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Mps1 Phosphorylates Borealin to Control Aurora B Activity and Chromosome Alignment

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SUMMARY

Maintenance of chromosomal stability relies on coordination between various processes that are critical for proper chromosome segregation in mitosis. Here we show that monopolar spindle 1 (Mps1) kinase, which is essential for the mitotic checkpoint, also controls correction of improper chromosome attachments. We report that Borealin/DasraB, a member of the complex that regulates the Aurora B kinase, is directly phosphorylated by Mps1 on residues that are crucial for Aurora B activity and chromosome alignment. As a result, cells lacking Mps1 kinase activity fail to efficiently align chromosomes due to impaired Aurora B function at centromeres, leaving improper attachments uncorrected. Strikingly, Borealin/DasraB bearing phosphomimetic mutations restores Aurora B activity and alignment in Mps1-depleted cells. Mps1 thus coordinates attachment error correction and checkpoint signaling, two crucial responses to unproductive chromosome attachments.

INTRODUCTION

Equal segregation of chromosomes during cell division depends on a coordinated effort to attach and align all chromosomes before onset of anaphase. Proper execution of these processes is monitored by the mitotic checkpoint that halts cell-cycle progression until all paired sister chromatids are attached via their kinetochores to opposite poles (bioriented) and aligned on the metaphase plate. The mitotic checkpoint responds to lack of attachment of kinetochores to spindle microtubules or lack of tension between kinetochores of sister chromatids. Checkpoint signal transduction from the kinetochore depends on several kinases including Bub1, BubR1, and Mps1, and culminates in production of an inhibitor of the E3 ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C), whose activity is required for anaphase onset (Kops et al., 2005).

The mitotic checkpoint is necessarily active when chromosomes establish bipolar attachments in order to align. Interestingly, some proteins essential for checkpoint signaling also contribute to attachment processes. For example, generation of stable attachments of kinetochores to spindle microtubules requires BubR1 (Ditchfield et al., 2003; Lampson and Kapoor, 2005), while Bub1 is essential for centromeric cohesion in prometaphase (Kitajima et al., 2005; Tang et al., 2004) and establishment of end-on attachments (Johnson et al., 2004; Meraldi and Sorger, 2005). Recently, TAO1/MARKK was found to be a novel kinase that is essential for both the mitotic checkpoint and chromosome alignment (Draviam et al., 2007). These kinases are therefore crucial activities in coordinating various mitotic processes, but direct substrates that exert control over these processes have yet to be identified for any of the kinases.

In early mitosis, as chromosomes attempt to biorient, various erroneous attachments are made that result in lack of tension between sister centromeres and that need to be corrected to allow proper chromosome alignment. This attachment error correction is controlled by the chromosomal passenger complex (CPC) of which the Aurora B kinase is the effector enzyme (Ditchfield et al., 2003; Hauf et al., 2003; Lampson et al., 2004; Tanaka et al., 2002). In vertebrates, the CPC facilitates error correction by Aurora B-dependent phosphorylation of the microtubule-binding Ndc80/Hec1 complex and the kinesin 13 microtubule depolymerase MCAK (Andrews et al., 2004; Cheeseman et al., 2006; DeLuca et al., 2006; Lan et al., 2004). Aurora B activity is also required for the checkpoint response to lack of tension, likely through creating unattached kinetochores during the correction process (Pinsky et al., 2006), but direct, microtubule-independent involvement of Aurora B in checkpoint function has also been suggested (Kallio et al., 2002; Morrow et al., 2005). At the metaphase-to-anaphase transition, Aurora B relocates from centromeres to the central spindle, where it conducts the final stages of cytokinesis. Besides Aurora B, the CPC includes INCENP, Survivin, and Borealin/DasraB (hereafter referred to as Borealin) (Adams et al., 2000; Bolton et al., 2002; Gassmann et al., 2004; Kaitna et al., 2000; Sampath et al., 2004). Although specific functions in the spatiotemporal control of Aurora B activity have been suggested for each of these auxiliary proteins

(Vader et al., 2006b), a clear picture for how Aurora B is localized and activated at centromeres is lacking.

In *Saccharomyces cerevisiae*, Mps1 controls spindle-pole body duplication (Winey et al., 1991), spindle assembly (Jones et al., 2005), and the spindle-assembly checkpoint (Weiss and Winey, 1996). Mutant Mps1 alleles or chemical inhibition in yeast have implicated the enzymatic activity of Mps1 in its control over these processes (Dorer et al., 2005; Jones et al., 2005; Schutz and Winey, 1998). In higher eukaryotes, the only undebated role for Mps1 during mitosis is in the mitotic checkpoint (Abrieu et al., 2001; Liu et al., 2003; Stucke et al., 2002), which in *Xenopus* egg extracts depends on its kinase activity (Abrieu et al., 2001). Mps1 has further been implicated in centrosome duplication, though this is controversial (Fisk et al., 2003; Stucke et al., 2002).

Using shRNA-based protein replacement, we set out to investigate the contribution of Mps1 kinase activity to mitotic progression in human cells. Here we show that Mps1 kinase activity is essential for chromosome alignment by enhancing Aurora B activity at the centromere, and we identify the Aurora B-regulatory protein Borealin/DasraB as an essential substrate that mediates this novel function of Mps1.

RESULTS

Mps1 Kinase Activity Is Essential for Mitotic Checkpoint Signaling and Chromosomal Stability

To investigate what mitotic processes in human cells rely on Mps1 kinase activity, endogenous Mps1 was replaced with a kinase-deficient mutant of Mps1 (D664A) (Stucke et al., 2002) in human cancer cell lines by simultaneous expression of plasmid-based Mps1 shRNA and RNAi-insensitive epitope-tagged Mps1 alleles (LAP-Mps1) (Cheeseman and Desai, 2005) (Figures 1A, 1B, and S1A; see Experimental Procedures for details). Depletion of Mps1 prevented cells from accumulating in mitosis upon treatment with the spindle poison nocodazole, confirming a role for Mps1 in mitotic checkpoint activation (Figures 1C and 1D) (Abrieu et al., 2001; Liu et al., 2003; Stucke et al., 2002). Similar results were obtained with taxol (Figure S1B). As reported previously (Fisk et al., 2003; Liu et al., 2003; Martin-Lluesma et al., 2002), the essential mitotic checkpoint proteins Mad1 and Mad2 but not CENP-E, BubR1 or Bub1 were absent from unattached kinetochores of cells lacking Mps1 (Table S1 and Figure S1C). Mitotic checkpoint signaling in response to nocodazole (Figures 1C and 1D) and taxol (Figure S1B), as well as Mad1 localization (Figures S1C and S1D) were restored by expression of wild-type but not kinase-dead Mps1 to similar levels. This proves that kinase activity of Mps1 is indispensable for the mitotic checkpoint in human cells. As expected from previous studies on mitotic checkpoint inhibition (Kops et al., 2004), Mps1 kinase activity was also essential for the maintenance of ploidy and survival of human cancer cells (Figures S1E and S1F).

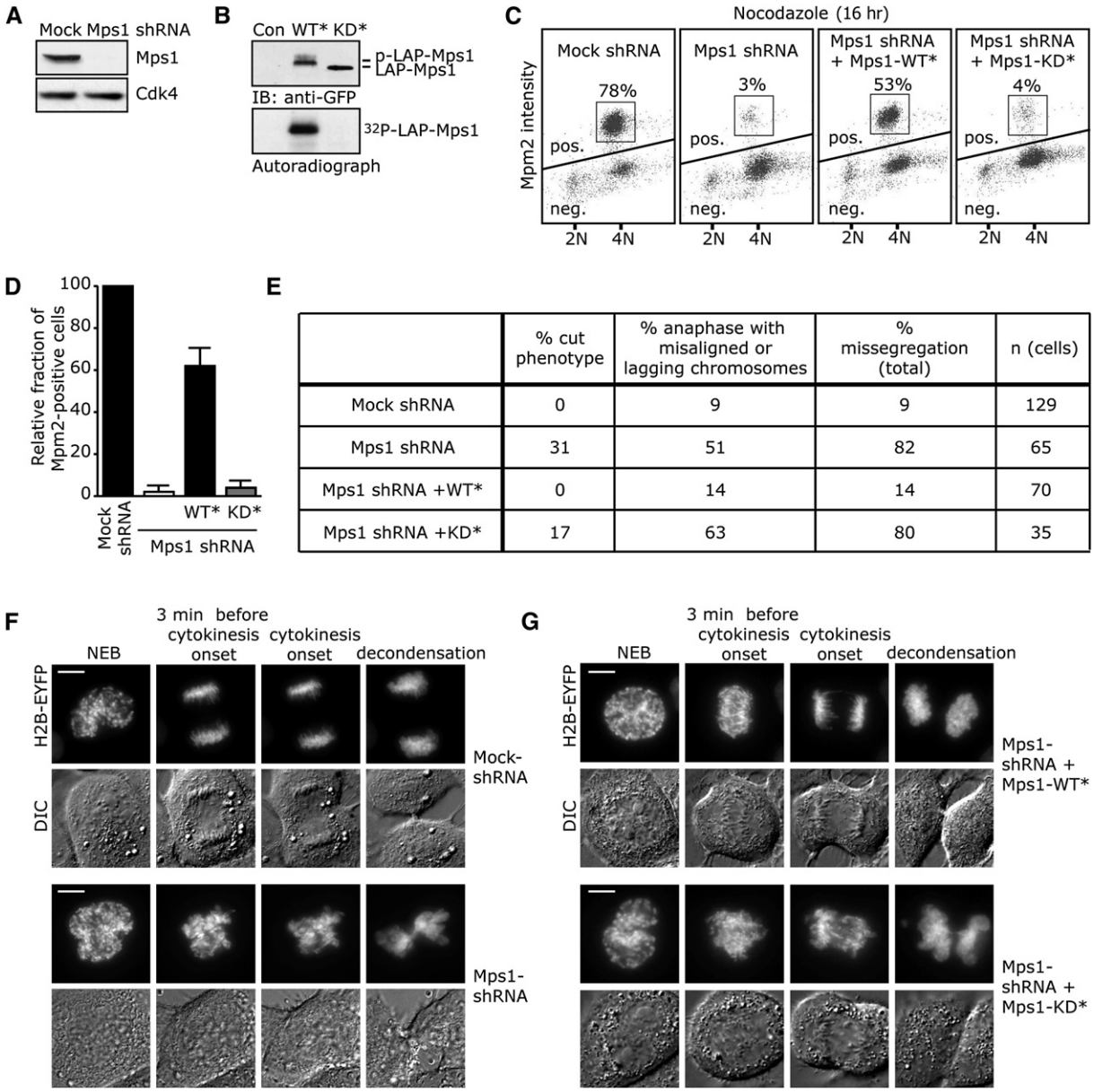
To get insight into the roles of Mps1 kinase activity during unperturbed mitosis, chromosome segregation was analyzed by time-lapse microscopy of chromosomes loaded with fluorescent histones (H2B-EYFP). Anaphase A movements were apparent in 69% of Mps1-depleted cells but the majority of those cells initiated anaphase with misaligned chromosomes (Figure 1E and Movie S1). In the remaining 31% of cells no metaphase plate was formed and no anaphase was noticeable before the onset of cytokinesis. Instead, cells displayed a ''cut'' phenotype: chromosomes remained condensed and hardly moved before the DNA pack was split in two by the incoming cleavage furrow during cytokinesis (Figures 1E, 1F, and Movie S2). Since Mps1 shRNA was transfected transiently, the difference in severity of the two observed phenotypes may be explained by differences in extent of knockdown of Mps1. Nevertheless, regardless of whether anaphase was observed or not, reducing Mps1 protein levels resulted in massive chromosome missegregation in 82% of all divisions analyzed (Figure 1E). This could be attributed specifically to inhibition of Mps1 kinase activity, as re-expression of shRNA-insensitive wild-type but not kinase-dead Mps1 restored proper chromosome segregation (Figures 1E and 1G).

Efficient Chromosome Alignment Requires Mps1

Initiation of chromosome segregation in the presence of misaligned chromosomes in cells lacking Mps1 kinase activity could simply have been due to premature APC/C activation, or may have been caused by problems in chromosome alignment. To discriminate between these possibilities, exit from mitosis was blocked by treatment with the proteasome inhibitor MG132, allowing cells more time to align their chromosomes. Strikingly, the majority of Mps1-depleted cells had misaligned chromosomes even after spending one hour in mitosis, while control cells had reached full alignment during this time (Figure 2A). These misalignments were independent of mitotic checkpoint inactivity, as cells depleted of Mad2 had no difficulty aligning all chromosomes (Figure 2B) (Lampson and Kapoor, 2005). Analysis of chromosome movements in real time further revealed that 85% of Mps1-depleted cells versus 10% of control cells showed misaligned chromosomes 30 min after entry into mitosis in the presence of MG132 (Figures 2C and 2D). After 2 hr, 52% of Mps1-depleted cells still contained one or more chromosomes that had not reached the metaphase plate compared to 3% of mock-shRNA cells. Replacement of endogenous Mps1 with a kinase-dead mutant showed that chromosome alignment required Mps1 kinase activity (Figure 2E). In agreement with this, simultaneous treatment of prophase cells with MG132 and SP600125, a small molecule that inhibits Mps1 in mitotic human cells (Schmidt et al., 2005), caused severe misalignments that persisted until removal of the inhibitor 75 min after addition (Figure 2F and Movie S3). Together, these data show that Mps1 activity contributes to alignment of chromosomes on the metaphase plate in mitosis.

Interactions between Kinetochores and Spindle Microtubules Are Stable in Cells Lacking Mps1

We next examined what process required for chromosome alignment was defective in Mps1-depleted cells. The following observations suggested that misalignments were not caused by general defects in spindle assembly or stable microtubule capture by the kinetochore. First, interkinetochore distances of aligned chromosomes in Mps1-depleted cells were similar to those of control cells (Figure 3A), showing that sufficiently strong attachments were generated that could impose normal tension between sister centromeres. Second, no obvious differences in spindle morphology or density of cold-stable kinetochore



	% cut phenotype	% anaphase with misaligned or lagging chromosomes	% missegregation (total)	n (cells)
Mock shRNA	0	9	9	129
Mps1 shRNA	31	51	82	65
Mps1 shRNA +WT*	0	14	14	70
Mps1 shRNA +KD*	17	63	80	35

Figure 1. Mps1 Kinase Activity Is Essential for Mitotic Checkpoint Signaling and Chromosome Segregation

(A) Immunoblots of lysates of U2OS cells transfected with the indicated shRNA plasmids.

(B) Kinase activity of anti-GFP immunoprecipitates of cells transfected with empty vector (Con) or RNAi-resistant (indicated by asteriks) LAP-tagged wild-type (WT*) or kinase-dead (KD*) Mps1.

(C and D) Flow cytometric analysis of mpm2 positivity within a population of cells transfected with mock or Mps1 shRNA plasmids along with the indicated RNAi-resistant Mps1 alleles and treated with nocodazole for 16 hr. Cells considered positive (pos.) or negative (neg.) for mpm2 are indicated. Graph in (D) represents averages of five independent experiments (± SD).

(E) Table summarizing data of time-lapse analyses as performed under (F) and (G). "Percent anaphase with misaligned or lagging chromosomes" indicates all abnormal segregations in which anaphase chromosome movements were observed (excluding "cut" phenotypes).

(F and G) Time-lapse analysis of chromosome segregation (H2B-EYFP) and morphology (DIC) of cells transfected with H2B-EYFP along with the indicated shRNA plasmids (F) in combination with either empty vector or the various RNAi-resistant Mps1 alleles (G). Stills from the time-lapse analyses that represent the indicated stages are presented. Scale bars are 5 μm.

microtubules were detected between mock- and Mps1-depleted cells (Figure 3B). As a control, Nuf2-depleted cells (Figure S6A) showed many misaligned chromosomes that lacked obvious interactions with spindle microtubules (Figure 3B). Third, alignment was maintained when SP600125 was added after chromosomes had reached full alignment (Figure 3C). These three measurements excluded fundamental defects in spindle assembly and stable microtubule capture by kinetochores in

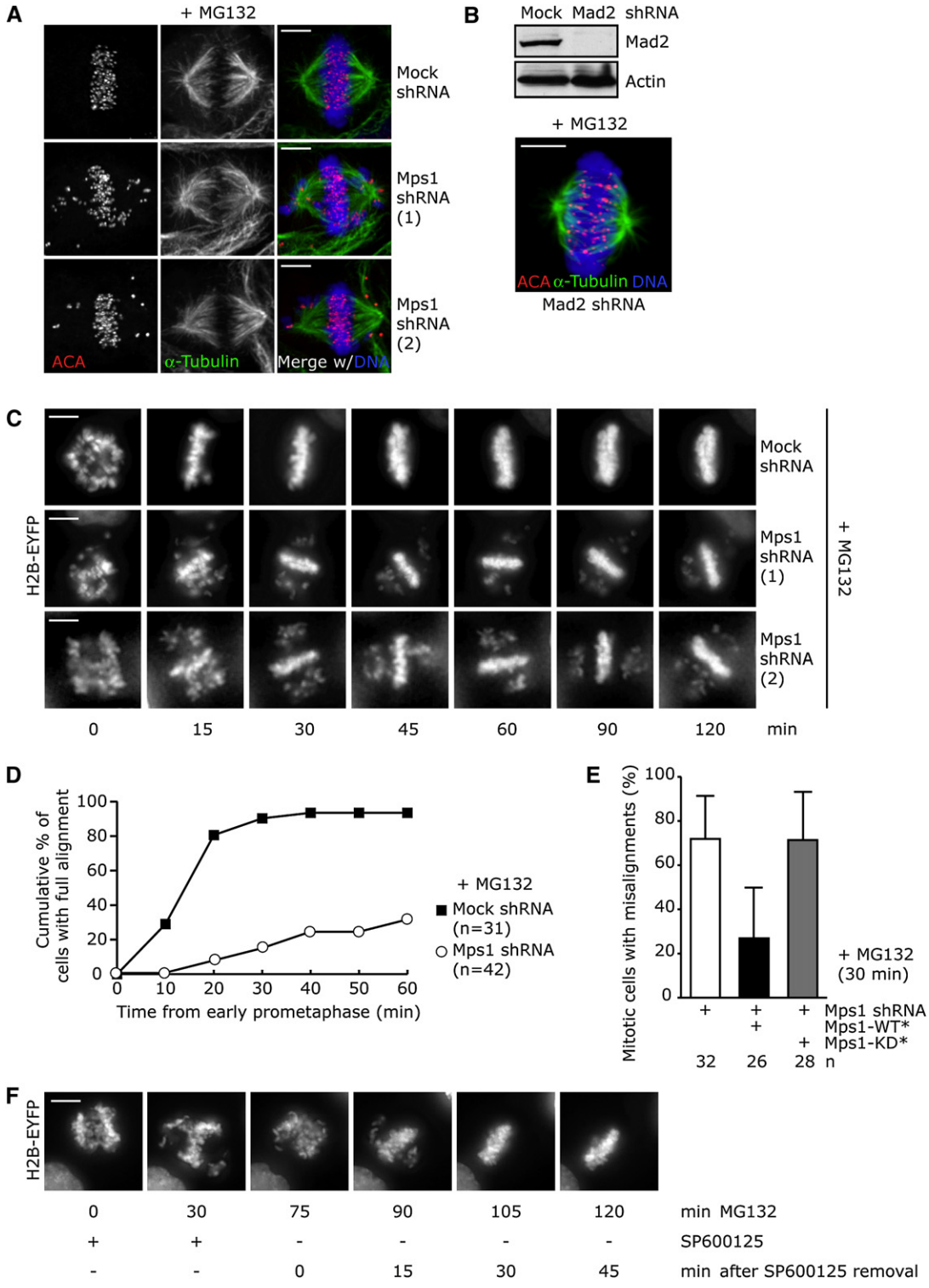


Figure 2. Lack of Mps1 Kinase Activity Causes Severe Chromosome Misalignments

(A and B) Chromosome alignment in HeLa cells transfected as in Figure 1F (A) or with Mad2 shRNA (B) and treated with MG132 for 1 hr. Cells were stained for α-tubulin, centromeres (ACA), and DNA (DAPI). Eighty-four percent of cells analyzed as in (A) had misaligned chromosomes (average of four experiments). Scale bars are 5 μm.

(C and D) Time-lapse analysis of chromosome alignment in cells transfected with H2B-EYFP along with the indicated shRNA plasmids and treated with MG132 2–5 min prior to start of image acquisition.

cells lacking Mps1. However, as they were primarily focused on the aligned chromosomes, we could not exclude the possibility that the misaligned chromosomes had experienced difficulties in microtubule capture. To examine this, kinetochores were analyzed for the presence of CLIP-170. This microtubule-binding protein localizes specifically to unattached kinetochores in a mitotic checkpoint-independent manner and leaves the kinetochore upon microtubule capture (Dujardin et al., 1998; Tanenbaum et al., 2006). Kinetochores of both aligned and misaligned chromosomes in MG132-treated cells lacking Mps1 had no detectable CLIP-170, whereas the occasional misaligned chromosome in MG132-treated, mock-shRNA-transfected cells had recruited high levels of CLIP-170 on at least one of its kinetochores (Figures 3D and 3E). Because binding of CLIP-170 to unattached kinetochores did not depend on Mps1 in nocodazole-treated or early prometaphase cells (Figures 3F and 3G and Table S1), absence of CLIP-170 on kinetochores of the misaligned chromosomes in the MG132-treated, Mps1-depleted cells was indicative of attachment of those kinetochores to microtubules. Although ultrastructural analysis of kinetochore-microtubule interactions is needed to rigorously exclude possible defects in stability of these interactions, our indirect analyses indicate that such defects as a cause for misalignments in cells depleted of Mps1 is unlikely.

Mps1 Activity Is Required for Efficient Correction of Erroneous Attachments

In most Mps1-depleted cells, some misaligned chromosomes were adjacent to the spindle poles (Figures 2A and 3B). This phenotype is reminiscent of cells depleted of CENP-E activity, a plus-end-directed kinesin required for efficient chromosome congression (reviewed in Cleveland et al., 2003). Nevertheless, the many misaligned chromosomes in MG132-treated, Mps1-depleted cells as well as the occasional misalignment in mock shRNA cells recruited high levels of CENP-E (Figures 4A and 4B and Table S1). Similar results were obtained with nocodazole-treated cells or with cells treated with the Eg5 inhibitor S-trityl-L-cysteine (STLC) (Skoufias et al., 2006), which causes monopolar spindles with mono-oriented chromosomes but leaves microtubule dynamics unaffected (Figure 4B and Table S1).

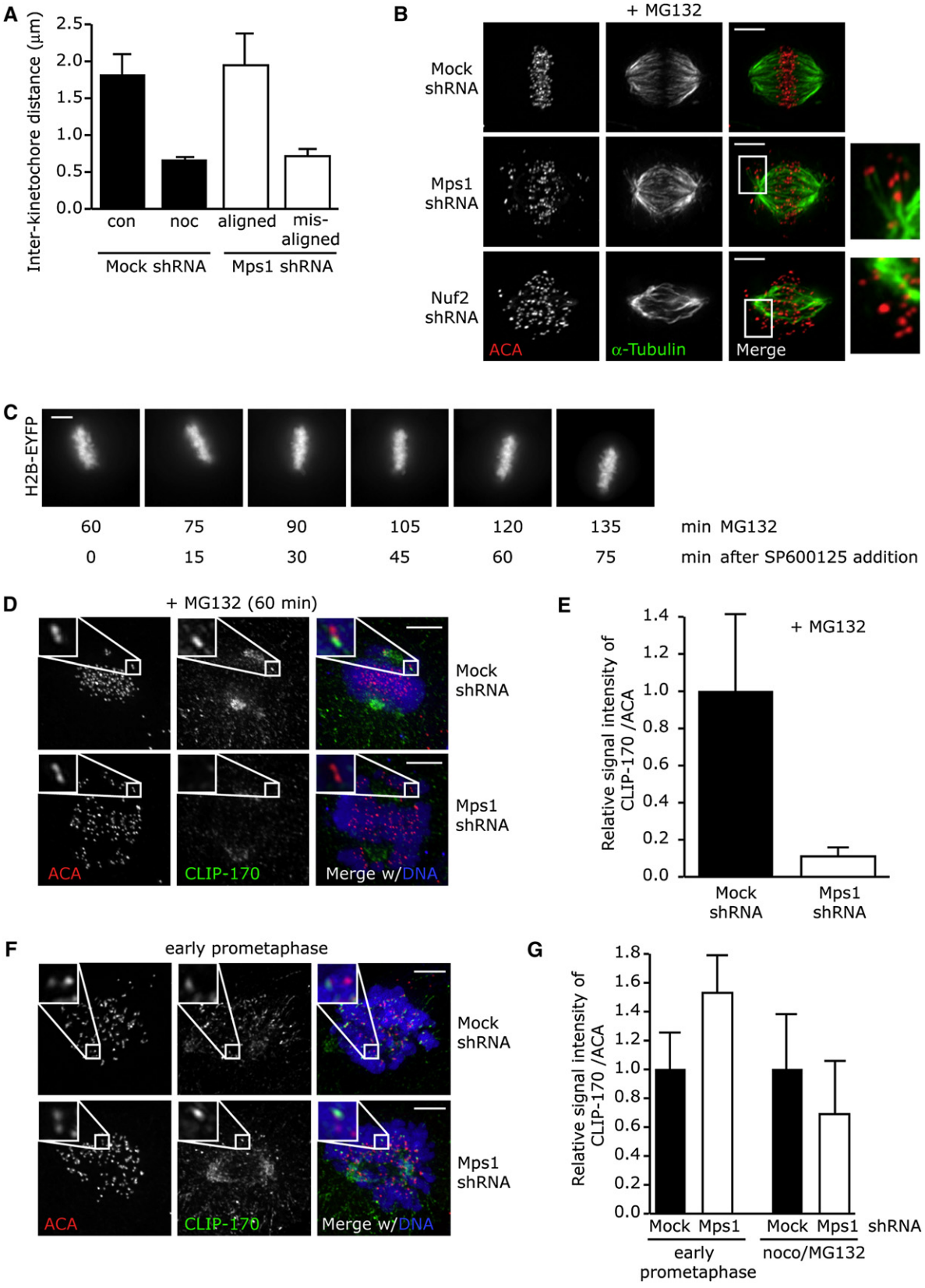
We next investigated the ability of Mps1-depleted cells to correct faulty attachments, a process that is controlled by Aurora B kinase activity at centromeres (Hauf et al., 2003; Lampson et al., 2004; Tanaka et al., 2002). To this end, Mps1-depleted cells were released from monastrol into MG132. Like STLC, monastrol causes monopolar spindles with large numbers of chromosomes that have syntelic or monotelic attachments (Kapoor et al., 2000). Unlike STLC, monastrol is efficiently removed from cells, which allows the formation of a bipolar spindle in which full chromosome alignment requires correction of the improper attachments by Aurora B (Khodjakov et al., 2003; Lampson et al., 2004). While complete alignment was achieved in control cells 90 min after release from monastrol, many misaligned chromosomes, a subset of which was clearly attached in a syntelic manner, were observed in almost all (97%) Mps1-depleted cells (Figure 4C). Similar results were obtained when SP600125 was used to inhibit Mps1 during release from monastrol (Figure S2A). Improper attachments lead to absence of tension between sister centromeres, causing Aurora B activity to destabilize kinetochore-spindle microtubule interactions, which results in unattached kinetochores (Ditchfield et al., 2003; Hauf et al., 2003; Lampson et al., 2004; Tanaka et al., 2002). To investigate if such destabilizations still took place in the absence of Mps1, CLIP-170 levels on kinetochores in cells treated with STLC were examined. Whereas 35% of kinetochores were unattached in mock-depleted cells, only 3% unattached kinetochores were detected in cells lacking Mps1 (Figures 4D and 4E). Together, these results support the hypothesis that attachment error correction by Aurora B is impaired when Mps1 is removed.

Mps1 Enhances Aurora B Activity at Centromeres

The contribution of Mps1 activity to attachment error correction was investigated by analyzing Aurora B localization and activity, which requires the auxiliary proteins INCENP, Survivin, and Borealin (Ruchaud et al., 2007; Vader et al., 2006b). Interestingly, Aurora B was present at normal levels on inner centromeres of chromosomes in Mps1-depleted cells (Figures 5A and 5C and Table S2). In agreement with a role for Survivin in targeting Aurora B to the inner centromere (Vader et al., 2006a), Survivin levels on inner centromeres and in Aurora B immunoprecipitates were unaffected by depletion of Mps1 (data not shown and see Figure 6H). As Survivin interacts with Aurora B indirectly by binding INCENP (Bolton et al., 2002; Vader et al., 2006a), this indicated that assembly of the CPC does not depend on Mps1 activity. In contrast, Aurora B kinase activity was diminished on centromeres of monastrol-treated, Mps1-depleted cells, as evidenced by low levels of phosphorylated CENP-A, an endogenous centromeric Aurora B substrate (Zeitlin et al., 2001) (Figures 5B and 5C and Table S2) and low levels of phosphorylated histone H3, another endogenous Aurora B substrate (Vader et al., 2006b), in mitotic extracts (Figure 5D). Moreover, in vitro kinase activity of Aurora B immunoprecipitated from mitotically arrested cells was 3-fold lower in Mps1-depleted cells than in control cells (Figures 5E, 5F, S2B, and S2C). In agreement with this, Aurora B autophosphorylation on Thr232 was substantially diminished in Mps1-depleted cells (Figure S3 and Table S2). Aurora B activity has been proposed to control Mps1 localization in *Xenopus* egg extracts (Vigneron et al., 2004), which would be at odds with our observation that Mps1 activity controls Aurora B function. However, efficient inhibition of Aurora B activity by the inhibitory compound ZM447439 (Ditchfield et al., 2003) did not affect Mps1 localization to unattached kinetochores in HeLa cells (Figure 5G). Thus, our results support the hypothesis that

(E) Percentage (± SD) of cells containing misaligned chromosomes 30 min after onset of mitosis in the presence of MG132 of cells transfected as in Figure 1G and treated with MG132 for 30 min.

(F) Time-lapse analysis of HeLa cells stably expressing H2B-EYFP and treated simultaneously with MG132 and SP600125. In most instances (five of six), severe chromosome misalignment was observed for the duration of this treatment. Seventy-five minutes after the initial treatment, cells were washed, and image acquisition of the same cells in media containing only MG132 continued for 45 min. All cells had reached alignment by that time.


A
Inter-kinetochore distance (μm)
2.5
2.0
1.5
1.0
0.5
0.0
con
noc
aligned
mis-aligned
Mock shRNA
Mps1 shRNA
B
+ MG132
Mock shRNA
Mps1 shRNA
Nuf2 shRNA
ACA
α-Tubulin
Merge
C
H2B-EYFP
60 75 90 105 120 135 min MG132
0 15 30 45 60 75 min after SP600125 addition
D
+ MG132 (60 min)
Mock shRNA
Mps1 shRNA
ACA
CLIP-170
Merge w/DNA
E
+ MG132
Relative signal intensity of CLIP-170 /ACA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Mock shRNA
Mps1 shRNA
F
early prometaphase
Mock shRNA
Mps1 shRNA
ACA
CLIP-170
Merge w/DNA
G
Relative signal intensity of CLIP-170 /ACA
1.8
1.6
1.4
1.2
1.0
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0.6
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Mock Mps1 Mock Mps1 shRNA
early prometaphase
noco/MG132

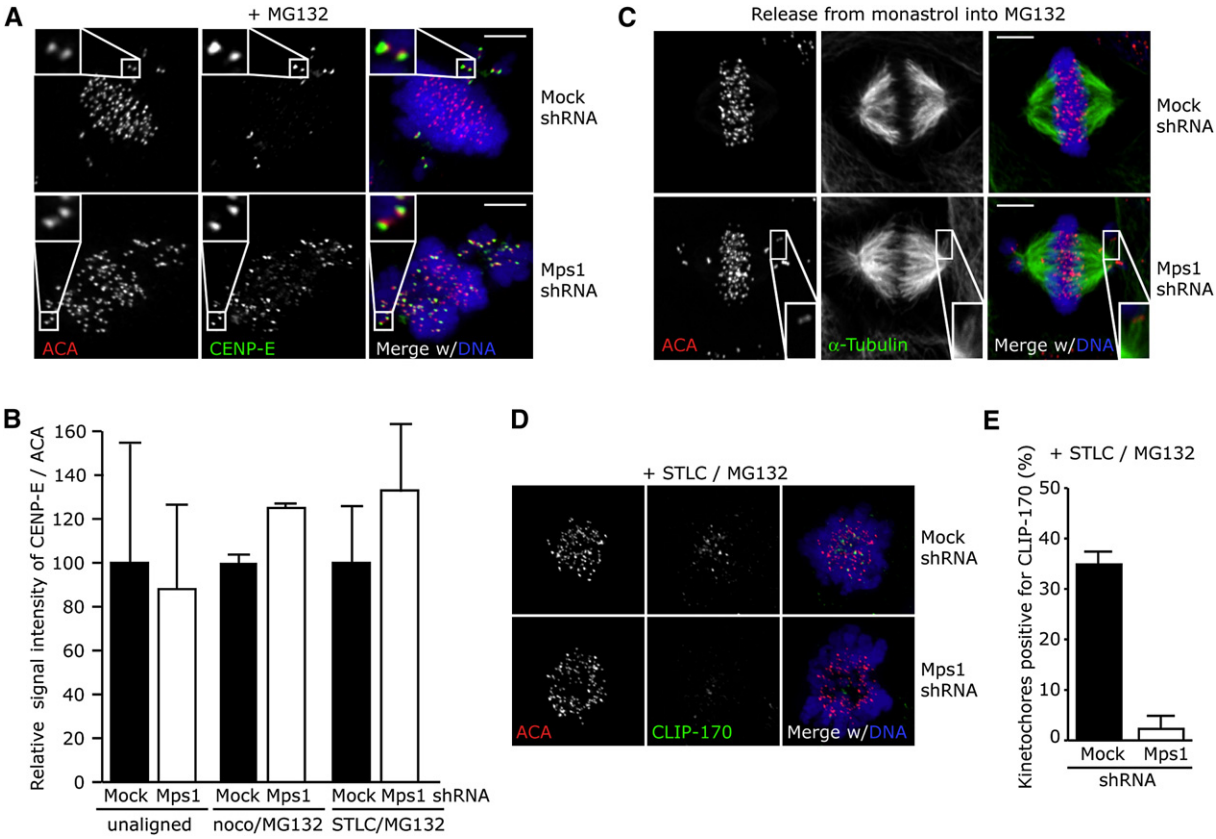

Figure 4. Mps1 Facilitates Attachment Error Correction

(A and B) Immunolocalization of CENP-E and centromeres (ACA) in cells transfected as in Figure 2A and treated with MG132 for 45 min (A) or with nocodazole/MG132 or STLC/MG132 (B) for 60 min. Details of graph (± SEM) in (B) are displayed in Table S1.

(C) Analysis of chromosome alignment in cells transfected with mock or Mps1 shRNA plasmids. Cells were treated with monastrol for 1 hr, after which MG132 was added for another hour to prevent Mps1-depleted cells from prematurely exiting mitosis. Cells were then released from monastrol into MG132 for 90 min. Enlarged region in Mps1 shRNA panel shows syntelic attachment of a pair of misaligned sister chromatids. Ninety-seven percent of Mps1-depleted cells showed misalignments in this assay (average of 40 cells from two experiments).

(D and E) Immunolocalization of CLIP-170 and centromeres (ACA) in cells transfected as in Figure 2A and treated with STLC/MG132 for 60 min. CLIP-170-positive kinetochores in (E) were defined as having levels of more than 2X background (± SEM). Scale bar is 5 μm.

Mps1 contributes to full Aurora B activity at inner centromeres of human cells without affecting its localization.

Direct Phosphorylation of the Aurora B Regulator Borealin by Mps1 Enhances Aurora B Activity and Is Essential for Chromosome Alignment

To examine if Mps1 could contribute to Aurora B function directly, various complex members were tested as substrate for recombinant Mps1 in an in vitro kinase assay. Whereas Aurora B and Survivin were untouched by Mps1, Borealin was efficiently phosphorylated (Figure 6A). Analysis of the phosphorylated GST-Borealin protein by mass spectrometry identified four Mps1-dependent phosphorylation sites (Figures 6A and S4). GST-Borealin in which all four sites were mutated to alanine (4TA) was a poor substrate for Mps1, showing that the majority of Mps1-dependent phosphorylation sites had been identified (Figure 6B). To investigate the contribution of phosphorylation by Mps1 to Borealin function, shRNA-resistant VSV-tagged Borealin-4TA or Borealin-4TD (in which the phosphorylated threonines were substituted for phosphomimetic aspartate residues)

Figure 3. Mps1 Depletion Has No Severe Effect on Kinetochore-Spindle Microtubule Interactions

(A) Interkinetochore distances (± SEM) of cells transfected and treated as in Figure 2A or treated with nocodazole for 30 min (noc) and immunostained for centromeres (ACA). Distances were measured for at least five pairs per cell, ten cells per treatment, three independent experiments. Scale bars are 5 μm.

(B) Calcium-resistant microtubules in cells transfected and treated as in (A) and immunostained for α-tubulin and centromeres (ACA). Quantification of tubulin levels revealed no difference between mock- and Mps1-depleted cells (not shown).

(C) Time-lapse analysis of HeLa cells stably expressing H2B-EYFP and treated with MG132. After 60 min, SP600125 was added and image acquisition continued. Thirty minutes after addition of SP600125, 86% (12 of 14) of cells had maintained alignment.

(D–G) Immunolocalization of CLIP-170 and centromeres (ACA) in cells transfected as in Figure 2A and either left untreated (F and G), treated with MG132 (D and E) or nocodazole/MG132 (G) for 30 min. Details of graphs (± SEM) in (E) and (G) are displayed in Table S1.

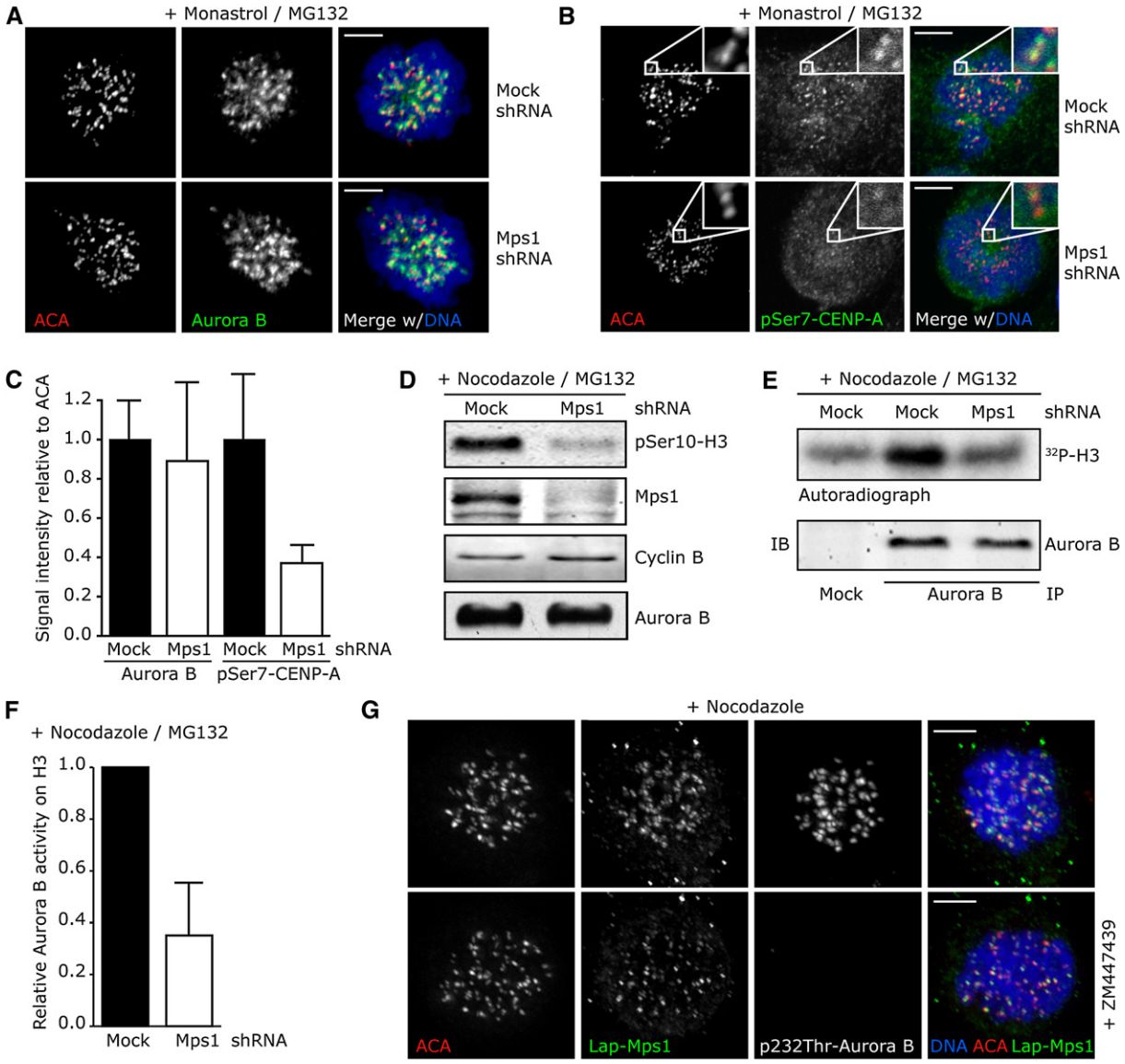

Figure 5. Full Aurora B Kinase Activity Requires Mps1

(A and B) Immunolocalization of Aurora B (A) or pSer7-CENP-A (B) and centromeres (ACA) in cells transfected with mock or Mps1 shRNA plasmids and treated with monastrol for 30 min. Scale bars are 5 μm.

(C) Quantification of pSer7-CENP-A intensities as a ratio of the ACA signal at kinetochores of cells treated as in (B). Details of graph (± SEM) are displayed in Table S2.

(D) Immunoblots of the indicated proteins in lysates of cells transfected with mock or Mps1 shRNA plasmids in combination with pBabe-puro, selected with puromycin and treated with nocodazole and MG132 for 2 hr.

(E and F) In vitro kinase activity toward recombinant histone H3 of Aurora B immunoprecipitated from cells transfected and treated as in (D). Graph in (F) represents average of three independent experiments (± SD).

(G) Immunolocalization of Mps1 (anti-GFP), pT232-Aurora B, and centromeres (ACA) of HeLa cells stably expressing LAP-Mps1 and treated with nocodazole ± ZM447439 for 1 hr.

were expressed in U2OS cells in the background of Borealin RNAi (Figures 6C and S5A) and fidelity of chromosome alignment was analyzed by treating cells with MG132 for 90 min. The severe defects in chromosome alignment upon Borealin depletion (Gassmann et al., 2004; Sampath et al., 2004) were rescued by expression of both shRNA-resistant wild-type Borealin (Borealin-WT) or Borealin-4TD (Figure 6C). On the other hand, Borealin-4TA, while correctly localized and expressed to similar levels as Borealin-WT (see Figures 6C, 6E, and S5B), was severely impaired in rescuing chromosome misalignments caused by Borealin depletion (Figure 6C). Therefore, residues of Borealin that are phosphorylated by Mps1 in vitro are critical for Aurora B function in vivo.

Like Borealin-WT, both Borealin-4TD and Borealin-4TA interacted with other members of the CPC (Figure 6D) and were able to direct Aurora B to inner centromeres in cells depleted

of endogenous Borealin (Figure 6E). However, similar to what was observed in cells lacking Mps1, Borealin-depleted cells expressing Borealin-4TA displayed poor centromeric Aurora B activation (Figures 6F, 6G, and Table S2). Importantly, the low in vitro activity of CPCs immunoprecipitated from mitotic, Mps1-depleted cells (see Figure 5E) could be enhanced by preincubation with purified active Mps1 prior to the in vitro kinase reaction (Figure 6H). These data strongly suggest that Mps1 enhances Aurora B activity by directly phosphorylating Borealin.

Phosphomimetic Mutations in Borealin Restore Chromosome Alignment in Cells Lacking Mps1

To investigate the importance of Borealin phosphorylation to the control of chromosome alignment by Mps1, alignment was examined in Mps1-depleted cells expressing the Borealin-4TD mutant to mimic a state of constitutive phosphorylation by Mps1. Strikingly, Borealin-4TD, but not Borealin-WT, was very efficient in restoring chromosome alignment caused by Mps1 depletion (Figure 7A). The rescue by Borealin-4TD of misalignments in Mps1-depleted cells was nearly as effective as restoring Mps1 expression itself in these cells (Figure 7A). The requirement for Mps1 activity in the process of chromosome alignment could therefore, at least in large part, be bypassed by expression of constitutively phosphorylated Borealin. The rescue of misalignments by Borealin-4TD was specific for signaling by Mps1, as this mutant was unable to restore alignment in BubR1- or Plk1-depleted cells (Figure 7B) (Ditchfield et al., 2003; Lampson and Kapoor, 2005; Sumara et al., 2004; van Vugt et al., 2004). Importantly, Aurora B-dependent phosphorylation of CENP-A (Figures 7C and 7D and Table S2) as well as Aurora B autophosphorylation (Figures S3B and S3C and Table S2) were restored in Mps1-depleted cells expressing Borealin-4TD. Finally, to examine if Borealin is an effector in the control of Mps1 over the mitotic checkpoint, checkpoint response in Borealin-4TD-expressing, Mps1-depleted cells was determined by flow cytometry. Whereas Borealin-4TD was able to restore checkpoint signaling in taxol-treated cells depleted of endogenous Borealin, it was unable to do so in either nocodazole- or taxol-treated cells lacking Mps1 (Figure 7E), showing that it cannot bypass the requirement of Mps1 activity for mitotic checkpoint signaling. Together, these data identify Borealin as a major effector of the Mps1 kinase in the control of attachment-error-correction and chromosome alignment.

DISCUSSION

A Conserved Role for Mps1 in Chromosome Alignment?

We have shown here that Mps1 kinase activity is indispensable for both the mitotic checkpoint and chromosome alignment in human cells (Figure 7F). A role for *Saccharomyces cerevisiae* Mps1 in spindle assembly was recently suggested and based on the observation that chemical inhibition of Mps1 resulted in improper spindle formation and chromosome positioning (Jones et al., 2005). A mitotic checkpoint-independent role for Mps1 in regulating correct chromosome segregation thus appears to be conserved. Interestingly, Aurora B/Ipl1 mutant yeast strains have certain phenotypes in common with strains exposed to chemical inhibition of Mps1. These include elongated spindles at metaphase and chromosome missegregations at anaphase (Biggins et al., 1999; Jones et al., 2005). In *S. cerevisiae*, evidence of a link between Mps1 and Aurora B/Ipl1 activities has been reported. Cell-cycle arrest in response to Mps1 overexpression depends on Aurora B activity (Biggins and Murray, 2001) and the yeast Mps1-inhibitor cincreasin at certain concentrations abrogates checkpoint signaling in response to lack of tension but not lack of attachment, very much like Aurora B/Ipl1 mutants (Biggins and Murray, 2001; Dorer et al., 2005). It is therefore possible that Mps1 also controls Aurora B activity in organisms other than mammals. Borealin orthologs have been identified in most model organisms (though not in yeast), some of which express two homologous Borealin-like proteins, related to the DasraA/B genes originally identified in *Xenopus laevis* (Gassmann et al., 2004; Sampath et al., 2004). In this respect, it is of interest to note that three of four residues (Thr169 is the exception) found phosphorylated by Mps1 are present in at least one of the Borealin-like proteins of most organisms.

A Novel Function for Borealin in the Regulation of Aurora B

Our data suggest that Mps1 is an upstream activator of Aurora B kinase activity and that Borealin contributes to stimulation of the intrinsic kinase activity of Aurora B. Maximal activation of Aurora B at the centromere is regulated on many levels, including phosphorylation by Chk1 (Zachos et al., 2007) and local clustering that triggers a chromatin-dependent autoactivation loop (Kelly et al., 2007; Sessa et al., 2005). Borealin has been proposed to facilitate this clustering as well as stabilize interactions between INCENP and Survivin (Klein et al., 2006; Vader et al., 2006a). We provide evidence that Borealin additionally contributes to Aurora B activation independent of its role in loading Aurora B onto centromeric chromatin, as Aurora B is properly localized yet not fully activated in Mps1-depleted cells.

Phosphorylation by Aurora B of the TSS motif in INCENP and autophosphorylation on T232 within its activation loop are essential for activating Aurora B (Adams et al., 2000; Honda et al., 2003; Sessa et al., 2005; Yasui et al., 2004). Phosphorylation of Borealin by Mps1 does not contribute to Aurora B activity on such a fundamental level, as it enhances Aurora B activity by 2- to 4-fold, as judged by pSer7-CENP-A immunolocalization. Interestingly, whereas regulation of Aurora B activity by Mps1 is important for its function at the centromere, it does not seem to impact the function of Aurora B at the central spindle, as we have seen no defect in cytokinesis in Mps1-depleted cells (data not shown). Perhaps cytokinesis can proceed with low levels of Aurora B activity, while error-correction needs that activity to be enhanced by Mps1, or perhaps a different mechanism ensures enhanced Aurora B activity on the central spindle. On the other hand, phosphorylated Borealin, on top of its role in regulating Aurora B activity, may contribute to establishing interactions with proteins that are specifically required for Aurora B to correct faulty attachments but not for Aurora B to contribute to cytokinesis. Clarifying the role of Borealin and its modifications in activation of Aurora B at the centromere will require in vitro reconstitution of the full complex from purified components and biochemical analysis of in vivo complexes containing the various Borealin mutants.

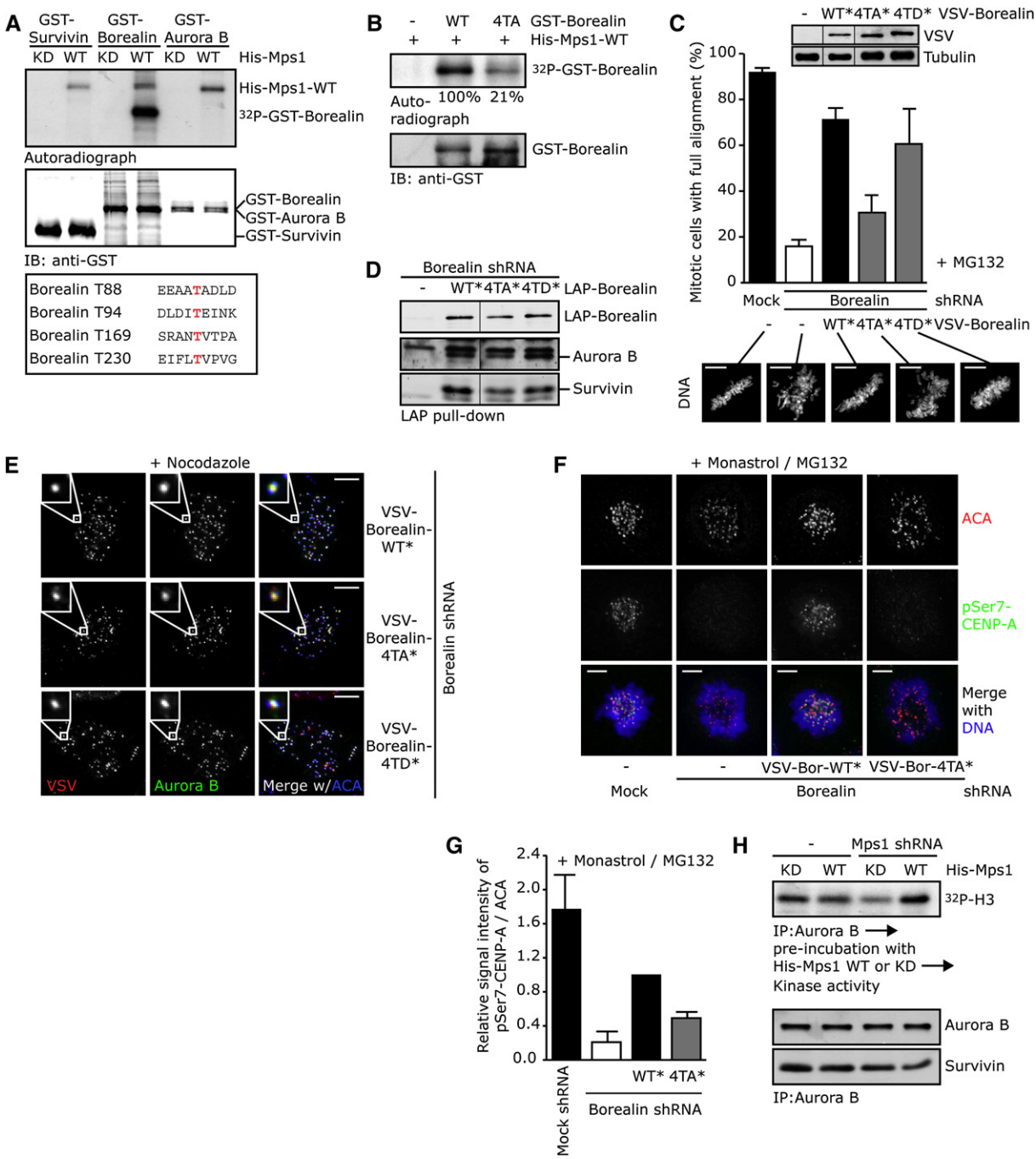

Figure 6. Phosphorylation of Borealin by Mps1 Contributes to Activation of Aurora B

(A) GST-tagged proteins were incubated with kinase-deficient (KD) or active (WT) recombinant Mps1 and analyzed for phosphate incorporation (upper panel) and protein levels (lower panel). The phosphorylation sites identified on in vitro phosphorylated GST-Borealin by mass spectrometry are shown in the boxed alignment.

(B) GST-Borealin-wild-type (WT) and -4TA were analyzed as in (A). Percentage of incorporated radiolabel relative to WT is indicated.

(C) Chromosome alignment in U2OS cells transfected with mock or Borealin shRNA plasmids along with the indicated VSV-tagged Borealin mutants (separation lines in immunoblots in C and D indicate that irrelevant lanes on the gel were removed from the image) and treated with MG132 for 90 min. Graph indicates percentage of cells with proper chromosome alignment (averages of three experiments, at least 60 mitotic cells counted per experiment [± SD]) and representative images are shown. Scale bars are 5 μm.

(D) Immunoblots of CPC members coprecipitating with the indicated LAP-Borealin mutants from U2OS cells treated with nocodazole for 16 hr.

(E) Immunolocalization of Aurora B, Borealin (anti-VSV) and centromeres (ACA) in U2OS cells transfected with Borealin shRNA and the indicated VSV-tagged Borealin mutants and treated with nocodazole for 60 min.

Mps1 Kinase Activity Is Essential for the Mitotic Checkpoint

In agreement with a recent report in which a small molecule inhibitor was used (Schmidt et al., 2005), our data using a mutant allele show that Mps1 kinase activity is essential for the checkpoint in human cells and, as a consequence, for survival of those cells. Studies using immunodepletion from *Xenopus* extracts have previously shown that Mps1 is required for proper recruitment of Bub1, BubR1, and CENP-E to unattached kinetochores (Abrieu et al., 2001; Vigneron et al., 2004; Zhao and Chen, 2006). In addition, Aurora B was shown to control the localization of Mps1 to kinetochores in this experimental system (Vigneron et al., 2004). In contrast, we and others have shown in human cells that depletion of Mps1 to levels sufficient to completely inhibit mitotic checkpoint signaling and induce severe misalignments leaves Bub1, BubR1, and CENP-E at kinetochores (this study and Fisk et al., 2003; Liu et al., 2003; Liu et al., 2006; Martin-Lluesma et al., 2002). Likewise, inhibition of Aurora B activity by RNAi (Stucke et al., 2004) or ZM447439 (this study) does not prevent Mps1 from binding kinetochores in human cells. What underlies the difference between these two systems with regards to interdependencies of kinetochore localization? The frog kinetochore in extracts may behave like an all-or-none system more so than the human kinetochore. It is newly assembled upon addition of the sperm DNA to the extract and may therefore be less mature than that of human mitotic cells. Perhaps the slight reduction in, for instance, Bub1 and BubR1 localization to kinetochores in human cells to cells depleted (see Table S1) can be more readily detected in the less rigidly structured kinetochores that must assemble and disassembly rapidly in the very short embryonic cell cycles.

Coordination between Chromosome Alignment and the Mitotic Checkpoint

Like Mps1, the other three kinases that have roles in the mitotic checkpoint, BubR1, Bub1, and TAO1, also contribute to chromosome alignment (Ditchfield et al., 2003; Draviam et al., 2007; Lampson and Kapoor, 2005; Meraldi and Sorger, 2005). BubR1 is required for establishment of stable attachments of chromosomes to spindle microtubules (Lampson and Kapoor, 2005) and Bub1 is required for formation of proper end-on attachments (Meraldi and Sorger, 2005). The mechanisms by which they exert these functions are unclear, but interestingly, BubR1 was proposed to inhibit Aurora B activity to allow stable attachments to be formed (Lampson and Kapoor, 2005). The data presented here add a new layer to the control of coordination between processes required for chromosome alignment and the mitotic checkpoint. A general principle is thus emerging in which kinases that set up the requirements for faithful chromosome segregation also signal to the cell-cycle machinery to halt until those requirements are met. These kinases are therefore crucial in the maintenance of chromosomal stability, and molecular insights into their activities will likely be valuable in our understanding of the origins of chromosomal instability in development and cancer.

EXPERIMENTAL PROCEDURES

Plasmids and shRNA-Based Protein Replacement

Construction of the various plasmids and mutants were carried out as described in Supplemental Data. Cells were cotransfected with a marker plasmid along with pSuper-Mps1 or pSuper-mock and shRNA-insensitive pCDNA3-LAP-Mps1-WT or -KD (D664A) in a 1:7:3 ratio. This ratio was based on the optimal functional rescue by wild-type, as determined by titration of the wild-type allele in relation to the shRNA. Borealin protein replacements were done similarly. Marker plasmids were pSpectrin-GFP for flow cytometry, pEYFP-H2B, or pH2B-dsRed for imaging and pBabe-Puro for colony outgrowth.

Tissue Culture, Transfections, and Treatments

U2OS and HeLa cells were grown in DMEM with 8% FBS, supplemented with pen/strep. Transfections were done using the calcium phosphate method (U2OS) or Effectene (QIAGEN) (UTRM10, HeLa). Thymidine (2.5 mM), nocodazole (200 $ng \cdot ml^{-1}$), taxol (1 μM), MG132 (10 μM), monastrol (200 μM), STLC (10 μM), and puromycin (1 $\mu g \cdot ml^{-1}$) were all from Sigma. SP600125 (10 μM) was from BioMol. ZM447439 (2 μM) was from Tocris Bioscience.

Flow Cytometry, Colony Outgrowth, and Immunoblotting

Cells were released from a 24 hr thymidine-induced block into nocodazole or taxol for 16 hr and analyzed as described (Kops et al., 2005). Flow cytometric analysis of transfected cells was based on Spectrin-GFP expression. Colony outgrowth analyses were done as described (Kops et al., 2004). As control, a fraction of cells was lysed 48 hr posttransfection and analyzed by immunoblotting for expression of exogenous Mps1. Immunoblotting was done using standard protocols, and the antibodies used are described in Supplemental Data.

Immunoprecipitations and In Vitro Kinase Assays

Conditions for immunoprecipitations of Aurora B using anti-Aurora B antibodies (Abcam) and for pull-downs of LAP-Borealin using S-protein-Agarose (Novagen) were copied from Gassmann et al. (2004), with minor modifications as described in Supplemental Data.

Immunofluorescence Microscopy and Live Cell Imaging

Immunofluorescence microscopy was carried out as described in Supplemental Data. For live cell imaging, cells were plated in 2-well chambered glass-bottom slides (LabTek), transfected and imaged in a heated chamber (37°C and 5% CO_2) using a 40X/1.3NA oil objective on a Zeiss Axiovert 200 M microscope equipped with a 0.55NA condensor and controlled by a lambda-DG4 (Roper Scientific) and MetaMorph software. Twelve bits DIC (25 msec exposure) and yellow fluorescent (75 msec exposure) images were acquired every 3 min using a Photometrics CoolSnap HQ CCD camera (Roper Scientific). Images were processed using MetaMorph software. Images of H2B-EYFP are maximum intensity projections of all Z planes.

Supplemental Data

Supplemental Data include six figures, Supplemental Experimental Procedures, Supplemental References, two tables, and three movies and can be found with this article online at http://www.cell.com/cgi/content/full/132/2/233/DC1/.

(F and G) Immunolocalization of pSer7-CENP-A and centromeres (ACA) in cells transfected with mock or Borealin shRNA plasmids along with the indicated VSV-tagged Borealin mutants and treated with monastrol and MG132 for 60 min. Details of graph (± SEM) in (G) are displayed in Table S2.

(H) Kinase activity toward recombinant histone H3 (^{32}P-H3) of Aurora B immunoprecipitated from UTRM10 cells treated with or without doxycycline for 3 days (see Supplemental Experimental Procedures) and nocodazole/MG132 for an additional 3 hr. Precipitates were preincubated with His6-Mps1-WT or -KD for 20 min after which Mps1 was removed by extensive washing. H3 and radiolabel were subsequently added.

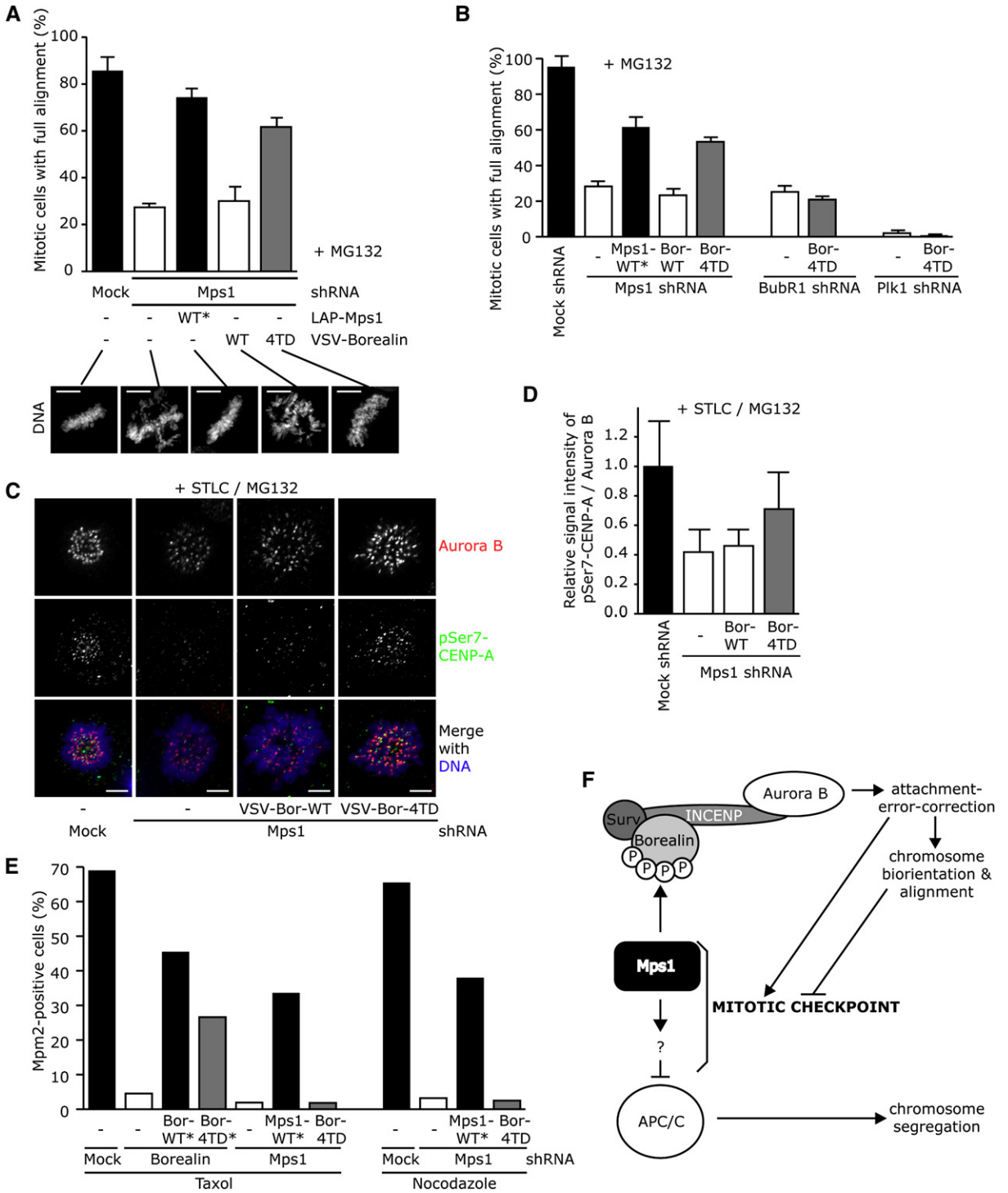

Figure 7. Phosphorylation of Borealin Is Essential in the Control of Chromosome Alignment by Mps1

(A and B) Chromosome alignment in cells transfected with the indicated shRNA plasmids along with LAP-Mps1-WT or the indicated VSV-tagged Borealin mutants and treated with MG132 for 45 min. Graphs indicate percentage of cells with proper chromosome alignment (averages of three experiments, at least 60 mitotic cells counted per experiment [± SD]) and representative images are shown for experiment in (A). Scale bars are 5 μm.

(C and D) Immunolocalization of pSer7-CENP-A and Aurora B in cells transfected with the indicated shRNA plasmids and VSV-Borealin-WT or -4TD and treated with STLC/MG132 for 60 min. Details of graph (± SEM) in (D) are displayed in Table S2.

(E) Flow cytometric analysis of mpm2 positivity in a population of cells transfected with the indicated shRNA plasmids and either LAP-Mps1-WT* or VSV-tagged Borealin mutants and treated with nocodazole or taxol for 16 hr.

(F) Model for the control of mitotic processes by Mps1 kinase activity. See text for clarification.

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Note Added in Proof

We call your attention to a recent paper that also showed a role for Mps1 in biorientation through attachment-error-correction in the budding yeast *S. cerevisiae*: Maure et al. (2007). Mps1 kinase promotes sister kinetochore biorientation by a tension-dependent mechanism. Curr. Biol. *17*, 2175–2182. 10.1016/j.cub.2007.11.032.