to microtubules from both spindle poles. Green, α-tubulin; red, CENP-B; blue, DNA. Insets show magnified centromeres. Scale bars: 5 μm. (B) Frequencies of anaphases with merotelic attachments. A minimum of 50 anaphases were analyzed for each of three independent experiments. (C) Frequencies of anaphases with lagging chromatin in wild-type (WT), Chk1<sup>−−</sup>, kinase-dead (KD) or revertant (Rev) DT40 cells. A minimum of 80 anaphases were analyzed for each of three independent experiments. (D,E) Frequencies of anaphases with merotelic attachments (D) or lagging chromosomes (E) in BE cells transfected as in A and analyzed in anaphase in the absence of drug treatment (~MG132) or after recovery from a 3-hour MG132 treatment (+MG132). A minimum of 50 (D) or 90 (E) anaphases were analyzed for each of three independent experiments; n, total number of anaphases tested. (F) Inhibition of Chk1 reduces sensitivity of kMTs to nocodazole. BE cells were treated with MG132 for 3 hours in the absence (control) or presence of UCN-01 and exposed to high concentrations of nocodazole. Microtubule-associated fluorescence intensities were normalized to the 2-minute time point. A minimum of 10 mitotic cells were analyzed for each time point. Values are mean ± s.d.
Furthermore, Chk1 depletion or treatment of cells with UCN-01 increased the frequency of anaphases with lagging chromosomes (15.1% and 14%, respectively) compared to controls (4.7%) and lagging chromosomes typically consisted of a single chromatid localized near the cell equator (supplementary material Fig. S1A,B).

To verify these observations in a different cell type, we used wild-type DT40 cells (WT), Chk1-deficient DT40 cells (Chk1−/−), Chk1−/− cells reconstituted with avian wild-type Chk1 protein (revertant cells, Rev), or Chk1−/− cells reconstituted with mutant aspartic acid 130 to alanine (D130A) avian Chk1 protein (kinase-dead cells, KD; supplementary material Fig. S1C). Chk1−/− or KD cells exhibited higher incidence of anaphases with lagging chromatins (30.3% and 33.8%, respectively) compared to WT (7.6%) or Rev (12.5%; Fig. 1C). Furthermore, 18/80 (22.5%) Chk1−/− cells in anaphase exhibited merotelic attachments compared to 1/80 (1.3%) WT and 3/80 (3.8%) Rev (supplementary material Fig. S1D). Taken together, these results show that Chk1 activity prevents anaphases with merotelic attachments and lagging chromosomes in vertebrate cells.

Chk1 is required for correction of merotelic attachments in metaphase-arrested cells

Lagging chromosomes can be caused by inefficient correction of merotelic attachments before anaphase. To investigate a role for Chk1 in error correction, control or Chk1-deficient cells were treated with the proteasome inhibitor MG132 for 3 hours to prolong metaphase (Cimini et al., 2003). At the end of this treatment, the drug was washed out and cells were released in fresh medium for the appropriate time to accomplish metaphase to anaphase transition, prior to fixation for analysis by confocal microscopy. In control BE cells, delayed anaphase onset by MG132 reduced the frequency of anaphases with merotelic attachments (0.6%) or lagging chromosomes (0.6%) compared to cells progressing into anaphase without MG132 delay (3.9% and 5.7%, respectively; Fig. 1D,E). In contrast, treatment of Chk1-depleted cells with MG132 did not reduce anaphases with merotelic attachments (17.2%) or lagging chromosomes (20.3%) compared to untreated (13.3% and 16.3%, respectively; Fig. 1D,E).

Furthermore, treatment of DT40 cells with MG132 reduced the frequency of anaphases with lagging chromosomes in WT cells (1%) compared to untreated (6.7%), but not in Chk1−/− cells compared to untreated (32.8% and 28.8%, respectively; supplementary material Fig. S1E). Collectively, these results suggest that Chk1 is required for correction of merotelic attachments before anaphase.

Inhibition of Chk1 leads to hyper-stable kinetochore microtubules

Hyper-stable kMTs associate with increased kinetochore mis-attachments (Cimini et al., 2006; Bakhoun et al., 2009). Importantly, BE cells arrested in metaphase with MG132 and treated with UCN-01 exhibited delayed depolymerization of kMTs in the presence of high doses of nocodazole, indicating hyper-stable kMTs, compared to controls (Fig. 1F; supplementary material Fig. S2A). These results suggest that Chk1 kinase activity is required for optimal stability of kMTs.

Chk1 is required for localization of MCAK and Kif2b to centromeres or kinetochores

MCAK and Kif2b destabilize kMTs. Confocal microscopy analysis of BE cells in prometaphase transiently expressing MCAK-GFP showed that depletion of Chk1 reduced MCAK-GFP staining at centromeres/kinetochores by ~78% compared to controls (P<0.001; Fig. 2A). As a positive control, treatment of cells with the Aurora-B inhibitor VX680 (VX) reduced MCAK-GFP staining at centromeres/kinetochores by 91% compared to controls (P<0.001; Fig. 2A). Also, in metaphase cells, depletion of Chk1 reduced MCAK-GFP staining at kinetochores by 86% compared to controls (P<0.001; Fig. 2B).

Furthermore, BE cells in prometaphase transiently expressing Kif2b-GFP exhibited reduced localization of Kif2b-GFP to kinetochores after Chk1 depletion or VX680 treatment by, respectively, 81% and 94% compared to controls (P<0.001; Fig. 2C). Significantly, MCAK-GFP or Kif2b-GFP levels per se were not affected by Chk1 depletion (Fig. 2D). Taken together, these results show that Chk1 is required for optimal localization of MCAK and Kif2b to centromeres or kinetochores during unperturbed mitosis, i.e. in the absence of spindle poisons.

Chk1 is required for Hec1 Ser55 and Ser44 phosphorylation

Phosphorylation of Hec1 at Ser55 (pS55) and Ser44 (pS44) destabilizes kinetochore-microtubule attachments. BE control cells in prometaphase exhibited phosphorylated Ser55 at kinetochores and this phosphorylation was reduced after Chk1 depletion or VX680 treatment by, respectively, 81% and 84% compared to controls (P<0.001; Fig. 3A; supplementary material Fig. S2B). Furthermore, control cells in metaphase exhibited reduced phosphorylation of Ser55 at kinetochores by 55% compared to prometaphase controls (P<0.001; Fig. 3B; supplementary material Fig. S2B). Significantly, depletion of Chk1 further reduced pS55 kinetochore staining in metaphase cells by 71% compared to controls (P<0.001; Fig. 3B; supplementary material Fig. S2B). In addition, Chk1-depleted or VX680-treated cells in prometaphase exhibited reduced Hec1 Ser44 phosphorylation by, respectively, 86% and 89% compared to controls (P<0.001; supplementary material Fig. S2C). Of note, Ser55 and Ser44 phospho-antibodies cross-reacted with the spindle poles and this staining was non-specific (DeLuca et al., 2011). Taken together, these results show that Chk1 is required for optimal phosphorylation of Hec1 Ser55 and Ser44 in mitosis.

Phosphorylation of Aurora-B Ser331 at kinetochores is high in prometaphase and decreases in metaphase cells

Chk1 phosphorylates Aurora-B on Ser331. Phosphorylated Ser331 colocalized with CENP-A and Hec1 at kinetochores in prometaphase and metaphase cells (Fig. 3C; supplementary material Fig. S3A). Significantly, quantification of pS331/CENP-A and pS331/Hec1 kinetochore fluorescence intensities revealed a, respectively, 53% and 57% decrease in phosphorylation of Hec1 Ser331 at kinetochores compared to prometaphase cells (P<0.001; supplementary material Fig. S3A). Of note, Ser55 and Ser44 phospho-antibodies cross-reacted with the spindle poles and this staining was non-specific (DeLuca et al., 2011).
Phosphorylation of Ser331 prevents merotelic attachments and lagging chromosomes in anaphase

To investigate a role for Ser331 phosphorylation in preventing merotelic attachments, CHO cells expressing 6x-Myc-tagged wild-type or S331A Aurora-B under control of a Tetracycline-induced promoter were analyzed by confocal microscopy (Petsalaki et al., 2011). After induction with Tetracycline, 67/300 (22.3%) of CHO S331A cells exhibited anaphases with merotelic kinetochore attachments compared to 3/300 (1%) of CHO WT cells (P<0.001; Fig. 3D). Furthermore, 109/300 (36.3%) of CHO S331A cells exhibited anaphases with lagging chromosomes compared to 17/300 (5.7%) of CHO WT cells (P<0.001; supplementary material Fig. S3C). Please note that CENP-B localizes to kinetochores in CHO cells (Cooke et al., 1990). These results suggest that phosphorylation of Aurora-B Ser331 prevents anaphases with merotelic attachments and lagging chromosomes.

Phosphorylation of Ser331 is required for localization of MCAK and Kif2b to centromeres or kinetochores

To investigate the significance of Ser331 phosphorylation for MCAK and Kif2b localization, CHO WT and CHO S331A cells transiently expressing MCAK:GFP or Kif2b:GFP were induced with Tetracycline and analyzed by confocal microscopy. In prometaphase cells, expression of Aurora-B S331A reduced localization of MCAK:GFP to centromeres or kinetochores by 72% compared to CHO WT (P<0.001; Fig. 4A). In comparison, treatment of CHO WT cells with VX680 or UCN-01 reduced MCAK:GFP staining at centromeres/kinetochores by, respectively, 82% and 80% compared to controls (P<0.001; Fig. 4A; supplementary material Fig. S3D). Also, CHO S331A cells in metaphase exhibited diminished localization of MCAK:GFP to kinetochores by 80% compared to CHO WT (P<0.001; Fig. 4B).

Furthermore, expression of Aurora-B S331A or treatment of cells with VX680 reduced localization of Kif2b:GFP to kinetochores in prometaphase by, respectively, 84% and 92% compared to controls (P<0.001; Fig. 4C). Taken together, these results suggest that Aurora-B Ser331 phosphorylation is required for optimal localization of MCAK and Kif2b to centromeres or kinetochores.

Phosphorylation of Ser331 is required for Hec1 phosphorylation

Furthermore, after induction with Tetracycline, CHO S331A cells in prometaphase exhibited reduced phosphorylation of Hec1 Ser55 at kinetochores by 73% compared to CHO WT (P<0.001; Fig. 4D). In contrast, treatment of CHO WT cells with VX680 or UCN-01 diminished phospho-Ser55 kinetochore staining by, respectively, 83% and 82% compared to controls (P<0.001; Fig. 4D; supplementary material Fig. S4A,C). In addition, CHO WT cells in metaphase exhibited reduced phosphorylation of Ser55 at kinetochores by 56% compared to prometaphase CHO WT (P<0.001; supplementary material Fig. S4B,C). Significantly, expression of Aurora-B S331A further reduced phospho-Ser55 kinetochore staining in metaphase by 67% compared to CHO WT (P<0.001; supplementary material Fig. S4B,C). Taken together, these results suggest that phosphorylation of Ser331 is required for optimal phosphorylation of Hec1 Ser55.
Additive effects from inhibition of Mps1 and Chk1 depletion

Mps1 is required for chromosome segregation. Inhibition of Mps1 activity by AZ3146 (AZ) in BE cells transfected with negative siRNA (control + AZ) increased the frequency of anaphases with merotelic attachments (64.6%) and lagging chromosomes (70%) compared to controls (2.6% and 5.2%, respectively; Fig. 5A; supplementary material Fig. S4D–F). Importantly, combined Chk1 depletion with AZ3146 treatment (siChk1 + AZ) further increased anaphases with merotelic attachments (76.4%) and lagging chromosomes (83.8%) compared to control + AZ cells (Fig. 5A; supplementary material Fig. S4F). These results show that inhibition of Mps1 and Chk1 depletion exhibit an additive effect on anaphases with merotelic attachments and lagging chromosomes.

Inhibition of Mps1 impairs localization of MCAK and Kif2b to centromeres or kinetochores

Treatment with AZ3146 or depletion of Mps1 by siRNA (siMps1; supplementary material Fig. S4G) reduced localization of MCAK:GFP to centromeres/kinetochores in prometaphase cells by 54% or 57%, respectively compared to controls (P<0.001; Fig. 5B; supplementary material Fig. S4F). Importantly, combined Chk1 depletion with AZ3146 treatment (siChk1 + AZ) further increased anaphases with merotelic attachments (76.4%) and lagging chromosomes (83.8%) compared to control + AZ cells (Fig. 5A; supplementary material Fig. S4F). These results show that inhibition of Mps1 and Chk1 depletion exhibit an additive effect on anaphases with merotelic attachments and lagging chromosomes.

Mps1 jointly regulate localization of MCAK to centromeres or kinetochores

Mps1 activity is required for optimal localization of MCAK to centromeres or kinetochores during unperturbed mitosis.

Furthermore, treatment of cells with AZ3146, siMps1 or combined Chk1 depletion with AZ3146 or siMps1 treatment reduced localization of Kif2b:GFP to kinetochores by 95–96% compared to controls (P<0.001; Fig. 5D; supplementary material Fig. S5B). Importantly, MCAK:GFP or Kif2b:GFP levels per se were not affected by AZ3146 treatment in the absence or presence of Chk1 siRNA (Fig. 6A). Taken together, these results show that Mps1 activity is required for optimal localization of MCAK and Kif2b to centromeres or kinetochores.

Mps1 activity is required for initial binding and Chk1 for maintenance of MCAK to centromeres

To investigate whether Chk1 and Mps1 are required for prolonged binding or initial recruitment of MCAK and Kif2b to centromeres or kinetochores, cells were incubated with taxol, a spindle drug that stabilizes kinetochore–microtubule attachments and reduces tension at kinetochores (Schiff and Horwitz, 1980; Yang et al., 2009). MG132 was also added to the culture medium to prevent mitotic exit of cells after Chk1 depletion or AZ3146 treatment (Schmidt et al., 2005; Zachos et al., 2007).

After treatment with taxol and MG132 for 6 hours, localization of MCAK:GFP to centromeres in control + AZ cells was reduced by 52% compared to controls (P<0.001; Fig. 6B,D). Furthermore, depletion of Chk1, combined Chk1 depletion with AZ3146 treatment, or combined transfection of negative siRNA with VX680 treatment (control + VX) reduced MCAK:GFP staining at centromeres by 90–96% compared to control cells (P<0.001; Fig. 6B,D).
Surprisingly, after treatment with taxol and MG132 for 1 hour, Chk1-depleted and control cells exhibited similar levels of MCAK:GFP at centromeres ($P = 0.48$; Fig. 6C,D). These results show that Chk1-depleted cells treated with taxol are capable of MCAK:GFP binding and that it is MCAK maintenance at centromeres, rather than its initial recruitment, that is affected in Chk1-depleted cells. In contrast, control + AZ, control + VX or siChk1 + VX cells treated with taxol and MG132 for 1 hour exhibited diminished MCAK:GFP staining at centromeres by 53–57% compared to controls ($P < 0.001$; Fig. 6C,D). These results suggest that Mps1 activity is required for optimal initial binding of MCAK to centromeres in the presence of taxol.

**Mps1 activity is required for initial recruitment and Chk1 for maintenance of Kif2b to kinetochores**

Furthermore, after treatment with taxol and MG132 for 6 hours, localization of Kif2b:GFP to kinetochores in Chk1-depleted, control + AZ, control + VX, or siChk1 + AZ cells was reduced by 89–93% compared to controls ($P < 0.001$; Fig. 6C,D). These results suggest that Mps1 activity is required for optimal initial binding of MCAK to centromeres in the presence of taxol.

**Mps1 is required for Hec1 Ser55 phosphorylation**

Furthermore, treatment of BE cells with AZ3146 or combined Chk1 depletion with AZ3146 treatment reduced Hec1 Ser55 phosphorylation at kinetochores by 74% in prometaphase and by 65–69% in metaphase compared to controls ($P < 0.001$; supplementary material Fig. S5C). In addition, in the presence of taxol and MG132 for 1 hour, phospho-Ser55 kinetochore staining in Chk1-depleted, control + AZ, or siChk1 + AZ cells was reduced by 78–81% compared to controls ($P < 0.001$; supplementary material Fig. S5D). Collectively, these results show that Mps1 activity is required for optimal phosphorylation of Hec1 at Ser55 during unperturbed mitosis or treatment with taxol.

**Chk1 is required for stable binding of Mps1 to kinetochores**

Inhibition of Mps1 significantly increases its own abundance at kinetochores (Hewitt et al., 2010). To investigate a role for Chk1 in Mps1 localization, BE cells were treated with AZ3146 and analyzed in prometaphase by confocal microscopy. Depletion of
Chk1 reduced Mps1 staining at kinetochores by 84% compared to controls (P<0.001; Fig. 8B). In comparison, treatment with VX680 diminished Mps1 staining at kinetochores by 90% compared to controls (P<0.001; Fig. 8B). These results show that Chk1 is required for optimal localization of Mps1 to kinetochores in the absence of spindle poisons.

Furthermore, after treatment with taxol, MG132 and AZ3146 for 1 hour, Chk1-depleted cells exhibited reduced Mps1 staining at kinetochores by 58% compared to controls (P<0.001; Fig. 8C) thus showing that kinetochores in Chk1-depleted cells are capable of weakened Mps1 binding. Significantly, treatment with taxol, MG132 and AZ3146 for 6 hours, diminished Mps1 kinetochore staining in Chk1-depleted cells by 94% compared to controls (P<0.001; supplementary material Fig. S5E). These results indicate that Chk1 is required for stable binding of Mps1 to kinetochores in the presence of taxol.

**Phosphorylation of Ser331 is required for Mps1 localization to kinetochores**

Furthermore, after induction with Tetracycline and treatment with AZ3146, CHO^SS31A cells transiently expressing Mps1:GFP exhibited reduced Mps1:GFP staining at kinetochores by 79% compared to CHO^WT (P<0.001; Fig. 8D). These results suggest that Aurora-B Ser331 phosphorylation is required for optimal localization of Mps1 to kinetochores. In contrast, treatment of BE cells with AZ3146 did not significantly alter Ser331 phosphorylation at kinetochores compared to controls (P=0.44; supplementary material Fig. S6). In comparison, UCN-01 treatment reduced phospho-Ser331 kinetochore staining by 89% compared to controls (P<0.001; supplementary material Fig. S6).

**Discussion**

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 weakens the mitotic arrest in the presence of many unattached kinetochores and suggest that Aurora-B contributes to spindle checkpoint signalling independently of error correction. This is an actively debated issue (Yang et al., 2009), it is therefore important to understand Aurora-B regulation and downstream signalling during error correction. We previously showed that Chk1 phosphorylates Aurora-B at Ser331 and this phosphorylation is required for optimal spindle checkpoint function (Zachos et al., 2007; Petsalaki et al., 2011). In the present study, we show that Chk1 protects against anaphases with merotelic attachments and lagging chromosomes and is required for correction of merotelic attachments in metaphase-delayed cells. Spindle checkpoint defects can result in anaphases with mono-attached or syntelically attached chromosomes and both sister chromatids delivered to one daughter cell (Kops et al., 2005). It is therefore
unlikely that spindle checkpoint failure accounts for anaphases with merotelic attachments in Chk1-deficient cells. Instead, our data suggest a novel role for Chk1 in correction of mis-attached kinetochores. Furthermore, we show that Chk1 activity is required for optimal stability of kMTs, for localization of MCAK and Kif2b to centromeres or kinetochores and for Hec1 Ser55 and Ser44 phosphorylation.

Aurora-B phosphorylated at Ser331 colocalizes with CENP-A and Hec1 at kinetochores, in agreement with recent studies showing a small population of Aurora-B at kinetochores (Posch et al., 2010; DeLuca et al., 2011; Petsalaki et al., 2011). Significantly, Aurora-B Ser331 phosphorylation is high in prometaphase and decreases in metaphase cells, thus suggesting a role for Ser331 phosphorylation in chromosome bi-orientation (DeLuca et al., 2011). Furthermore, using cells overexpressing wild-type or non-phosphorylatable mutant S331A Aurora-B we propose that Ser331 phosphorylation and complete Aurora-B activation is required for optimal localization of MCAK and Kif2b to centromeres or kinetochores and mitotic Hec1 phosphorylation during correction of merotelic attachments (Petsalaki et al., 2011).

We also show that inhibition of Mps1 induces merotelic attachments and anaphase lagging chromosomes and exhibits an additive effect with Chk1 depletion. To our knowledge, this is the first report describing a role for Mps1 in preventing merotic attachments. Furthermore, inhibition of Mps1 diminishes localization of MCAK and Kif2b to centromeres or kinetochores and reduces Hec1 Ser55 phosphorylation during unperturbed mitosis. Aurora-B phosphorylates Hec1 Ser55 and MCAK and Kif2b localization depend on Aurora-B kinase activity (Andrews et al., 2004; DeLuca et al., 2006; Bakhoum et al., 2009). One possibility is that Mps1 phosphorylates Borealin to enhance Aurora-B activity towards specific substrates (such as Hec1) or after certain treatments (Jelluma et al., 2008b; Sliedrecht et al., 2010) but not others (Hewitt et al., 2010; Maciejowski et al., 2010; Santaguida et al., 2010); however, further experiments are required to fully support this idea.

Furthermore, using taxol, a drug that stabilizes kinetochore–microtubule attachments, we demonstrate that Mps1 activity is required for initial recruitment and Chk1 for maintenance of MCAK:GFP to centromeres. (A) Western blot analysis of total GFP, Chk1 and actin. BE cells expressing MCAK:GFP or Kif2b:GFP were transfected with negative siRNA (control) or Chk1 siRNA (siChk1) and treated with AZ3146 for 3 hours. (B,C) Localization of MCAK:GFP. BE cells expressing MCAK:GFP were transfected as in A and treated with taxol and MG132 in the absence or presence of AZ3146 (AZ) or VX680 (VX) for 6 hours (B) or for 1 hour (C). Boxed values show mean GFP/CENP-B fluorescence intensity. Green, GFP; red, CENP-B; blue, DNA. Values in brackets represent kinetochore pairs quantified, followed by the number of cells analyzed. Insets show magnified centromeres. Scale bars: 5 μm. (D) GFP/CENP-B fluorescence intensity at centromeres in cells from B and C. Values are mean ± s.d.
activity and Aurora-B Ser331 phosphorylation are required for stable binding of Mps1 to kinetochores, in agreement with recent findings that Aurora-B regulates localization of Mps1 (Hewitt et al., 2010; Santaguida et al., 2010). Our results are consistent with a feedback mechanism between Mps1 and Aurora-B rather than a strictly linear pathway in which one kinase is upstream of the other (Jelluma et al., 2008b; Hewitt et al., 2010; Santaguida et al., 2010). Significantly, inhibition of Mps1 did not reduce phosphorylated Ser331 at kinetochores (Jelluma et al., 2008b; Santaguida et al., 2010; van der Waal et al., 2012b).

On the basis of those findings, we propose the following model for the role of Chk1 and Mps1 in correction of merotelic attachments (Fig. 8E). Chk1 phosphorylates Aurora-B Ser331 and this phosphorylation is required for sustained binding of MCAK and Kif2b to centromeres or kinetochores, optimal phosphorylation of Hec1 at Ser44 and Ser55 and correction of merotelic attachments before anaphase. Furthermore, Chk1 promotes stable binding of Mps1 to kinetochores by phosphorylating Aurora-B Ser331. In turn, Mps1 is required for initial recruitment of MCAK and Kif2b to centromeres or kinetochores and mitotic Hec1 phosphorylation, perhaps through phosphorylating Borealin. Our model raises several important questions regarding regulation of MCAK and Kif2b by Mps1 and Chk1, the significance of Ser331 phosphorylation for specific correction of kinetochore mis-attachments and the potential role of Chk1 and Mps1 in tumour cell killing by anti-mitotic agents.

Mps1 and Chk1 regulate initial binding and maintenance, respectively, of MCAK and Kif2b to centromeres or kinetochores. One possibility is that Chk1 and Mps1 regulate MCAK/Kif2b localization through modulating Aurora-B catalytic activity (Jelluma et al., 2008b; Petsalaki et al., 2011) and that different levels of Aurora-B activity are required for recruitment versus maintenance of MCAK and Kif2b. However, inhibition of Mps1, Chk1 or simultaneous inhibition of both kinases resulted in similar levels of Aurora-B activity as judged by Hec1 Ser55 or CENP-A Ser7 phosphorylation in the presence of taxol (Petsalaki et al., 2011). Alternatively, phosphorylation of Borealin by Mps1 and increased binding of Survivin to the CPC by Ser331 phosphorylation may influence substrate affinity of Aurora-B (Vader et al., 2006; Petsalaki et al., 2011). Furthermore, an Aurora-B-independent role for Mps1 in MCAK/Kif2b localization and error correction cannot be formally excluded. Mps1 phosphorylation targets include yeast Mad1 (Hardwick et al., 1996), yeast Ndc80/Hec1 (Kemmler et al., 2009) and vertebrate CENP-E mitotic proteins (Kim et al., 2010); however, a role for these phosphorylations in error correction has not been established.

Furthermore, Chk1-deficient cells treated with taxol for 1 hour exhibit MCAK and Kif2b staining at centromeres and
kinetochores despite reduced Mps1 kinetochore levels. One possibility is that the amount of Mps1 that remains bound to kinetochores is sufficient to recruit MCAK and Kif2b. Alternatively, Mps1 may regulate MCAK and Kif2b localization without being present at the kinetochore (Maciejowski et al., 2010; Liu and Winey, 2012).

How does Aurora-B specifically destabilize erroneous kMT attachments? One model proposes that Aurora-B creates a phosphorylation gradient by diffusing away from the centromere and can only reach its outer kinetochore substrates when sister kinetochores are under reduced tension (Cheeseman et al., 2002; Pinsky et al., 2006; Liu et al., 2009; Wang et al., 2011). Phosphorylation at Ser331 may therefore contribute to formation of a steep Aurora-B activity gradient between inner and outer kinetochore to fine-tune regulation of kinetochore–microtubule interactions (van der Waal et al., 2012a). Furthermore, reduced phosphorylation of Ser331 from prometaphase to metaphase may facilitate formation of amphitelic attachments by reducing phosphorylation of outer kinetochore Aurora-B substrates such as Hec1, while still allowing some levels of kMT turnover to ensure error correction late in mitosis, for example by diminishing the inhibitory phosphorylation of MCAK (Andrews et al., 2004; Lan et al., 2004; DeLuca et al., 2011).

Several kinases including Chk1, Mps1 and Aurora-B are required for error correction and optimal spindle checkpoint signalling, thus suggesting that mechanisms that regulate and monitor kinetochore–microtubule attachments are intertwined at the molecular level (Ditchfield et al., 2003; Santaguida et al., 2010; Elowe, 2011). Importantly, reducing the levels of certain proteins that have dual roles in checkpoint activation and chromosome bi-orientation can sensitise tumour cells to low levels of anti-mitotic drugs by enhancing chromosome mis-segregation (Kops et al., 2005; Janssen et al., 2009). Further understanding of the role of Chk1 and Mps1 in error correction may provide new prospects for improving cancer treatment.

Materials and Methods

Antibodies and plasmids

Monoclonal antibody against Chk1 (G-4) and polyclonal antibodies against CENP-B, GFP (FL) and TTK (Mps1; C-19) were from Santa Cruz Biotechnology. Monoclonal anti-Hec1 (9G3) and polyclonal anti-Aurora-B (ab2254) antibodies were from Abcam. Monoclonal antibodies against α-tubulin (DM1A) and actin (AC-40) were from Sigma, polyclonal antibody against avian CENP-O was from MBL and monoclonal anti-CENP-A (3-19) was from GeneTex. Anti-pS331 rabbit polyclonal antisera against phosphorylated Ser331 of human Aurora-B was previously described (Petsalaki et al., 2011). Anti-pS55 and anti-pS44 rabbit polyclonal antibodies against phosphorylated Ser55 and Ser44 of Hec1 were gifts from J. DeLuca (DeLuca et al., 2011) and SMP1.1 sheep polyclonal antisera against Mps1 was from S. Taylor (Tighe et al., 2008).

Plasmid MCAK:GFP coding for human MCAK fused to GFP was a gift from J. Swedlow (Andrews et al., 2004) and Kif2b:GFP plasmid coding for human Kif2b was from B. Orr and D. Compton (Bakhoum et al., 2009). Plasmid Mps1:GFP coding for human Mps1 fused to GFP was a gift from S. Taylor (Hewitt et al., 2010).
Cell culture and treatments

Chk1-deficient avian B-lymphoma DT40 cells (Chkl<sup>−−</sup>) or Chkl<sup>+/−</sup> cells expressing exogenous avian wild-type Chk1 encoded by a transfected transgene (revertant cells) or Chkl<sup>+/−</sup> cells reconstituted with DI30A avian Chk1 (kinase-dead cells) were as described (Zachos et al., 2003). The DI30A mutant Chk1 protein shows a shorter electrophoretic mobility but sequencing of the expression construct has confirmed that the DI30A is the only mutation (Bourke et al., 2007). Human hamster ovary CHO<sup>−−</sup> and CHO<sup>S331A</sup> expressing human wild-type or S331A mutant Aurora-B respectively under control of Tetracycline-inducible transgenes were previously described (Petsalaki et al., 2011).

Human colon carcinoma BE cells were grown in DMEM ( Gibco) containing 10% foetal bovine serum and CHO cells in Ham’s F12 (GIBCO) supplemented with 10% foetal bovine serum, at 37°C, 5% CO<sub>2</sub>, DT40 cells were cultured in DMEM containing 10% foetal bovine serum, 1% chicken serum, 10<sup>−3</sup>M β-mercaptoethanol, at 39.5°C, 5% CO<sub>2</sub>.

Cells were treated with 25 nM taxol (Applichem), 10 μg/ml MG132 (Merck), 300 nM VX680 (Selleckchem), 300 nM UCN-01 (Sigma), or 2 μM AZ3146 (Axon) as appropriate. To induce expression of Aurora-B transgenes, CHO<sup>−−</sup> or CHO<sup>S331A</sup> cells were treated with 17 ng/ml or 30 ng/ml Tetracycline (Sigma) respectively for 16 hours prior to analysis or further treatment with drugs (Petsalaki et al., 2011). Addition of Tetracycline stimulated accumulation of 6xMyc-Aurora-B<sup>WT</sup> or 6xMyc-Aurora-B<sup>S331A</sup> at approximate levels 10-fold higher than the endogenous protein and this level of expression was shown to disrupt endogenous Aurora-B functions while maintaining correct localization of 6xMyc-Aurora-B to centromeres (Petsalaki et al., 2011).

Negative siRNA or siRNA duplexes designed to repress human TTK (Mps1) or Chkl (Dharmacon) were transfected into BE cells 24–48 hours prior to analysis or reagents.

Recovery from MG132

Cells were treated with 10 μg/ml MG132 (Merck) for 3 hours. At the end of the treatment, cells were washed three times for 5 minutes with 37°C medium, cultured in fresh medium for 30–60 minutes and fixed as described below. Preliminary experiments had shown that the cells had accomplished metaphase to anaphase transition in 30–60 minutes and were observed in anaphase.

Nocodazole sensitivity assay

Cells were treated with 10 μg/ml MG132 for 3 hours in the absence or presence of 300 nM UCN-01. At the end of the treatment, 500 ng/ml nocodazole was added to cells for a further hour in nocodazole-free media while still exposed to MG132. Cells were fixed in paraformaldehyde in cytosol buffer and analysed by confocal microscopy as described below. Images were collected at 0.2 μm stacks over 10-μm depth, merged using Leica LCS Lite software and microtubule-associated fluorescence for each mitotic cell was quantified by analyzing an equal image area using Image J (NIH). To investigate stability of kMTs, microtubule-associated fluorescence values were normalized to the 2 minute time point because, after 2 minutes in nocodazole, the majority of non-kinetochore-microtubules is depolymerized as determined by confocal microscopy (supplementary material Fig. S2A).

Indirect immunofluorescence microscopy

For phospho-Hec1 (pS55 or pS44) staining, cells were pre-fixed in pre-warmed (37°C) 4% paraformaldehyde in Hec1-PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl<sub>2</sub>, 2 mM MgEGTA, 5 mM PIPES, 5 mM Glucose, pH 6.1) for 10 seconds at room temperature, permeabilized in pre-warmed (37°C) 4% paraformaldehyde in Phem buffer for 5 minutes at room temperature, fixed with cold methanol for 5 minutes at −20°C, washed twice with PBS and immunostained (Petsalaki et al., 2011).

To depolymerize the majority of non-kinetochore-microtubules and visualize merotelic attachments, cells were incubated in ice-cold medium for 15 minutes at 4°C, pre-fixed in pre-warmed (37°C) 4% paraformaldehyde in Phem buffer (60 mM PIPES, 25 mM HEPES pH 7.0, 10 mM EGTA, 4 mM MgSO<sub>4</sub>) for 10 seconds at room temperature, permeabilized in pre-warmed (37°C) Phem supplemented with 0.5% Triton X-100 for 5 minutes at room temperature, fixed in pre-warmed (37°C) 4% paraformaldehyde in Phem for 20 minutes at room temperature, washed twice with PBS and immunostained (Silkworth et al., 2009).

For all other fluorescence microscopy applications including nocodazole sensitivity assays, 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, 5 mM Glucose, pH 6.1) for 5 minutes at 37°C, permeabilised in 0.5% Triton X-100 in cytoskeleton buffer at room temperature and immunostained as appropriate (Zachos et al., 2007).

Fluorescein- (FITC) or Rhodamine-TRITC-conjugated secondary antibodies (Jackson ImmunoResearch) were used as appropriate, 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 Leica TCS SP2 laser scanning spectral confocal microscope, Leica LCM Lite software and a 63×1.40 Apochromat objective. The Leica 11513859 low fluorescence immersion oil used was and imaging was performed at room temperature. Average projections of image stacks were obtained using the Leica LCM Lite software.

To analyze fluorescence intensities, background readings were subtracted and fluorescence intensities quantified using Leica LCS Lite. The GFP values were normalized against the CENP-B or the Hec1 signal, the pS55 and pS44 against the Hec1 signal, the pS331 against the CENP-A signal and the Mps1 against the Hec1 signal. Several kinetochore pairs per cell from a minimum of three cells per experiment from two independent experiments were analyzed for each treatment.

Western blotting

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, 1 mM DTT, 5 μg/ml Leupetin, 50 μg/ml PMSF, 1 mM Benzamidine, 5 μg/ml Aprotinin, 1 mM Na<sub>3</sub>VO<sub>4</sub>) for 30 minutes on ice. Lysates were cleared by centrifugation at 15,000 g for 10 minutes and analyzed by SDS PAGE.

Statistical analysis

The P-values were calculated using the Student’s t-test.

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Author contributions

E.P. performed the experiments and analysed the results. G.Z. designed the experiments and wrote the paper.

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References


Fig. S1. Chk1-deficient cells exhibit anaphases with lagging chromosomes and merotelic attachments. (A) Examples of anaphases in BE cells transfected with negative siRNA (control), Chk1 siRNA (siChk1), or treated with UCN-01 for 3 h. Red: CENP-A; Blue: DNA. (B) Frequencies of anaphases with lagging chromosomes in BE cells treated as in (A). Error bars show the standard deviation from the mean from three independent experiments. A minimum of 50 anaphases were analyzed for each experiment. (C) Western blot analysis of total Chk1 or actin in wild-type (WT), Chk1-/-, kinase-dead (KD) or revertant (Rev) DT40 cells. (D) Examples of anaphases in DT40 cells. In Chk1-/-, a single kinetochore is connected to microtubules from both spindle poles. Green: CENP-O; Red: α-tubulin; Blue: DNA. Mis-segregated chromosomes are indicated by arrows. Insets show magnified kinetochores. Bars, 5 μm. (E) Frequencies of anaphases with lagging chromatin. Wild-type (WT) or Chk1-/- DT40 cells were analyzed in anaphase in the absence of drug treatment (-MG132) or after recovery from a 3 hour MG132 treatment (+MG132). Error bars show the standard deviation from the mean from three independent experiments. A minimum of 80 anaphases were analyzed for each experiment. n, total number of anaphases tested.
**Fig. S2.** Inhibition of Chk1 reduces sensitivity of kMTs to nocodazole and diminishes Hec1 Ser55 and Ser44 phosphorylations. 

(A) BE cells were treated with MG132 for 3 h in the absence (control) or presence of UCN-01 and exposed to high concentrations of nocodazole for the indicated times. 

(B) Mean pS55/Hec1 fluorescence intensity values in prometaphase (prometaph) and metaphase (metaphase) kMTs in BE cells treated as in (A). Error bars show the standard deviation from the mean.

(C) Phosphorylation of Hec1 Ser44 in BE cells treated as in (B). Boxed values show mean pS44/Hec1 fluorescence intensity. Green: pS44; Red: Hec1; Blue: DNA. Values in brackets represent kinetochore pairs quantified followed by the number of cells analyzed. Insets show magnified kinetochores. Bars, 5 μm.
Fig. S3. Phosphorylation of Aurora-B Ser331 at kinetochores is high in prometaphase and decreases in metaphase cells. (A) Ser331 phosphorylation. Boxed values show mean pS331/Hec1 fluorescence intensity. Green: pS331; Red: Hec1; Blue: DNA. (B) Aurora-B localization. Boxed values show mean Aurora-B/CENP-A fluorescence intensity. Green: Aurora-B; Red: CENP-A; Blue: DNA. (C) Mis-segregated chromosomes. Tetracycline-induced CHO<sup>WT</sup> or CHO<sup>S331A</sup> cells in anaphase are shown. Mis-segregated chromosomes are indicated by arrows. Red: CENP-B; Blue: DNA. (D) Localization of MCAK:GFP. Tetracycline-induced CHO<sup>WT</sup> expressing MCAK:GFP were untreated or treated with UCN-01 for 3 h. Boxed values show mean GFP/CENP-B fluorescence intensity. Green: GFP; Red: CENP-B; Blue: DNA. Values in brackets represent kinetochore pairs quantified followed by the number of cells analyzed. Insets show magnified kinetochores. Bars, 5 μm.
**Fig. S4. Expression of S331A Aurora-B diminishes phosphorylation of Hec1 Ser55.** (A,B) Tetracycline-induced CHO<sup>WT</sup> or CHO<sup>S331A</sup> cells were untreated or treated with UCN-01 for 3 h. Hec1 Ser55 phosphorylation in prometaphase (A) and metaphase (B) cells is shown. Boxed values show mean pS55/Hec1 fluorescence intensity. Green: pS55; Red: Hec1; Blue: DNA. Values in brackets represent kinetochore pairs quantified followed by the number of cells analyzed. (C) Mean pS55/Hec1 fluorescence intensity values at kinetochores. Tetracycline-induced CHO<sup>WT</sup> and CHO<sup>S331A</sup> cells were untreated, treated with UCN-01 or VX680 (VX) for 3 h. Error bars show the standard deviation from the mean. (D,E) Example of anaphases with lagging chromosomes (D) and a merotelic attachment (E) in BE cells transfected with negative siRNA (control) and treated with AZ3146 (AZ) for 3 h. (D) Lagging chromosomes are indicated by arrows. Red: CENP-A; Blue: DNA. (E) Green: α-tubulin; Red: CENP-B; Blue: DNA. Insets show magnified kinetochores. Bars, 5 μm. (F) Frequencies of anaphases with lagging chromosomes. BE cells transfected with negative siRNA (control) or Chk1 siRNA (siChk1) were untreated or treated with AZ3146 (AZ) for 3 h and analyzed in anaphase. Error bars show the standard deviation from the mean from three independent experiments. A minimum of 60 anaphases were analyzed for each experiment. n, total number of anaphases tested. (G) Western blot analysis of total Mps1 and actin in BE cells transfected with negative siRNA (control) or Mps1 siRNA (siMps1).
Fig. S5. Inhibition of Mps1 diminishes Hec1 Ser55 phosphorylation. (A,B) Localisation of MCAK:GFP (A) and Kif2b:GFP (B) in BE cells transfected with Mps1 siRNA (siMps1). Boxed values show mean GFP/CENP-B fluorescence intensity. Green: GFP; Red: CENP-B; Blue: DNA. (C,D) Hec1 Ser55 phosphorylation. (C) BE cells transfected with negative siRNA (control) or Chk1 siRNA (siChk1) were treated with AZ3146 (AZ) for 3 h and analysed in metaphase. (D) BE cells transfected as in (C) were treated with AZ3146 (AZ), taxol and MG132 for 1 h. Boxed values show mean pS55/Hec1 fluorescence intensity. Green: pS55; Red: Hec1; Blue: DNA. (E) Localization of Mps1. BE cells transfected as in (C) were treated with AZ3146 (AZ), taxol and MG132 for 6 h. Boxed values show mean Mps1/Hec1 fluorescence intensity. Green: Mps1; Red: Hec1; Blue: DNA. Values in brackets represent kinetochore pairs quantified followed by the number of cells analyzed. Insets show magnified kinetochores. Bars, 5 μm.
Fig. S6. Inhibition of Mps1 does not reduce Ser331 phosphorylation. BE cells were untreated, treated with AZ3146 ( AZ) or UCN-01 for 3 h. Boxed values show mean pS331/CENP-A fluorescence intensity. Green: pS331; Red: CENP-A; Blue: DNA. Values in brackets represent kinetochore pairs quantified followed by the number of cells analyzed. Insets show magnified kinetochores. Bars, 5 μm.