ARTICLE
Journal of Cellular Biochemistry 111:1291–1298 (2010)

Journal of Cellular Biochemistry

Regulation of Borealin by Phosphorylation at Serine 219

Harpreet Kaur, Mike E. Bekier, and William R. Taylor*

Department of Biological Sciences, University of Toledo, 2801 W. Bancroft Street, MS 601, Toledo, OH 43606

ABSTRACT

The chromosomal passenger complex consisting of Borealin, INCENP, Survivin, and Aurora B follows a dynamic pattern of localization to perform its role as a regulator of chromosome alignment, aspects of the spindle assembly checkpoint, and cytokinesis. Post-translational modifications of chromosomal passenger proteins play an important role in regulating the localization and function of the complex. Borealin displays a slower electrophoretic mobility during mitosis as a result of phosphorylation. Here we show that phosphorylation at S219 is responsible for this mobility shift. An S219A mutant of Borealin that cannot be phosphorylated at this site displays a defect in centromere localization that is evident in cells arrested in mitosis with nocodazole. Further, the S219A form of Borealin is unable to efficiently rescue mitotic defects that occur upon knock-down of the endogenous protein. These defects are correlated with a reduction in the intensity of Mad2 staining at kinetochores in cells expressing the S219A form of Borealin. These results highlight an important role for phosphorylation of Borealin at S219 in the proper progression through mitosis. J. Cell. Biochem. 111: 1291–1298, 2010.

KEY WORDS: MITOSIS; CHECKPOINT; CENTROMERE; CHROMOSOMAL PASSENGER COMPLEX

The chromosomal passenger complex (CPC) is an essential regulator of chromosome segregation and cytokinesis [Vagnarelli and Earnshaw, 2004; Ruchaud et al., 2007b]. To perform these diverse roles, the CPC follows a characteristic pattern of localization concentrating at the centromere in metaphase, migrating to the midzone in anaphase and accumulating at the midbody from telophase through cytokinesis [Vagnarelli and Earnshaw, 2004; Ruchaud et al., 2007b]. When positioned at the centromere, the CPC activates the spindle assembly checkpoint (SAC) in response to defects in tension at the kinetochore/centromere region [Liu et al., 2009]. CPC located at the spindle midzone in anaphase activates centralspindlin contributing to the contraction of the actomyosin ring [Yuce et al., 2005]. The CPC remains attached to microtubules as the cleavage furrow collects the midzone microtubules into the midbody. INCENP plays a primary role in targeting the CPC to the microtubules, whereas the centromere targeting of the CPC is not completely understood [Vagnarelli and Earnshaw, 2004; Ruchaud et al., 2007b].

Post-translational modifications of CPC proteins play an important role in regulating the complex. For example, phosphorylation of INCENP at S197 during mitosis is essential for targeting INCENP to the midbody [Yang et al., 2007]. Borealin can be phosphorylated by Aurora B kinase in vitro [Gassmann et al., 2004]. This phosphorylation occurred on S165 and possibly other sites [Gassmann et al., 2004]. T88, T94, T169, and T230 in Borealin can be phosphorylated by purified Mps1 kinase [Jelluma et al., 2008]. A phosphomimetic form of Borealin targeting these sites was found to relieve a chromosome alignment defect observed upon Mps1 knock-down, suggesting that the phosphorylation of these sites is functionally important [Jelluma et al., 2008]. However, studies with an analogue sensitive version of Mps1 suggests that this kinase regulates progression through mitosis independently of Aurora B [Maciejowski et al., 2010]. In another study, S154, S219, S275, and S278 of Borealin were phosphorylated by Aurora B kinase in vitro [Hayama et al., 2007]. Cells exposed to a cell-permeable wild-type peptide corresponding to the region of Borealin that contains the four predicted phosphorylation sites contained lower levels of endogenous Borealin. One explanation for this observation is that the peptide competes for Borealin kinases, leading to lower levels of Borealin phosphorylation. This would imply that phosphorylation regulates the stability of the Borealin protein. In addition, Borealin was shown to be phosphorylated at S219 in vivo by mass spectrometric analysis of proteins bound to the mitotic spindle microtubules [Nousiainen et al., 2006].

Abbreviations used: CPC, chromosomal passenger complex; SAC, spindle assembly checkpoint; UTR, untranslated region; PMSF, phenylmethane sulfonyl fluoride; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Additional supporting information may be found in the online version of this article.

Grant sponsor: National Institutes of Health; Grant number: R15GM084410-01.

*Correspondence to: William R. Taylor, Department of Biological Sciences, University of Toledo, 2801 W. Bancroft Street, MS 601, Toledo, OH 43606. E-mail: william.taylor3@utoledo.edu

Received 14 May 2010; Accepted 11 August 2010 • DOI 10.1002/jcb.22853 • © 2010 Wiley-Liss, Inc.
Published online 27 August 2010 in Wiley Online Library (wileyonlinelibrary.com).

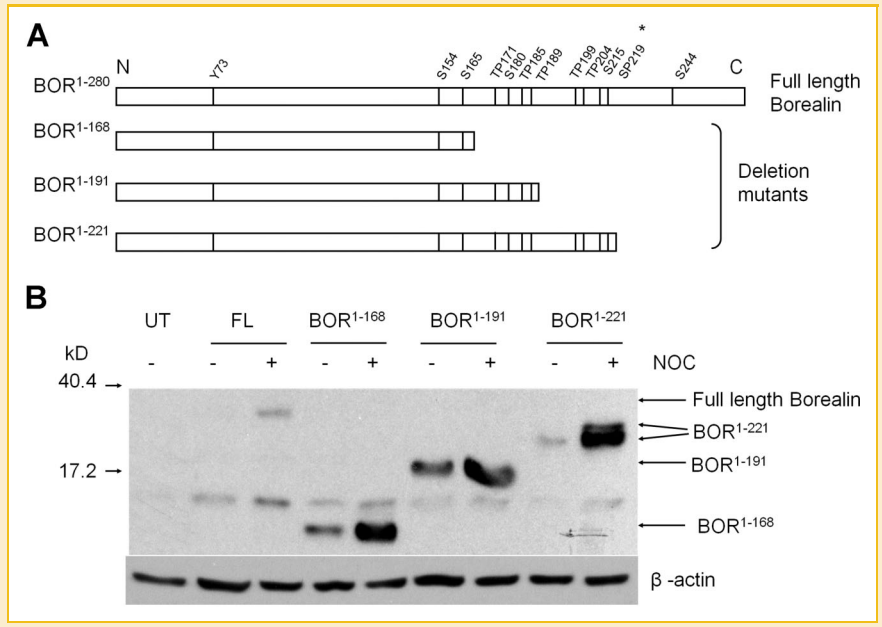

Fig. 1. Schematic and mobility shift analysis of deletion mutants of Borealin. a: Schematic shows the three flag-tagged deletion mutants of Borealin compared to full length Borealin. Amino acid regions of Borealin were analyzed using Netphos and 12 candidate phosphorylation sites were found conserved in all the species studied (*Homo sapiens*, *Macaca mulatta*, *Bos Taurus*, *Canis familiaris*, *Rattus norvegicus*, *Mus musculus*, *Xenopus laevis*, and *Danio rerio*). *S219 was shown to be phosphorylated in vivo by mass spectrometric analysis of proteins bound to the mitotic spindle [Nousiainen et al., 2006]. b: Analysis of truncation mutants by Western blotting. The mobility shift was studied by transiently transfecting HelaM cells with full length Borealin or the three truncation mutants. Cells were either left untreated or treated with nocodazole for 18 h to block cells in mitosis. Lysates were analyzed by Western blotting with antibodies to the Flag-tag. β-actin was used as loading control. UT, untransfected; FL, full-length.

We observed that Borealin exhibits a slower mobility during mitosis as a result of phosphorylation [Kaur et al., 2007]. The phosphorylated form of Borealin is abundant in INCENP immunoprecipitates from mitotic cells suggesting that this phosphorylated form is part of the mitotic CPC [Kaur et al., 2007]. Here we show that the mobility shift of Borealin is due to phosphorylation at S219. Converting this residue to alanine produces a form of Borealin with reduced mitotic functions.

MATERIALS AND METHODS

CELL CULTURE CONDITIONS, TRANSFECTIONS, AND DRUGS

Cells were grown in Dulbecco's modified Eagle's medium (Mediatech) with penicillin/streptomycin and 10% fetal bovine serum in a humidified atmosphere of 10% CO_2 at 37°C. HelaM cells, a subline of Hela were used for all experiments [Tiwari et al., 1987]. Nocodazole (Sigma) was used at a concentration of 200 ng/ml and puromycin was used at 1 μg/ml. Transient transfections were done using either Fugene 6 (Roche) or Expressfect (Denville Scientific, Inc.) with similar results.

WESTERN BLOTTING

Western blotting was conducted as previously described [Kaur et al., 2007] using antibodies to the Flag-tag directly conjugated to horseradish peroxidase (Bethyl Laboratories). Briefly, cells were lysed in a buffer containing 10 mM TRIS (7.4), 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethane sulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), protease inhibitors (1 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin) and phosphatase inhibitors (1 mM sodium fluoride and 1 mM sodium vanadate). Lysates were separated using either 12.6% or 15% acrylamide with a ratio of 29.2:0.8 acrylamide/bisacrylamide. Extended electrophoresis was required to resolve the phosphorylated form of Borealin. The proteins were subsequently transferred to polyvinylidenefluoride membranes and probed with antibodies (Millipore, Bedford, MA). Antibodies to β-actin (Neomarkers) were used and endogenous Borealin was detected using anti-Borealin antisera [Kaur et al., 2007]. Goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Santa Cruz) were used and detected by enhanced chemiluminescence (Thermo Scientific).

BOREALIN CONSTRUCTS

The deletion mutants of Borealin were constructed by amplifying the corresponding regions of a cDNA encoding human Borealin protein by PCR. The PCR products encoding Flag-tag at the C-terminus were cloned into pcDNA 3.2 using TOPO cloning (Invitrogen). Point mutants of Borealin were created using the Quik Change multisite directed mutagenesis kit (Stratagene). To create GFP-tagged Borealin, the open-reading frame of the wild-type gene was amplified by PCR and inserted, using Topo-cloning into pENTR. The open-reading frame was transferred to pDEST47 by gateway cloning to create a C-terminal GFP fusion. This process was repeated with the S219A form of Borealin. In order to express shRNA against *borealin*, a fragment of the *borealin* mRNA was cloned into pSUPER. The shRNA targeted the 3′-untranslated region (UTR) of *borealin* mRNA

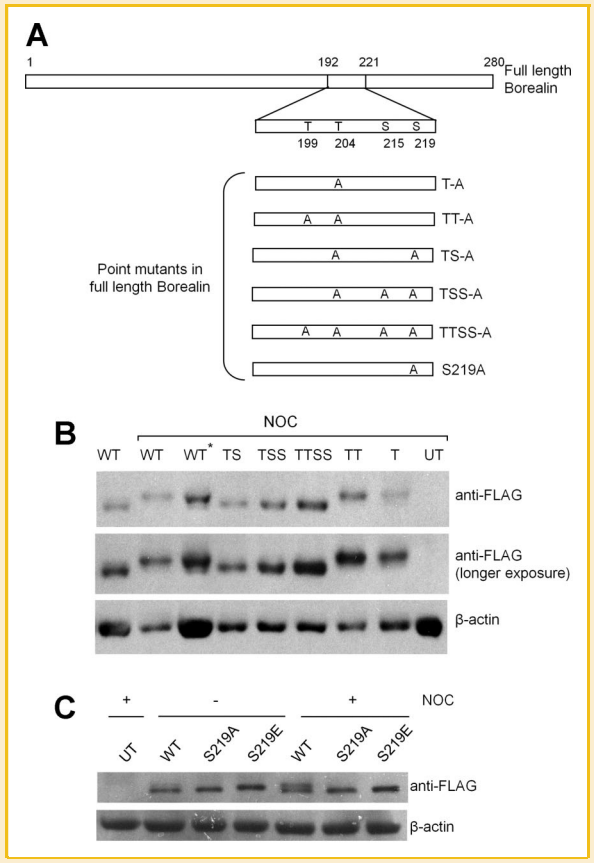

Fig. 2. Schematic and mobility shift analysis of point mutants of Borealin. a: Schematic showing the point mutants of Borealin in which the indicated threonine or serine residues were mutated to alanine. b: Mobility shift of point mutants. HelaM cells were transiently transfected with full length Borealin or the five point mutants. The cells were then either left untreated or treated with nocodazole for 18 h to block them in mitosis. Transfected proteins from the entire culture were detected by Western blotting using antibodies to the Flag-tag. β-actin was used as loading control. UT, untransfected. *Two different amounts of wild type lysate were loaded to visualize the mobility shift clearly. β-actin was used as loading control. UT, untransfected; WT, wild type. c: Mobility shift of S219A and S219E forms of Borealin. HelaM cells were transfected with either wild type, S219E or S219A forms of Borealin. Transfected cells were blocked in mitosis by treating with nocodazole for 18 h. Transfected proteins were detected by Western blotting.

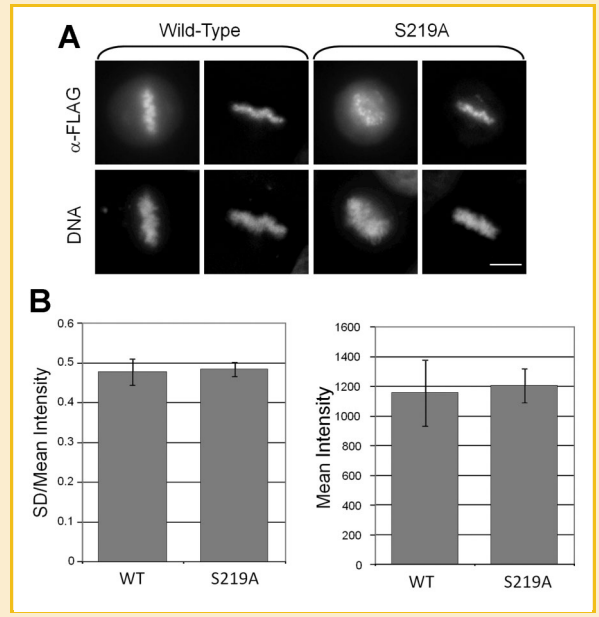

Fig. 3. Localization of Borealin during metaphase. HelaM cells were transfected with Flag-tagged wild-type or S219A Borealin and analyzed by immunofluorescence. a: Examples of cells in metaphase. Bar = 10 μm. b: Quantitation of staining uniformity. Cells expressing either wild-type (n = 12) or S219A (n = 13) Borealin at least twofold above background were analyzed. The standard deviation (SD) of pixel intensities in the entire cell was divided by the mean intensity of the same cell to derive a corrected SD. Mean intensities are also shown. Bars represent average values with standard errors indicated.

and was constructed using the following oligonucleotide sequence: 5′-AGGTAGAGCTGTCTGTTCAdTdT-3′ [Klein et al., 2006]. Top and bottom strand oligonucleotides (Invitrogen) were annealed and ligated between *Xho*1 and *Bgl*II sites in the pSuper vector. All constructs were confirmed by DNA sequencing.

IMMUNOFLUORESCENCE AND TIME-LAPSE MICROSCOPY

For immunofluorescence microscopy, HelaM cells grown on coverslips were transiently transfected with the indicated constructs. Twenty-four hours post-transfection, cells were fixed with 2% formaldehyde in PBS. Cells were permeabilized with 150 mM NaCl, 10 mM Tris (pH 7.7), 0.1% Triton-X 100 (v/v) and 0.1% BSA (w/v) and then blocked with PBS containing 0.1% BSA for 16 h at 4°C. In Figures 3 and 4, transfected Borealin was detected using rabbit anti-ECS antibody that recognizes the Flag-tag (Bethyl Laboratories) while Histone H3 phosphorylated at serine 10 was detected with a monoclonal antibody (Cell Signaling). These primary antibodies were detected with a mixture of goat–anti-rabbit antibodies conjugated to Alexafluor-568 (Molecular Probes) and goat–anti-mouse antibodies conjugated to Alexafluor-488 (Molecular Probes). Endogenous Borealin in Figure 5 was detected with a rabbit antiserum [Kaur et al., 2007] followed by goat–anti-rabbit antibodies conjugated to Alexafluor-568. Microtubules in Figure 6 were detected using mouse anti-α-tubulin (Sigma) followed by goat–anti-mouse Alexafluor-647 (Molecular Probes) secondary antibodies. In Figure 7, Mad2 was detected with a rabbit antiserum (Covance) followed by goat–anti-rabbit antibodies conjugated to Alexafluor-568, while Borealin was fused with GFP and was detected without the need for indirect staining. Coverslips were mounted using Vectashield (Vector Laboratories) and analyzed using an Axiophot fluorescence microscope. For multinucleation phenotype assay, fluorescence microscopy was performed as above with the exception that cells were fixed 48 h post-transfection and microtubules were detected using goat anti-mouse Alexafluor-568 (Molecular Probes) secondary antibodies. For time-lapse microscopy, 24 h post-transfection, cells were plated into slide flasks. Twenty-four hours post-plating, 10 μM Taxol was added to the medium of untransfected and transfected cells. Phase contrast images were taken every 30 min for 72 h using a Leitz Diavert microscope fitted with a stage heated to 37°C.

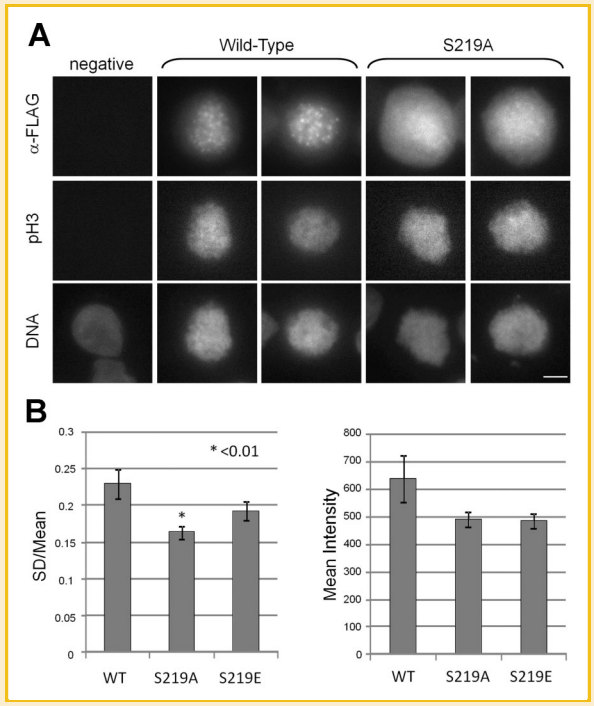

Fig. 4. Localization of Borealin in cells blocked in prometaphase with nocodazole. HelaM cells were transfected with Flag-tagged wild-type or S219A Borealin. Cells were then exposed to nocodazole for 4 h and analyzed by immunofluorescence to detect the Flag tag and Histone H3 phosphorylated at serine 10 ("pH3") as a marker for mitosis. a: Examples of cells expressing wild-type or S219A Borealin. Bar = 10 μm. b: Quantitation of staining uniformity. Cells expressing either wild-type (n = 13), S219A (n = 21) or S219E (n = 11) Borealin at least twofold above background were analyzed. Staining uniformity (SD/mean) was assessed as described in Figure 3. Bars represent average values with standard errors indicated. All significant differences compared to wild type are shown.

RESULTS AND DISCUSSION

Borealin shows a mobility shift due to phosphorylation during mitosis [Kaur et al., 2007]. To find the sites of phosphorylation responsible for this mobility shift, we created three C-terminal truncation mutants of Flag-tagged Borealin (Fig. 1a). Mutants were based on the positions of several well-conserved predicted phosphorylation sites. The mobility shift of these mutants was analyzed by western blotting of transiently transfected HelaM cells blocked in mitosis using nocodazole. Bor^{1-168} and Bor^{1-191} failed to show a mobility shift whereas a shifted band was detected with the Bor^{1-221} mutant (Fig. 1b). These observations indicated that T199, T204, S215, and S219 may be phosphorylated in vivo. To determine which site was phosphorylated, we mutated these sites in full-length Borealin to nonphosphorylatable alanine in different combinations (Fig. 2a). When expressed in HelaM cells, the mutants TS-A, TSS-A, TTSS-A, and S219A migrated faster than wild-type (WT) Borealin in the presence of nocodazole (Fig. 2b,c) implying that phosphorylation of S219 is responsible for the mitotic mobility shift of Borealin. The S219E mutant of Borealin, which mimics phosphorylation at this residue, showed a slightly slower migration compared to wild type Borealin in the absence of nocodazole (Fig. 2c). In Figure 2c, Borealin plasmids were combined with a vector that expresses an shRNA directed against *borealin* (pSup-Bor; see below). Similar results on mobility were observed in the presence or absence of pSup-Bor (data not shown). These observations indicate that phosphorylation of S219 is responsible for the mitotic mobility shift of Borealin.

We have previously observed that when mitotic cells are collected by shake-off, the vast majority of Borealin migrates with the slower mobility [Kaur et al., 2007]. In the experiments shown in Figure 2b,c, cells were exposed to nocodazole for 18 h and the entire culture used for Western blotting. Under these conditions, some cells may still be in interphase which might explain the presence of the fast migrating band in some nocodazole-treated samples. In particular, Figure 2c shows similar amounts of fast and slowly migrating wild-type Borealin after exposure to nocodazole suggesting that the nocodazole block was less efficient in this particular experiment. Nonetheless, S219A shows none of the slow migrating band indicating that this site is required for the mobility shift. We have also observed that nocodazole treatment increases the total level of either endogenous or Flag-tagged Borealin [Kaur et al., 2007]. This effect is particularly evident in Figures 1b and 5a. In other experiments (Fig. 2c) the increase is less evident presumably reflecting the efficiency of mitotic arrest.

Proper localization of the CPC is essential for its various mitotic functions. The S219A mutant form of Borealin appeared to localize normally in cells progressing through mitosis (Fig. 3a). Localization to the midzone and midbody was indistinguishable from wild-type Borealin (data not shown). Punctate staining of S219A was also observed in cells in metaphase (Fig. 3a). In some metaphase cells, we observed increased localization of the mutant to the cytoplasm in metaphase; however, this pattern could also be observed with wild-type Borealin (Fig. 3a). To quantify this effect, we measured the standard deviation (SD) of pixel intensities of Borealin staining within the boundaries of a transfected mitotic cell. This SD value was divided by the average pixel intensity of the same cell to obtain the "corrected SD." The corrected SDs from multiple transfected cells were used to derive an average corrected SD. This analysis confirmed that the distribution of S219A Borealin was indistinguishable from the wild type (Fig. 3b). Interestingly, S219A appeared to exhibit a more uniform staining pattern in cells exposed to nocodazole (Fig. 4a). Consistent with this observation, we detected a significant reduction in the corrected SD of staining with the S219A mutant compared to wild-type Borealin (Fig. 4b).

To determine the functional significance of the phosphorylation of Borealin at S219, the S219A and S219E mutant forms of Borealin were analyzed in a background of low endogenous Borealin by knocking down the endogenous protein with a shRNA targeting the 3′-UTR of the *borealin* mRNA (Fig. 5a). In this experiment, the entire population of cells that were transiently transfected was analyzed. The presence of residual Borealin may be derived either from untransfected cells, or from incomplete knock-down. Immunofluorescence analysis after transfection with the Borealin shRNA plasmid indicated that some cells expressed undetectable levels of endogenous Borealin (Fig. 5b). To reconstitute Borealin expression, cells were co-transfected with the shRNA vector along

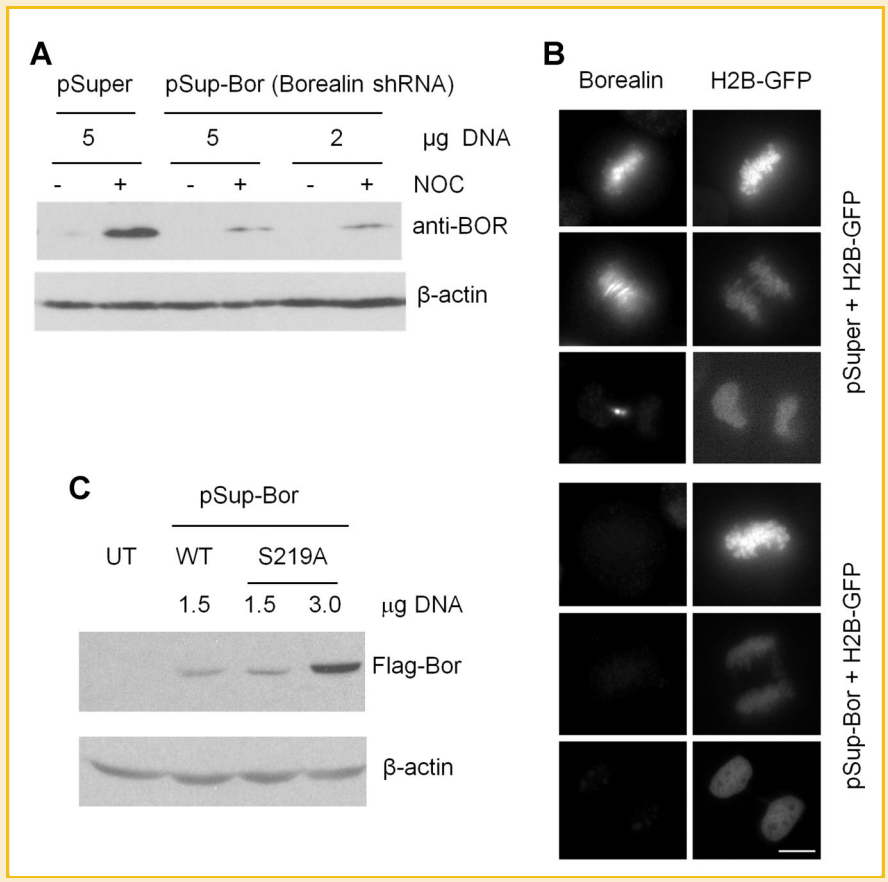

Fig. 5. Knockdown of endogenous Borealin and reconstitution with Borealin-Flag. HelaM cells were transfected with a pSuper-derived plasmid expressing an shRNA directed against the 3′-UTR of *borealin* (pSup-bor). To reconstitute exogenous Borealin, cells were transfected with Flag-tagged versions of *borealin* lacking the 3′-UTR. a: Knockdown of Borealin. HelaM cells were transiently transfected with the indicated quantities of either pSuper (empty vector) or Borealin shRNA. Cells were either left untreated or blocked in mitosis by exposing to nocodazole for 18 h. Cell lysates were separated by SDS–polyacrylamide gel electrophoresis followed by Western blot analysis with anti-Borealin antisera. b: Single cell analysis of Borealin knockdown. HelaM cells were co-transfected with H2B-GFP and either pSuper or pSup-Bor. Transfected cells were analyzed by immunofluorescence using a rabbit antiserum to detect the endogenous protein. Images of H2B-GFP positive cells in metaphase, anaphase or telophase are shown. Bar = 10 μm. c: Reconstitution with Borealin-Flag. HelaM cells were co-transfected with pSuper-Bor, pBABE-puro and the indicated quantities of Borealin-Flag. Transiently expressed exogenous Borealin in puro-selected populations was detected by Western blotting with an anti-Flag antibody. UT, untransfected.

with Flag-tagged wild-type or S219A Borealin, both of which lack the 3′-UTR. For this experiment, cells were co-transfected with pBABE-puro to eliminate untransfected cells. The expression of equal levels of exogenous wild type and mutant Borealin proteins in the knock-down cells was confirmed by immunoblotting with antibodies to the Flag-tag (Fig. 5c). As expected, transfecting with more exogenous S219A plasmid resulted in an increase in expression of the mutant protein (Fig. 5c).

HelaM cells transfected with our shRNA construct targeting *borealin* showed an increase in the number of cells with multiple or abnormally shaped nuclei (Fig. 6a,b). Similar results were obtained when we transfected HelaM cells with a Dharmacon Smart-pool targeting *borealin* (Supplemental Fig. 1). For all subsequent experiments we used our shRNA construct to knock-down Borealin. To test the role of phosphorylation of Borealin at S219 in the multinucleation phenotype, Borealin-depleted HelaM cells were reconstituted with either wild type or mutant forms of Borealin and the number of cells that retained normal nuclei was counted. The number of cells with normal nuclei after expressing either the wild type, or S219E forms of Borealin was similar to the GFP control lacking shRNA, and much higher than shRNA control (Fig. 6a). This suggests that defects occurring upon reduction of endogenous Borealin are rescued with exogenous wild-type Borealin. However, the S219A mutant, even when transfected at twice the amount of the wild type, was unable to completely rescue this phenotype (Fig. 6a). Expressing the TTSS-A mutant of Borealin in the knock-down cells produced as many normal looking nuclei as simply knocking down Borealin (Fig. 6a). These results suggest that phosphorylation of Borealin at S219 is necessary for the proper progression of cells through an unperturbed mitosis. The presence of multiple nuclei may be a result of defects in chromosome segregation or cytokinesis, both of which are disrupted upon knock-down of Borealin [Gassmann et al., 2004].

One of the major roles of the CPC is to regulate the SAC [Ruchaud et al., 2007a]. During mitosis, initiation of anaphase is delayed by the SAC until all kinetochore-microtubule misattachments are resolved and chromosomes are aligned at the metaphase plate [Musacchio and Salmon, 2007]. Aurora B is required for the

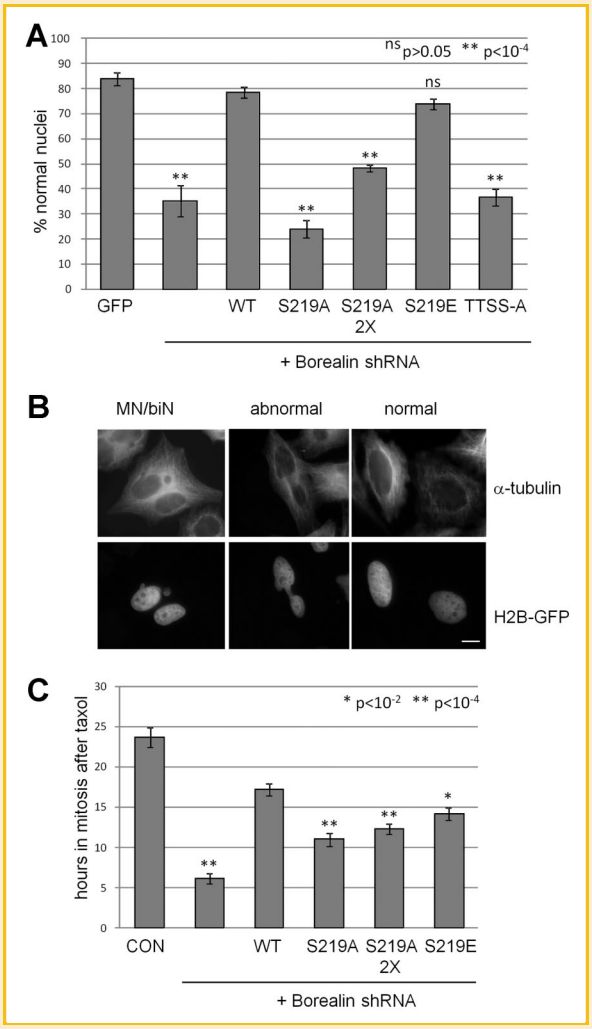

Fig. 6. Defective mitosis in the presence of S219A mutant of Borealin. HelaM cells were transfected with pSup-Bor to knock-down endogenous Borealin and then reconstituted with wild-type or mutant Flag-tagged Borealin. The various experimental conditions were compared to wild type (WT) using a Student's *t*-test. *P* values are indicated. a: Inability of S219A to rescue the multinucleation phenotype induced by Borealin depletion. HelaM cells were cotransfected with H2B-GFP, Borealin shRNA and either Bor^{WT}-flag, Bor^{S219A}-flag, or Bor^{S219E}-flag. Cells were transfected with empty vector (either pSuper or GFP) as a negative control. In each experiment, ≥300 H2B-GFP positive cells were analyzed by fluorescence microscopy for the presence of multiple or abnormal nuclei. The average number of cells retaining normal nuclear morphology is shown. "S219A 2X" indicates transfection with twice the μg quantity of exogenous DNA compared to the wild type. The results are representative of two independent experiments performed in triplicate. Average values with standard errors are shown. b: Examples of multinucleated and binucleated cells (MN, multinucleated; biN, binucleated). Bar = 10 μm. c: Role of S219 in the SAC triggered by Taxol. HelaM cells were cotransfected with H2B-GFP, Borealin shRNA and either Bor^{WT}-flag, Bor^{S219A}-flag, or Bor^{S219E}-flag and progression through mitosis was monitored using time-lapse microscopy. Untransfected cells and H2B-GFP positive cells in transfected samples were analyzed for time spent in mitosis. The results are combined numbers from at least two independent time-lapse movies. Average values with standard errors are shown.

resolution of improper kinetochore-microtubule attachments [Ruchaud et al., 2007a]. Borealin is also essential for the SAC since cells treated with Taxol (a spindle poison) fail to arrest in mitosis when endogenous Borealin is knocked down [Jelluma et al., 2008]. To determine whether phosphorylation of Borealin at S219 is important for the SAC, HelaM cells depleted of endogenous Borealin were reconstituted with wild type or mutant forms of Borealin and then treated with 10 μM Taxol. Transfected cells were monitored by time-lapse microscopy to determine the time spent in mitosis (e.g., see Supplemental Videos 1–4). Knocking down Borealin reduced the Taxol-induced block from ~24 to ~6 h (Fig. 6c). Under similar conditions, we observed that inhibiting Aurora B with ZM447439 allowed HelaM cells to arrest for only ~1.5 h in response to Taxol [Bekier et al., 2009]. This indicates that there may be residual checkpoint activity in our Borealin knock-down experiment. However, this checkpoint activity is clearly much lower than that observed in cells with normal levels of endogenous Borealin (Fig. 6c). Borealin-depleted cells transfected with the S219A form of Borealin spent ~11 h in mitosis whereas those transfected with wild type Borealin spent an average of ~17 h in mitosis (Fig. 6c). Therefore, the S219A form of Borealin can partially rescue the checkpoint arrest, but does show a significant defect compared to wild-type Borealin.

To begin to investigate how the phosphorylation of Borealin at S219 may regulate progression through mitosis we analyzed the recruitment of Mad2 to kinetochores. Mad2 associates with unattached kinetochores where it initiates a "wait-anaphase" signal. This signal, also known as the SAC, inhibits the APC keeping the cell in mitosis until defective kinetochore attachments are fixed. HelaM cells were transfected with the shRNA against Borealin and either wild-type or S219A Borealin-Flag. Mad2 showed variable levels of staining, but on average was significantly lower in cells reconstituted with the S219A form of Borealin (Fig. 7a,b). This effect was only observed in untreated cultures while Taxol-treated cultures showed similar intensities of Mad2 positive dots under all conditions tested (Fig. 7b). These experiments show that Mad2 can still be recruited to kinetochores in cells expressing S219A Borealin; however, the amount of Mad2 at kinetochores is lower when compared to cells expressing wild-type Borealin.

Overall, our results indicate that the mitotic mobility shift of Borealin is dependent on phosphorylation at S219. It is possible that this is a priming site, and that secondary phosphorylation generates the mobility shift; however, we currently have no direct evidence to support this idea. Converting S219 to non-phosphorylatable alanine results in a protein that shows a subtle, yet significant defect in localization. This is most clearly seen in cells blocked in prometaphase with nocodazole, where the S219A localizes to the cytoplasm as well as the chromatin. In contrast, wild-type Borealin localizes more strongly to the chromatin under similar conditions. Localization of the CPC to the centromere is not completely understood although a role for the C-terminus of Borealin has been described [Jeyaprakash et al., 2007]. Specifically, a truncated version of Borealin extending to amino acid 110, did not allow the CPC to travel to the centromere. In contrast, this truncation did not disrupt the normal localization of the CPC to the spindle midzone and midbody. Our results show that phosphorylation of S219 is not

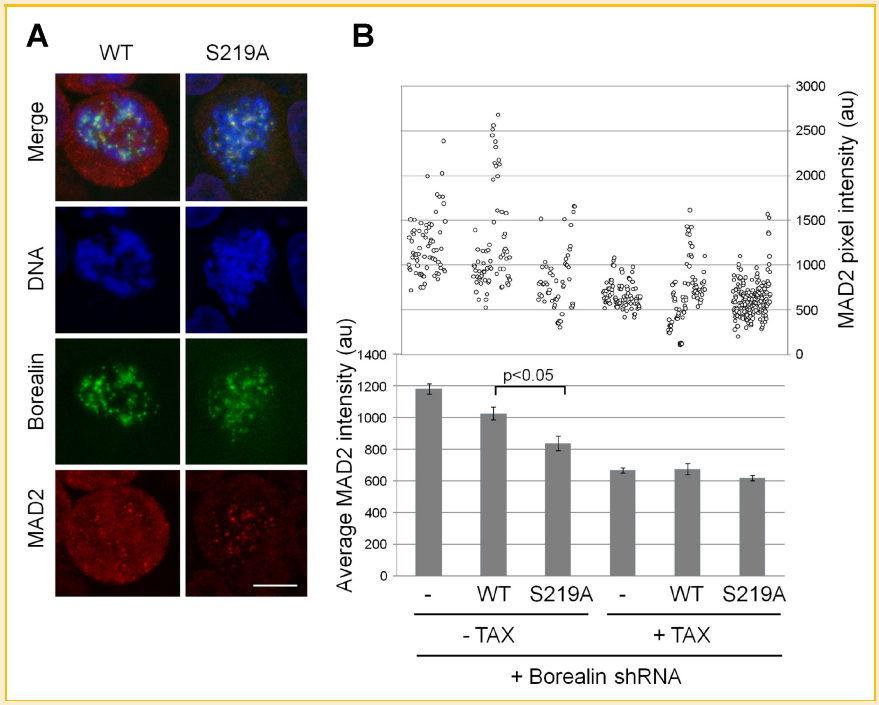

Fig. 7. Lower levels of Mad2 in cells reconstituted with S219A-Borealin. HelaM cells were transfected with pSup-Bor to knock-down endogenous Borealin and then reconstituted with wild-type or mutant GFP-tagged Borealin. Cells were analyzed by immunofluorescence with antibodies to Mad2. a: Examples of stained cells. Cells expressing Borealin were identified based upon GFP fluorescence. Bar = 10 μm. b: Intensity of Mad2 positive dots. Digital images were analyzed to determine the intensity of individual Mad2 dots from at least eight cells per condition. The highest levels of Mad2 staining (usually >2,000 units) were observed in prometaphase cells. To avoid skewing data by variable numbers of prometaphase cells, average pixels intensities (and *t*-test) included only values below 2,000 units. All of the data collected are shown in the top panel.

absolutely required for centromere localization of Borealin. Instead, phosphorylation at this site may reduce the stability of the interaction allowing the protein to more easily dissociate from this location in cells exposed to nocodazole.

Consistent with a defect in centromere localization, the S219A mutant is unable to fully rescue the SAC defect in Borealin depleted cells. Also, this mutant is unable to rescue defective alterations in nuclear morphology that occur upon Borealin knock-down. Neither of these defects could be rescued even in cells transfected with twice the amount of S219A mutant Borealin as compared to the wild type suggesting that the effects are not due to expression levels. Consistent with these defects, cells reconstituted with the S219A form of Borealin showed a significantly lower intensity of Mad2 staining at kinetochores. Mad2 associates with unattached kinetochores and is required to inhibit the APC thereby keeping the cell in mitosis [Mapelli and Musacchio, 2007]. The CPC, including Borealin, plays an essential role in creating unattached kinetochores on chromosomes that display defective attachment to the spindle [Vader et al., 2006]. Phosphorylation of Borealin at S219 may be required for this activity of the CPC. All together, these results indicate that phosphorylation of Borealin at S219 is essential for its normal function as a chromosomal passenger protein.

ACKNOWLEDGMENTS

We are grateful to Song-Tao Liu for help with microscopy. This work was supported by NIH grant R15GM084410-01 to W.R.T.

REFERENCES

Bekier ME, Fischbach R, Lee J, Taylor WR. 2009. Length of mitotic arrest induced by microtubule-stabilizing drugs determines cell death after mitotic exit. Mol Cancer Ther 8:1646–1654.

Gassmann R, Carvalho A, Henzing AJ, Ruchaud S, Hudson DF, Honda R, Nigg EA, Gerloff DL, Earnshaw WC. 2004. Borealin: A novel chromosomal passenger required for stability of the bipolar mitotic spindle. J Cell Biol 166:179–191.

Hayama S, Daigo Y, Yamabuki T, Hirata D, Kato T, Miyamoto M, Ito T, Tsuchiya E, Kondo S, Nakamura Y. 2007. Phosphorylation and activation of cell division cycle associated 8 by aurora kinase B plays a significant role in human lung carcinogenesis. Cancer Res 67:4113–4122.

Jelluma N, Brenkman AB, van den Broek NJ, Cruijsen CW, van Osch MH, Lens SM, Medema RH, Kops GJ. 2008. Mps1 phosphorylates Borealin to control Aurora B activity and chromosome alignment. Cell 132:233–246.

Jeyaprakash AA, Klein UR, Lindner D, Ebert J, Nigg EA, Conti E. 2007. Structure of a Survivin-Borealin-INCENP core complex reveals how chromosomal passengers travel together. Cell 131:271–285.

Kaur H, Stiff AC, Date DA, Taylor WR. 2007. Analysis of mitotic phosphorylation of borealin. BMC Cell Biol 8:5.

Klein UR, Nigg EA, Gruneberg U. 2006. Centromere targeting of the chromosomal passenger complex requires a ternary subcomplex of Borealin, Survivin, and the N-terminal domain of INCENP. Mol Biol Cell 17:2547–2558.

Liu D, Vader G, Vromans MJ, Lampson MA, Lens SM. 2009. Sensing chromosome bi-orientation by spatial separation of aurora B kinase from kinetochore substrates. Science 323:1350–1353.

Maciejowski J, George KA, Terret ME, Zhang C, Shokat KM, Jallepalli PV. 2010. Mps1 directs the assembly of Cdc20 inhibitory complexes during

interphase and mitosis to control M phase timing and spindle checkpoint signaling. J Cell Biol 190:89–100.

Mapelli M, Musacchio A. 2007. MAD contortions: Conformational dimerization boosts spindle checkpoint signaling. Curr Opin Struct Biol 17:716–725.

Musacchio A, Salmon ED. 2007. The spindle-assembly checkpoint in space and time. Nat Rev Mol Cell Biol 8:379–393.

Nousiainen M, Sillje HH, Sauer G, Nigg EA, Korner R. 2006. Phosphoproteome analysis of the human mitotic spindle. Proc Natl Acad Sci USA 103: 5391–5396.

Ruchaud S, Carmena M, Earnshaw WC. 2007a. The chromosomal passenger complex: One for all and all for one. Cell 131:230–231.

Ruchaud S, Carmena M, Earnshaw WC. 2007b. Chromosomal passengers: Conducting cell division. Nat Rev Mol Cell Biol 8:798–812.

Tiwari RK, Kusari J, Sen GC. 1987. Functional equivalents of interferon-mediated signals needed for induction of an mRNA can be generated by double-stranded RNA and growth factors. EMBO J 6:3373–3378.

Vader G, Medema RH, Lens SM. 2006. The chromosomal passenger complex: Guiding Aurora-B through mitosis. J Cell Biol 173:833–837.

Vagnarelli P, Earnshaw WC. 2004. Chromosomal passengers: The four-dimensional regulation of mitotic events. Chromosoma 113:211–222.

Yang F, Camp DG II, Gritsenko MA, Luo Q, Kelly RT, Clauss TR, Brinkley WR, Smith RD, Stenoien DL. 2007. Identification of a novel mitotic phosphorylation motif associated with protein localization to the mitotic apparatus. J Cell Sci 120:4060–4070.

Yuce O, Piekny A, Glotzer M. 2005. An ECT2-centralspindlin complex regulates the localization and function of RhoA. J Cell Biol 170:571–582.