Chromatin Protein HP1α Interacts with the Mitotic Regulator Borealin Protein and Specifies the Centromere Localization of the Chromosomal Passenger Complex*

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Background: Borealin is a conserved centromere protein essential for mitosis.
Results: Borealin interacts with HP1α, and this interaction recruits the CPC to the centromere.
Conclusion: The HP1α–borealin interaction specifies CPC localization in the centromere.
Significance: A physical link between CPC and HP1α in the centromere orchestrates chromosome segregation and accurate cell division.

Accurate mitosis requires the chromosomal passenger protein complex (CPC) containing Aurora B kinase, borealin, INCENP, and survivin, which orchestrates chromosome dynamics. However, the chromatin factors that specify the CPC to the centromere remain elusive. Here we show that borealin interacts directly with heterochromatin protein 1α (HP1α) and that this interaction is mediated by an evolutionarily conserved PXVXL motif in the C-terminal borealin with the chromo shadow domain of HP1α. This borealin–HP1α interaction recruits the CPC to the centromere and governs an activation of Aurora B kinase judged by phosphorylation of Ser-7 in CENP-A, a substrate of Aurora B. Consistently, modulation of the motif PXVXL leads to defects in CPC centromere targeting and aberrant Aurora B activity. On the other hand, the localization of the CPC in the midzone is independent of the borealin–HP1α interaction, demonstrating the spatial requirement of HP1α in CPC localization to the centromere. These findings reveal a previously unrecognized but direct link between HP1α and CPC localization in the centromere and illustrate the critical role of borealin–HP1α interaction in orchestrating an accurate cell division.

The kinetochore is a supermolecular complex assembled at each centromere in eukaryotic cells. Kinetochore provide a chromosomal attachment point for spindle microtubules and power the spatiotemporal dynamics for initiating, controlling, and monitoring the movements of chromosomes during mitosis. Accurate chromosome segregation is essential for cell plasticity, and aberrant mitosis contributes to tumorigenesis (1, 2). The chromosomal passenger complex (CPC), which comprises of Aurora B, INCENP, survivin, and borealin, regulates multiple events during mitotic progression, including spindle assembly, chromosome alignment, and cytokinesis (3, 4). The accurate execution of CPC function depends on its spatiotemporal dynamics, which are highly dynamic and finely regulated during the cell cycle. From later G2 phase to prophase, the CPC localizes to the pericentromeric heterochromatin and chromosome arms. During prometaphase and metaphase, the CPC localizes at the inner centromere and relocates to the centrosome at anaphase onset (3). In budding yeast, relocation of the CPC from the centromere to the central spindle at the metaphase–anaphase transition is triggered by the dephosphorylation of INCENP by phosphatase Cdc14 (5). In mammals, the mitotic kinesin Mklp2 is responsible for translocating the CPC from the centromere to the central spindle at anaphase onset (6). In addition, Mklp2 also recruits Cdc14A to the central spindle, and the dephosphorylation of INCENP by Cdc14A may contribute to the relocation of the CPC (6, 7). Using a FRET-based kinase sensor, our previous study showed that Aurora B kinase activation requires PLK1-mediated phosphorylation of survivin as a priming factor (8). However, the molecular mechanisms underlying CPC localization to the centromere and the temporal control of its activity have remained elusive.

Several lines of evidence demonstrate that the correct localization of CPC components is interdependent (9–12). In addition, structural biological analyses of the survivin-borealin10–109-INCENP1–57 core complex indicate a mutual dependence underlying the localization of CPC components to the centromere (13).
Recently, several lines of evidence have suggested multiple mechanisms that mediate centromere localization of the CPC. Survivin can bind to the histone H3 phosphorylated by haspin kinase at threonine 3 (14, 15); Bub1 kinase phosphorylates histone H2A (at Thr-120) and, thereafter, phosphorylated H2A (phospho-Thr-120) recruits Sgo1 protein (16); and Cdk1/Cyclin B phosphorylates borealin to facilitate its binding with Sgo1 (17). Consistent with a previous study (18), borealin10–109 can localize to the central spindle and midbody correctly but fail to localize to the centromere (13). These results imply that the C-terminal region of borealin may specify the centromere localization of the CPC.

To explore the molecular mechanism underlying CPC targeting to the centromere, we searched for proteins interacting with HP1α, given its critical role in centromere specification. Here we show that borealin contains three P/LXXVI/V motifs, the chromo shadow domain (CSD) binding consensus, at its C terminus. Borealin interacts with the CSD of HP1α via its third PXVXL motif. Mutation of the PXVXL motif abolishes the localization of borealin and other CPC components to the centromere and perturbs accurate chromosome segregation in mitosis.

**MATERIALS AND METHODS**

**Cell Culture and Synchronization—**HeLa and HEK293T cells (ATCC) were maintained as subconfluent monolayers in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (Hyclone, Logan, UT) and 100 units/ml penicillin plus 100 μg/ml streptomycin (Invitrogen) at 37 °C with 8% CO₂. Cells were synchronized at G1/S phase with 2 mM thymidine for 12–16 h, washed with PBS three times, and cultured in thymidine-free medium. Nocodazole and MG132 were used at a final concentration of 100 ng/ml and 20 μM, respectively.

**Plasmid Construction and Recombinant Protein Production—**The cDNA of borealin (NM_018101.2) was provided by Dr. William Earnshaw (University of Edinburgh, UK). The shRNA plasmid targeting borealin and shRNA-resistant LAP-borealin plasmids were a gift from Dr. Geert Kops (University Medical Center Utrecht, The Netherlands). To generate GFP-tagged, FLAG-tagged, mCherry-tagged, and bacterial expression plasmids, PCR-amplified cDNAs were cloned into pEGFP-C3, p3XFLAG-myc-CMV24, pcDNA3.1B-mCherry, and pGEX-5X-3 (Amersham Biosciences) vectors by EcoRI and XhoI digestion, respectively. The PCR-amplified cDNA of the H2B targeting sequence was inserted into pEGFP-C3 by XhoI/HindIII digestion. MBP-HP1α mutant was obtained by site-directed mutagenesis by PCR, and the HP1αWT/HP1αV174E mutant was obtained by site-directed mutagenesis by PCR, and the HP1αWT/HP1αV174E was inserted into the GFP-H2B plasmid constructed above. PCR-amplified cDNA of the HP1α targeting sequence was inserted into the pEGFP-C3 vector by XhoI/HindIII digestion. MBP-HP1α was obtained by subcloning HP1α cDNA into pMAL-C2 by BamHI/HindIII digestion. HP1α cDNA was also subcloned into the p3XFLAG-myc-CMV24 vector (Sigma). Mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) according to the instructions of the manufacturer. All constructs were verified by sequencing.

**In Vitro Pulldown Assay—**MBP-HP1α-bound Sepharose beads were used as an affinity matrix to testify and measure the intensity of the interaction between borealin and HP1α. Briefly, purified GST-borealinWT, GST-borealinV174E, GST-borealinWT/HP1α, and two deletions, GST-borealin N (amino acids 1–140) and GST-borealin C (amino acids 141–280) were eluted from GST beads with a reduced glutathione solution and incubated with MBP-HP1α for 2 h at 4 °C. Beads were washed five times with MBP column buffer with 0.1% Triton X-100 and then boiled in SDS-PAGE sample buffer, followed by 6–14% SDS-PAGE gradient gel and Western blot detection using an anti-GST antibody. Immunoreactive signals were detected with an ECL kit (Pierce) and visualized by autoradiography on Kodak BioMax film. The intensity of bands was quantified by ImageJ (National Institutes of Health).

**Immunoprecipitation and Western Blotting—**293T cells transfected with corresponding plasmids were collected and lysed in lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM MgCl₂, 1 mM EGTA, 0.2% Triton X-100, 1 mM DTT, 10% glycerol, and protease inhibitor mixture (Sigma Chemicals)). Cell lysates were clarified using centrifugation at 13,000 rpm for 20 min at 4 °C, and the supernatants were mixed with FLAG-M2 affinity matrix (Sigma). After being washed five times with the lysis buffer, beads were boiled in SDS-PAGE sample buffer. Subsequently, the samples were subjected to SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, and probed with the indicated antibodies, followed by detection with ECL (Pierce).

**Antibodies and RNA Interference—**Anti-INCENP (Cell Signaling Technology), anti-Aurora B (BD Biosciences), anti-survivin (Epitomics), and anti-HP1α (Cell Signaling Technology) antibodies were obtained commercially. The borealin antibody was provided by Dr. Eric A. Nigg (University of Basel, Switzerland). The siRNAs against borealin and HP1α were from Dharmacon. All siRNAs were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen).

**Chromosome Spread—**For the chromosome spread, mitotic HeLa cells were collected after 16-h treatment with nocodazole, swollen in PEM buffer (5 mM PIPES (pH 7.2), 0.5 mM EDTA, 5 mM MgCl₂, and 5 mM NaCl), and then cytopspun onto the slides at 1000 rpm for 5 min. The immunofluorescence experiments were carried out as described below.

**Chromosome Fractionation—**Chromosomes isolation from mitotic HeLa cells for biochemical characterization has been described previously (19). Briefly, logarithmically growing HeLa cells transiently transfected to express GFP-H2B-HP1α and GFP-H2B-HP1αV174A proteins were treated with 100 ng/ml nocodazole for 18 h. After arrest, mitotic HeLa cells were harvested by mitotic shakeoff and washed with ice-cold PBS. Mitotic HeLa cells were hypotonically swollen for 5 min at room temperature in 10 volumes of PEM buffer containing 5 mM PIPES (pH 7.2), 0.5 mM EDTA, 5 mM MgCl₂, and 5 mM NaCl, and protease inhibitor mixture (1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin A, and 2 μg/ml leupeptin). The hypotonically swollen cells were harvested by centrifugation and homogenized in PEM buffer containing 0.1% digitonin (Sigma). The homogenates were clarified to remove nuclei, and the supernatant was loaded onto a stepwise gradient containing 40, 60, and 80% sucrose in PEM buffer and centrifuged (5000 × g for 15 min) at 4 °C. After centrifugation, a visible, flocculent band...
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migrating at the 50 – 60% sucrose interphase was harvested and suspended in 3 volumes of PEM buffer.

**Immunofluorescence Microscopy**—Immunofluorescence, image acquisition, and processing were performed as described previously (20). For time-lapse microscopy, cells were cultured in a glass-bottom culture dish (MatTek) with CO2-independent medium (Invitrogen) at 37 °C and examined with a DeltaVision microscopy system. Images were acquired at 3-min intervals and presented in Photoshop (Adobe).

**Protein Preparation for the NMR Study**—The HP1α CSD (amino acids110–180) was subcloned into a modified pET-28a plasmid using Ndel and Xhol restriction sites as an N-terminal His6-tagged fusion protein. The fusion protein was expressed in *Escherichia coli* BL21 (DE3) Gold cells induced with 0.5 mM isopropyl 1-thio-β-d-galactopyranoside at 16 °C overnight. Uniformly $^{15}$N- or $^{15}$N/$^{13}$C-labeled fusion proteins were produced by growing the bacteria in minimal medium using $^{15}$NH$_4$Cl (0.5 g/liter) and/or $^{13}$C$_6$-glucose (2.5 g/liter) as the sole nitrogen and carbon sources. The fusion proteins were first purified on a nickel-chelating column and further by size exclusion column chromatography. NMR samples (0.8 or 1.4 mM) were buffered in 40 mM sodium phosphate (pH 6.4), 200 mM NaCl, 1.5 mM TCEP, 1 mM EDTA, and 1 mM NaN$_3$ in 90% H$_2$O and 10% D$_2$O. The HP1α CSD$_{1139K}$ mutant was obtained by QuikChange mutagenesis (Takara) and confirmed by DNA sequencing. The mutant was expressed and purified following the same procedures as those used for the wild type. Borealin$^{223–240}$ peptide was synthesized chemically at Gel BioTech Co. Ltd. Peptide was dissolved in NMR buffer at 8 mM and stored at −80 °C.

**NMR Spectroscopy**—All NMR spectra were recorded at 310 K on a Bruker DMAX500 (with a cryoprobe) spectrometer. The backbone resonance assignments of HP1α CSD$_{1139K}$ were achieved by using triple-resonance CBCA(CO)NH, CBCANH, C(CO)NH-TOCSY, and $^{15}$N-NOESY spectra recorded on a uniformly $^{15}$N/$^{13}$C-labeled protein (1.4 mM). NMR titration of HP1α CSD$_{1139K}$ with borealin peptide was performed on $^{15}$N-labeled protein (dimer, 0.8 mM) by recording a series of $^1$H,$^{15}$N-HSQC in the presence of different amounts of peptide (0–3.2 mM). NMR spectra were processed with NMRPipe and NMRDraw (21). The spectra assignment and analysis were performed with Sparky software.

**Fluorescence Intensity Quantification**—The fluorescence intensity of kinetochore-associated protein labeling was measured using an Applied Precision Deltavision deconvolution microscope as described by Yuan et al. (20). In brief, the average pixel intensities of Aurora B from various treated cells with at least 20 kinetochore pairs from five cells were measured, and background pixel intensities were subtracted. The pixel intensities at each kinetochore pair were then normalized against ACA (anti-centromere antibody) pixel values to account for any variations in staining or image acquisition. The values of specific shRNA-treated and mutant borealin-expressing cells were then plotted as a percentage of the values obtained from cells transfected with a control siRNA duplex.

**Data Analyses**—To determine significant differences between means, unpaired Student’s t test assuming unequal variance was performed and evaluated using GraphPad software. Statistical analysis was considered significant when the two-sided p value was less than 0.05.

**RESULTS**

**Borealin Interacts Directly with HP1α via Its PXVXL Motif**—To search for the molecular mechanism underlying CPC localization to the centromere, we computed for a pentapeptide sequence (P/L)XVX(I/V) that would interact with the CSD (22). Our computational analyses of CSD binders in the centromere and the kinetochore proteome identified borealin as a strong interacting protein for HP1α, an evolutionarily conserved centromere protein essential for mitosis (23).

Borealin is a regulatory component of key mitotic machinery, the CPC, which governs the spatiotemporal dynamics of Aurora B activity in mitosis. Previous studies had shown that both borealin$^{1–140}$ and borealin$^{10–109}$, capable of forming a ternary complex with survivin and INCENP, could localize to the spindle midzone and midbody correctly (13). However, these deletion mutants lost their ability to localize to the inner centromere. We reasoned that the C terminus of borealin, borealin$^{141–280}$, contains an element that specifies the centromere localization of CPC. Our analyses of the primary amino acid sequence of borealin reveal that the borealin C terminus contains three (P/L)XVX(I/V) motifs. The first two (P/L)XVX(I/V) motifs (PAVGR, 172–176, and LEVSM, 177–181) are less conserved evolutionarily, whereas the third one (LTVPV, 229–233) is highly conserved among different species (Fig. 1A).

To test whether borealin interacts with HP1α, we carried out an MBP pulldown assay in which MBP-HP1α protein was purified on maltose-agarose beads and used as an affinity matrix to absorb recombinant GST-borealin proteins (wild type and mutants) purified from bacteria. As shown in Fig. 1B, lane 11, MBP-HP1α binds with GST-borealin but not GST protein, demonstrating a specific binding between HP1α and borealin. To map the region of borealin responsible for its interaction with HP1α, several deletion mutants, such as GST-borealin N (1–140) and GST-borealin C (141–280) were purified and used as inputs (Fig. 1B, lanes 4–6) for the MBP pulldown assay together with full-length (FL) borealin. Consistent with our prediction, MBP-HP1α pulled down full-length and C-terminal borealin (Fig. 1B, lane 13, GST-Bor-C) rather than N-terminal borealin (Fig. 1B, lane 12, GST-Bor-N). As shown in Fig. 1B (bottom panel), Western blotting using an anti-GST antibody confirmed that both full-length and C-terminal borealin proteins bind directly to HP1α.

Because all three CSD readers are located at the C-terminal of borealin (Fig. 1A), we attempted to delineate their requirements for CSD binding. To this end, we generated three Val mutants of borealin, V174E, V179E, and V231E, and tested their ability to bind with HP1α. As shown in Fig. 1C, all mutant borealin proteins exhibited a similar integrity as that of the wild-type protein (lanes 3–6), and borealin$^{V174E}$ mutant bound to HP1α indistinguishably from that of wild-type borealin (lanes 12 and 13). However, the binding of borealin$^{V179E}$ to HP1α was reduced, and the binding of borealin$^{V231E}$ to HP1α was abolished (Fig. 1C, lanes 14 and 15, respectively). We then quantified the binding efficiency of borealin mutants to HP1α from four independent experiments and expressed it as a per-

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percentage of the binding efficiency of wild-type borealin. As shown in Fig. 1D, mutation of borealin Val-231 resulted in a virtual loss of HP1α-binding, whereas the impact on the borealinV174E-HP1α and borealinV179E-HP1α interactions was much milder (quantification shown in Fig. 1E). Therefore, we conclude that Val-231 is critical for borealin-HP1α interaction.

To validate whether Val-231 is critical for formation of the borealin-HP1α complex in vivo, aliquots of HEK293T cells were transiently transfected to express GFP-tagged borealin (wild type and three mutants) and FLAG-tagged HP1α. 24 h after transfection, transfected cells were harvested and lysed, followed by centrifugation. The aliquots of soluble lysates were incubated with anti-FLAG antibody coupled with agarose beads. The affinity matrix was subjected to extensive washes, followed by boiling in SDS-PAGE sample buffer. The bound proteins were then fractionated by SDS-PAGE and transferred onto a nitrocellulose membrane, followed by probing with FLAG (top panel), GFP (center panel), and tubulin (bottom panel) antibodies.
with the fact that HP1α forms a cognate complex with borealin. Importantly, mutation of Val-231, but not Val-174 or Val-179, disrupted the complex (Fig. 1F; lane 8). Therefore, our studies show that HP1α interacts physically with borealin in vitro and in vivo.

Nuclear Magnetic Resonance Spectroscopic Analysis Mapping of the Borealin-HP1α Binding Interface—To further define the borealin-HP1α interaction and its physical contact, we used NMR spectroscopy to monitor the chemical shift perturbation upon borealin peptide (amino acids 223–240) binding to HP1α. Theoretically, the chemical environment of the interacting surface will be perturbed when two proteins are getting closer, which can lead to substantial shifts of resonance frequencies of neighboring residues as well as spectrum split resonances, which results in broad line widths. Therefore, residues located in interacting surface can be predicted on the basis of chemical shifts of resonance frequencies and line widths. In solution, the HP1α-CSD molecule existed as a mixture of dimer and higher-order oligomers that obviously decay the NMR signal. After a series of trial experiments with various mutants, we used the single site mutant L139K for the NMR study (Fig. 2D). In solution, HP1α-CSD-L139K mutant proteins were present as consistent dimers and did not affect peptide binding.

NMR data of the HP1α CSD were collected with or without the borealin PXVXL motif and analyzed as described previously (24). Analysis of the 1H,15N-HSQC spectra of HP1α-CSD-L139K in the presence of increasing amounts of borealin peptide revealed that several resonances were perturbed (Fig. 2, A and E). The line widths of residues Glu-169, Glu-170, Arg-171, Leu-172, Trp-174, and His-175 became so broad that their cross-peaks could not be observed from the beginning of the titration. These residues are likely in intermediate exchange relative to the NMR timescale, confirming a direct interaction with the peptide. Residues Ala-129, Thr-130, Asp-131, Ser-132, and Met-137 exhibited large chemical shift changes in both the proton and nitrogen dimensions. These residues are in fast chemical exchange and may contact the flanking residues of the peptide. The migration pattern of those peaks indicated that the interaction is highly similar to HP1α/PXVXL motif recognition (the peptide binds in an extended conformation across the symmetry axis of the HP1α-CSD-L139K dimer, sandwiching with the C-terminal tail from each monomer) (24). The information from the chemical shift perturbation was used to map the borealin peptide-binding surface on the HP1α-CSD-L139K dimer (Fig. 2, B and C). All perturbed residues were clustered together spatially and formed a contiguous and extensive binding surface. The denoted residues were all located in monomer A. The C-terminal tail of each monomer formed a hydrophobic pocket at the dimer interface, which was critical for peptide binding.

HP1α Is Essential for CPC Localization to the Inner Centromere—During mitosis, most HP1α proteins become dissociated from chromosome arms because they do not bind to methylated lysine 9 of histone H3 after the neighbor serine 10 is phosphorylated by Aurora B kinase (25–28). Interestingly, HP1α concentrated at the inner centromere during mitosis, whereas HP1β and HP1γ did not (29, 30). Moreover, HP1α recruits human Sgo1 to the centromere through its interaction with Sgo1 (31). Therefore, we tested whether HP1α is required for the centromere recruitment of the CPC. To this end, we first introduced siRNA oligonucleotides of HP1α into HeLa cells to suppress the HP1α protein level. We confirmed by immunoblotting that our siRNA treatment typically caused an ~87 ± 5% reduction in the amount of HP1α protein after 24 h, whereas the levels of both tubulin and Aurora B showed no fluctuations in HP1α siRNA-treated cells (Fig. 3A; quantification shown in Fig. 3B). We next examined CPC localization of chromosome spreads in HP1α-depleting cells. The chromosome spreads were chosen because centromere localization and quantification can be better achieved in a flattened mitotic chromosome. As shown in Fig. 3E, top row, in chromosome spreads of control siRNA-transfected cells, it is readily apparent that both HP1α and Aurora B localized to the centromere, as indicated by arrows in the magnified montage. However, the Aurora B signal at the centromere of the chromosome spreads is almost undetectable in HP1α siRNA-transfected cells (Fig. 3E, bottom row, arrow). However, Aurora B signals were seen occasionally in the chromosome arms (Fig. 3E, bottom row, arrowheads), suggesting that Aurora B failed to reach a stable localization to the centromere in the absence of HP1α.

Next, we tested whether HP1α was a direct recruiting factor of the CPC. To this end, we created an artificial GFP-H2B-HP1α construct to see whether expression of H2B-HP1α fusion protein will result in a gross distribution of borealin and other CPC components on chromosomes. As shown in Fig. 3C, exogenously expressed GFP-H2B and GFP-H2B-HP1α were expressed at the right sizes. In addition, the exogenously expressed GFP-H2B and GFP-H2B-HP1α proteins did not alter the expression level of endogenous proteins such as borealin (Fig. 3, C and D). Consistent with our expectations, the CPC components borealin and Aurora B were brought to chromosome arms in these GFP-H2B-HP1α-expressing cells because of the association of borealin with HP1α (Fig. 3, F and G). However, the localization of the kinetochore protein Hec1 was intact in GFP-H2B-HP1α-expressing cells (Fig. 3H), suggesting a physical interaction of borealin with HP1α. Quantitative analyses of chromosome spreads confirmed that the suppression of HP1α by siRNA abolished the localization of Aurora B to the centromere in a similar pattern as that of GFP-H2B-HP1α expression (Fig. 3I). As a control, expression of GFP-H2B did not alter the centromere localization of Aurora B, as judged by the chromosome spread assay (Fig. 3G).

To validate whether the CSD of HP1α specifies borealin recruitment to the centromere, we also generated a GFP-H2B-HP1αW174A mutant that would perturb the HP1α-borealin interactions via the CSD-LXVXL motif recognition. As shown in Fig. 4A, our immunoblot analyses of the isolated chromosome showed that both the GFP-H2B-HP1α and the GFP-H2B-HP1αW174A mutant were expressed at comparable levels. We then examined the centromere localization of borealin in GFP-H2B-HP1α and GFP-H2B-HP1αW174A mutant-expressing cells. As shown in Fig. 4C, center row, GFP-H2B-HP1α recruits borealin to the chromosome arms. In contrast, the GFP-H2B-HP1αW174A mutant, with a disrupted borealin-HP1α interaction, failed to recruit borealin to the chromosome arms. Instead, the localization of borealin remained concentrated in the centromere region in the GFP-H2B-HP1αW174A mutant (Fig. 4C, bottom row), indicating a critical role of Trp-174 in mediating a physical interaction between borealin and HP1α.

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As a control, expression of GFP-H2B did not alter the centromere localization of borealin (Fig. 4C, top row).

If the CPC relocates to the chromosome arms from the centromere because of H2B-HP1α overexpression, the centromere-associated Aurora B kinase activity would diminish. Because phosphorylation of CENP-A at Ser-7 is an exquisite reporter of Aurora B kinase activity, we performed immunofluorescence of Ser(P)-7-CENP-A. As shown in Fig. 4A, bottom panel, lanes 1 and 2, our immunoblot analyses of isolated chromosome expressing the GFP-H2B-HP1α and GFP-H2B-HP1α<sup>W174A</sup> mutants showed that the level of Ser(P)-7-CENP-A was reduced greatly in GFP-H2B-HP1α-expressing chromosomes compared with those of the GFP-H2B-HP1α<sup>W174A</sup> mutant. Quantitative analyses showed that the relative level of Ser(P)-7-CENP-A (the ratio of band density of Ser(P)-7-CENP-A/CENP-A) was reduced significantly in the GFP-H2B-HP1α-expressing chromosome fraction compared with that of the GFP-H2B-HP1α<sup>W174A</sup> mutant-expressing chromosomes (Fig. 4B).
FIGURE 3. HP1α is essential for CPC localization to the inner centromere. A, Western blot showing the efficiency of HP1α protein knockdown by siRNA. Control (lane 1) and HP1α-siRNA-treated (lane 2) cells were harvested 24 h after siRNA transfection. The HP1α protein level was decreased by 90%, whereas Aurora B and tubulin levels were not changed by siRNA treatment. B, quantification of HP1α protein suppression by siRNA. Data represent the means ± S.E. (error bars) from three independent experiments. **, p < 0.001 compared with scramble-transfected cells; ns, no significant difference. Cure B, Aurora B. C, Western blot analysis showing the efficiency of GFP-H2B and GFP-H2B-HP1α protein expression. GFP-H2B- (lane 1) and GFP-H2B-HP1α-expressing cells (lane 2) were harvested 24 h after the transient transfection. GFP-H2B and GFP-H2B-HP1α protein levels were probed with an anti-GFP mouse antibody. Note that overexpression of GFP-H2B and GFP-H2B-HP1α proteins does not interfere with borealin levels. D, quantification of borealin protein suppression levels in cells transfected with HP1α siRNA, scramble siRNA and the GFP-H2B and GFP-H2B-HP1α plasmids. Data represent the means ± S.E. (error bars) from three independent experiments. E, depletion of HP1α abrogates the centromere localization of CPC components. HeLa cells were transfected with scramble and HP1α siRNA for 24 h, followed by chromosome spread, fixation, and immunofluorescence staining. Both HP1α and Aurora B localize to the centromere of scramble-transfected (top row, arrows) but not HP1α-depleted cells (bottom row, arrow). Scale bars = 10 μm. F, expression of H2B-HP1α fusion protein abrogates the centromere localization of borealin. HeLa cells were transiently transfected to express H2B-HP1α fusion protein, followed by chromosome spread, fixation, and immunofluorescence staining. Borealin is localized to the centromeres of exogenously H2B-expressing cells but not H2B-HP1α fusion protein-expressing cells. Borealin is readily apparent on the entire chromosome in H2B-HP1α-expressing cells. Scale bars = 10 μm. G, expression of H2B-HP1α fusion protein abrogates the centromere localization of the CPC component Aurora B. HeLa cells were transiently transfected to express H2B-HP1α fusion protein followed by chromosome spread, fixation, and immunofluorescence staining. Aurora B is localized to the centromeres of exogenously H2B expressing cells but not H2B-HP1α fusion protein-expressing cells. Scale bars = 10 μm. H, expression of H2B-HP1α fusion protein does not alter Ndc80/Hec1 localization to the kinetochore. HeLa cells were transiently transfected to express H2B-HP1α fusion protein, followed by chromosome spread, fixation, and immunofluorescence staining. Hec1 is localized to the kinetochore regardless of the presence or absence of HP1α in the centromere. Scale bars = 10 μm. I, quantification of centromere localization of Aurora B and Hec1 in the absence of HP1α (siRNA treatment) or presence of exogenously expressed GFP-H2B and GFP-H2B-HP1α proteins. Values represent the means ± S.E. (error bars) from three independent experiments. **, p < 0.001 compared with scramble-transfected cells). Note that the centromere localization of Aurora B is a function of the accurate localization of HP1α protein.
Consistent with our biochemical characterization, our immunofluorescence analyses confirmed that the Aurora B activity was reduced dramatically in GFP-H2B-HP1α-expressing cells because little Ser(P)-7-CENP-A signal was detected in the centromere of GFP-H2B-HP1α-expressing cells (Fig. 4D, center row). In contrast, Aurora B activity is intact in GFP-H2B-HP1αW174A-expressing cells (Fig. 4D, bottom row) and GFP-H2B-expressing cells (Fig. 4D, top row). Quantitative analyses of centromere localization of the Ser(P)-7-CENP-A-positive chromosome spread indicates that expression of GFP-H2B-HP1α diminishes Aurora B activity from the centromere, as seen in Fig. 4E. Therefore, we conclude that HP1α plays an essential role in recruiting the CPC to the centromere via a direct interaction with borealin and that this function depends on its conserved Trp-174 residue within the CSD.

**Borealin Is Required for Correct Localization and Function of the CPC**—To test the function of the PXVXL motif of borealin, we first examined the localization of borealin mutants. To this end, HeLa cells were transiently transfected to express mCherry-tagged, wild-type borealin and mutant borealinV231E, followed by immunocytochemical staining. As shown in Fig. 5A, wild-type borealin localized to the centromere correctly in mitotic cells on the basis of its colocalization with centromere marker ACA (Fig. 5A, top row, Merge). In contrast, borealinV231E failed to localize in the centromeres of mitotic cells (Fig. 5A, bottom row). Moreover, borealinV231E exhibits
a diffusive staining pattern in the cytoplasm, confirming that
the HP1α-borealin interaction is critical for a stable localiza-
tion of borealin and the CPC to the centromere.

The CPC is important protein machinery that exhibits dynamic
spatiotemporal regulation in mitosis. To test whether the HP1α-
borealin interaction is responsible for the localization of borealin
to other mitotic structures, such as the midzone, we examined
anaphase cells expressing mCherry-borealinWT and the mutant
borealinV231E. Although borealinV231E failed to localize to the cen-
tromere, it targeted to the midzone correctly and exhibited no
difference from borealinWT (Fig. 5B). Examination of other CPC
components, such as Aurora B and survivin, in borealinV231E-ex-
pressing cells revealed no alteration (data no shown). Therefore,
these data demonstrate that the PXVXL motif specifies borealin
and CPC localization to the centromere but not to other mitotic
structures, such as the midzone.

FIGURE 5. The PXVXL motif of borealin is required for the correct localization and function of the CPC. A, mutation of the PXVXL motif of borealin abrogates chromosome alignment in the equator because of its inability to target to the centromere. HeLa cells were transiently transfected to express borealinWT or mutant borealinV231E. Overexpression of mCherry-borealinV231E exhibited a perturbed localization to the centromere because of the inability to bind HP1α and affect the chromosome misalignment phenotype. ACA, anti-centromere antibody. Scale bar = 10 μm. B, mutation of the PXVXL motif of borealin did not alter CPC localization to the midzone. HeLa cells were transfected and processed as outline above. Although borealinV231E failed to localize to the centromere, it targeted correctly to the spindle midzone. Scale bar = 10 μm. C, expression of shRNA-resistant borealinWT rescued the centromere localization of Aurora B and corrected the chromosome misalignment in borealin-depleted cells. HeLa cells were transfected with shRNA and shRNA-resistant borealin plasmids (wild-type and mutant V231E) plus the mCherry vector. 24 h after the transfection, HeLa cells were fixed and subjected to immunofluorescence staining. Aurora B localized to the centromere of shRNA-resistant borealinWT-expressing cells without endogenous borealin, whereas the localization of Aurora B to the centromere was impaired, and chromosomes were misaligned in shRNA-resistant borealinV231E-expressing cells. Scale bar = 10 μm. D, quantitative analysis of the fluorescence intensity of Aurora B in the centromere compared with that of ACA as shown in C. The data represent mean ± S.E. from three independent experiments. More than 20 kinetochore pairs (from five cells) were quantified for each group. Student’s t test was used to calculate the p value for comparison of the indicated groups. Bor, borealin. E, borealin mutant V231E remains associated with Aurora B as wild-type borealin. FLAG-borealin wild-type and the V231E mutant were transiently transfected to HeLa cells, which were synchronized afterward. Immunoprecipitates (IP) were fractionated by SDS-PAGE and transferred onto a nitrocellulose membrane, followed by probing with FLAG (top panel), HP1α (center panel), and Aurora B (bottom panel) antibodies. Both wild-type and V231E mutant borealin immunoprecipitated Aurora B, whereas V231E borealin failed to bind HP1α (lane 4). F, quantitative analysis of immunoprecipitated Aurora B by borealin as shown in E. Data represent the mean ± S.E. from three independent experiments. **, p < 0.01 compared with scramble-transfected cells; ns, no significant difference. G, quantitative analysis of misaligned kinetochores from the chromosome misalignment phenotype as shown in C.
To better illustrate the function of the PXVXL-mediated borealin-HP1α interaction in chromosome segregation, we next examined the localization of Aurora B in borealin V231E-expressing cells lacking endogenous borealin, which was achieved by expressing shRNA-resistant borealin (32, 33). As shown in Fig. 5C, shRNA-mediated knockdown efficiently suppressed the expression of borealin because little borealin signal is visualized at the centromere. Importantly, a very low level of Aurora B is found at the centromere, supporting the role of borealin in targeting Aurora B to the centromere (Fig. 5C, first panel). In contrast, the scrambled mock shRNA did not affect the expression and localization of borealin (Fig. 5C, first panel). As predicted, expression of shRNA-resistant, wild-type borealin indeed restored its localization to the centromere (Fig. 5C, third panel). In addition, the centromere localization of Aurora B became apparent in shRNA-resistant cells (Fig. 5C, third panel). Nevertheless, expression of shRNA-resistant borealin V231E failed to rescue Aurora B localization to the centromere of a misaligned chromosome (Fig. 5C, fourth panel). Interestingly, Aurora B appeared to be targeted correctly to the centromeres of some chromosomes in shRNA-resistant borealin V231E-expressing cells (Fig. 5C, fourth panel), validating that there are additional mechanisms responsible for the localization of Aurora B to the centromere. Consistent with the critical role of the CPC in mitosis, Aurora B-positive centromeres appeared to be aligned properly at the equator. We then conducted statistical analyses of four independent experiments to quantify the relative fluorescence intensity of Aurora B and present those analyses in Fig. 5D. Our analyses suggest that centromere targeting of borealin is required for accurate localization of Aurora B because the V231E mutant perturbs the localization of Aurora B.

To test whether the borealin V231E mutant affects borealin association with Aurora B, we carried out an immunoprecipitation experiment in which FLAG-borealin and the V231E mutant were transiently transfected to express borealin proteins. As shown in Fig. 5E, both wild-type and V231E mutant borealin immunoprecipitated Aurora B, whereas V231E borealin failed to retain HP1α protein. The statistical analyses from three independent experiments shown in Fig. 5F suggest that borealin mutant V231E remains associated with Aurora B.

If targeting borealin to centromere is necessary for accurate chromosome segregation, delocalization of borealin from the centromere would cause aberrant chromosome segregation, in particular in chromosome congression to the equator, because a functional CPC is required for proper kinetochore-spindle attachments. To validate this hypothesis, HeLa cells were transiently transfected to express the borealin wild type and V231E mutant, as indicated in Fig. 5C. Transfected cells were fixed 24 h after transfection. Treated cells were then stained with the appropriate antibodies before examination (data not shown). As shown in Fig. 5G, suppression of borealin expression by shRNA or expression of V231E altered chromosome alignment. Therefore, we conclude that the HP1α-borealin interaction specifies the localization of the CPC to the centromere, which governs accurate chromosome segregation in mitosis.

**DISCUSSION**

In this study, we reported that HP1α specifies the inner centromere localization of the CPC through its interaction with the CSD binding motifs within the C terminus of borealin. The most prominent CSD-binding interface is mapped to Val-231 of borealin on the basis of solid phase biochemical characterization and NMR analyses in solution. The functional importance of this HP1α-borealin interaction was demonstrated by the requirement of borealin for stable CPC localization to the centromere. This HP1α-dependent localization is essential for Aurora B kinase activity in the centromere and accurate chromosome segregation in mitosis.

The assembly of the centromere in mammalian cells involves several parallel but interactive pathways. We noticed that another CPC component, INCENP, also interacts with HP1α in chicken cells (34). However, the function of this interaction for targeting INCENP (and the CPC) was ruled out because the INCENP molecule lacking the HP1α binding domain localizes to the centromere correctly (34). Moreover, INCENP1–58 can effectively target survivin and borealin in the absence of endogenous INCENP (10), suggesting that INCENP may target to the centromere independent of its interaction with HP1α. Recently, the INCENP-HP1α interaction was revisited. In a proteomics search for HP1α-binding proteins, Nozawa et al. (35) identified the CPC components Aurora B, borealin, and INCENP in HP1α immunoprecipitates. Although a PXVXL motif (167–171) of human INCENP was required for its interaction with HP1α, the INCENP molecule lacking the wild-type PXVXL motif still localized to centromeres in metaphase chromosomes, consistent with our finding that the HP1α-borealin interaction specifies the CPC to the centromere. Therefore, the identification of borealin in HP1α immunoprecipitates by Nozawa et al. (35) supports our finding of a borealin-HP1α interaction. Our characterization of a direct physical interaction between borealin and HP1α revealed a previously unrecognized link between CPC and HP1α, and our NMR study of the CSD-PXVXL interaction delineates the structural basis of CPC localization to the centromere. On the other hand, Kang et al. (36) have shown that HP1α is targeted to mitotic centromeres by INCENP via binding of the HP1α CSD and a PXVXL/I motif in INCENP. Their studies also suggest that the HP1α-INCENP interaction is required for the recruitment of HP1α to mitotic centromeres, which prevents HP1α-Sgo1 binding (36). This recent study indicates another aspect of HP1α-CPC interaction that remains complicated and elusive.

During the course of our study, several other studies reported that there are alternative mechanisms responsible for CPC centromere localization. Survivin mediates the centromere localization of the CPC by binding phosphorylated histone pT-H3 (14, 15). Tsukahara et al. (17) also showed that phosphorylation of borealin by Cdk1 facilitates its association with Shugoshin proteins, hence its centromere localization. Our study demonstrates that borealin contributes to CPC centromere targeting via a new pathway, binding with CSD of HP1α. We reason that these multiple pathways of CPC centromere targeting are not mutually exclusive and that eukaryotic cells evolved an elaborate centromere plasticity control machinery to ensure faithful
chromosome biorientation, attachment error correction, and accurate segregation in mitosis. It is also likely that these pathways cooperate to orchestrate the spatiotemporal dynamics of CPC distribution and precise control of Aurora B activity, priming, and activation (37).

We envision that a multiple pathway model of the centromere localization of CPC is emerging (Fig. 6). Specifically, HP1α contributes to targeting the CPC through its direct interaction with borealin; borealin, phosphorylated by Cdk1, enables its association with Shugoshin, whose centromere localization is also dependent on HP1α (17, 31); and Haspin kinase-phosphorylated histone H3 recruits survivin (14, 15). In line with the key function of mitotic kinases in orchestrating mitosis, Cdk1, Haspin, and Bub1 play important regulatory roles in the centromere targeting of the CPC via the regulation of a survivin-dependent pathway (16, 17, 37). It would be of great interest to delineate how those pathways act in concert to ensure chromosome dynamics and plasticity during cell division and what happens in response to genotoxic stress. It would ensure chromosome dynamics and plasticity during cell division.

FIGURE 6. Hypothetic model accounting for CPC assembly and plasticity in the centromere. Several pathways have been involved in recruitment of the CPC to centromeres during early mitosis. With our findings in this study, we propose following scenarios. 1) The CPC is targeted to the centromere through the interaction of borealin with HP1α. HP1α binds to adjacent trimethylated Lys-9 on H3 (H3K9me3) via its chromo domain (CD) and, thereafter, recruits borealin through Trp-174 in its CSD and Val-231 in the borealin C terminus. HP1α can also recruit Sgo1 via its CSD. 2) Phosphorylated H3T3 is generated by haspin kinase and binds with the baculovirus IAP repeat (BIR) domain of survivin. 3) Phosphorylated borealin by cyclin-dependent kinase 1 (Cdk1) enables its binding with Sgo1, which interacts with phosphorylated histone H2A at Thr-120 generated by Bub1. Future studies will ascertain their respective contributions to chromosome dynamics and plasticity during the cell division cycle.

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REFERENCES
1. Holland, A.J., and Cleveland, D.W. (2009) Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. Nat. Rev. Mol. Cell Biol. 10, 478–487
2. Storchova, Z., and Pellman, D. (2004) From polyplody to aneuploidy: genome instability and cancer. Nat. Rev. Mol. Cell Biol. 5, 45–54
3. Ruchaud, S., Carmena, M., and Earnshaw, W.C. (2007) Chromosomal passengers: conducting cell division. Nat. Rev. Mol. Cell Biol. 8, 798–812
4. Carmena, M., Ruchaud, S., and Earnshaw, W.C. (2009) Making the Aurora glow: regulation of Aurora A and B kinase function by interacting proteins. Curr. Opin. Cell Biol. 21, 796–805
5. Pereira, G., and Schiebel, E. (2003) Separase regulates INCENP-Aurora B anaphase spindle function through Cdc4. Science 302, 2120–2124
6. Gruneberg, U., Neef, R., Honda, R., Nigg, E.A., and Barr, F.A. (2004) Relocation of Aurora B from centromeres to the central spindle at the metaphase to anaphase transition requires MKlp2. J. Cell Biol. 166, 167–172
7. Yuan, K., Hu, H., Guo, Z., Fu, G., Shaw, A.P., Hu, R., and Yao, X. (2007) Phospho-regulation of HsCdc14A By Polo-like kinase 1 is essential for mitotic progression. J. Biol. Chem. 282, 27414–27423
8. Chu, Y., Yao, P.Y., Wang, W., Wang, D., Wang, Z., Zhang, L., Huang, Y., Ke, Y., Ding, X., and Yao, X. (2011) Aurora B kinase activation requires survivin priming phosphorylation by PLK1. J. Mol. Cell Biol. 3, 260–267
9. Honda, R., Körner, R., and Nigg, E.A. (2003) Exploring the functional interactions between Aurora B, INCENP, and survivin in mitosis. Mol. Biol. Cell 14, 3325–3341
10. Klein, U.R., Nigg, E.A., and 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
11. Lens, S.M., Wolthuis, R.M., Klompmaker, R., Kauw, J., Agami, R., Brummelkamp, T., Kops, G., and Medema, R.H. (2003) Survivin is required for a sustained spindle checkpoint arrest in response to lack of tension. EMBO J. 22, 2934–2947
12. Vader, G., Kauw, J.J., Medema, R.H., and Lens, S.M. (2006) Survivin mediates targeting of the chromosomal passenger complex to the centromere and midbody. EMBO Rep. 7, 85–92
13. Jeyaprakash, A.A., Klein, U.R., Lindner, D., Ebert, J., Nigg, E.A., and Conti, E. (2007) Structure of a Survivin-Borealin-INSEN core complex reveals how chromosomal passengers travel together. Cell 131, 271–285
14. Kelly, A.E., Gheneou, C., Xue, J.Z., Zierhut, C., Kimura, H., and Funahashi, H. (2010) Survivin reads phosphorylated histone H3 threonine 3 to activate the mitotic kinase Aurora B. Science 330, 235–239
15. Wang, F., Dai, J., Daum, J.R., Niedzialkowska, E., Banerjee, B., Stukenberg, P.T., Gorbsky, G.J., and Higgins, J.M. (2010) Histone H3 Thr-3 phosphorylation by Haspin positions Aurora B at centromeres in mitosis. Science 330, 231–235
16. Yamagishi, Y., Honda, T., Tanno, Y., and Watanabe, Y. (2010) Two histone marks establish the inner centromere and chromosome bi-orientation. Science 330, 239–243
17. Tsukahara, T., Tanno, Y., and Watanabe, Y. (2010) Phosphorylation of the CPC by Cdk1 promotes chromosome bi-orientation. Nature 467,
cycle behavior of human HP1α subtypes: distinct molecular domains of HP1 are required for their centromeric localization during interphase and metaphase. J. Cell Sci. 116, 3327–3338

30. Minc, E., Allory, Y., Worman, H. I., Courvalin, J. C., and Buendia, B. (1999) Localization and phosphorylation of HP1 proteins during the cell cycle in mammalian cells. Chromosoma 108, 220–234

31. Yamagishi, Y., Sakuno, T., Shimura, M., and Watanabe, Y. (2008) Heterochromatin links to centromeric protection by recruiting shugoshin. Nature 455, 251–255

32. Jelluma, N., Brenkman, A. B., van den Broek, N. J., Cruijsen, C. W., van Osch, M. H., Lens, S. M., Medema, R. H., and Kops, G. J. (2008) Mps1 phosphorylates Borealin to control Aurora B activity and chromosome alignment. Cell 132, 233–246

33. Zhu, T., Dou, Z., Qin, B., Jin, C., Wang, X., Xu, L., Wang, Z., Zhu, L., Liu, F., Gao, X., Ke, Y., Wang, Z., Aikhionbare, F., Fu, C., Ding, X., and Yao, X. (2013) Phosphorylation of microtubule-binding protein Hec1 by mitotic kinase Aurora B specifies spindle checkpoint kinase Mps1 signaling at the kinetochore. J. Biol. Chem. 288, 36149–36159

34. Ainsztein, A. M., Kandels-Lewis, S. E., Mackay, A. M., and Earnshaw, W. C. (1998) INCENP centromere and spindle targeting: identification of essential conserved motifs and involvement of heterochromatin protein HP1. J. Cell Biol. 143, 1763–1774

35. Nozawa, R. S., Nagao, K., Masuda, H. T., Iwasaki, O., Hirota, T., Nozaki, N., Kimura, H., and Obuse, C. (2010) Human POGZ modulates dissociation of HP1e from mitotic chromosome arms through Aurora B activation. Nat. Cell Biol. 12, 719–727

36. Kang, J., Chaudhary, J., Dong, H., Kim, S., Brautigam, C. A., and Yu, H. (2011) Mitotic centromeric targeting of HP1 and its binding to Sgo1 are dispensable for sister-chromatid cohesion in human cells. Mol. Biol. Cell 22, 1181–1190

37. Kawashima, S. A., Yamagishi, Y., Honda, T., Ishiguro, K., and Watanabe, Y. (2010) Phosphorylation of H2A by Bub1 prevents chromosomal instability through localizing shugoshin. Science 327, 172–177

38. Carmena, M., Wheelock, M., Funabiki, H., and Earnshaw, W. C. (2012) The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis. Nat. Rev. Mol. Cell Biol. 13, 789–803

39. van der Waal, M. S., Hengeveld, R. C., van der Horst, A., and Lens, S. M. (2012) Cell division control by the chromosomal passenger complex. Exp. Cell Res. 318, 1407–1420

40. Kelley, L. A., and Sternberg, M. J. (2009) Protein structure prediction on the Web: a case study using the Phyre server. Nat. Protoc. 4, 363–371