The Spatiotemporal Dynamics of Chromatin Protein HP1α Is Essential for Accurate Chromosome Segregation during Cell Division*

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Background: HP1α is a heterochromatin protein essential for chromosome plasticity in mitosis.

Results: HP1α localization to the centromere depends on two distinct structural determinants in interphase and mitotic cells.

Conclusion: The centromere localization of HP1α is determined by its binding to H3K9me2/3 in interphase but to PXVXL motifs in mitosis.

Significance: The context-dependent spatiotemporal dynamics of HP1α is essential for accurate mitosis.

Heterochromatin protein 1α (HP1α) is involved in regulation of chromatin plasticity, DNA damage repair, and centromere dynamics. HP1α detects histone dimethylation and trimethylation of Lys-9 via its chromodomain. HP1α localizes to heterochromatin in interphase cells but is liberated from chromosomal arms at the onset of mitosis. However, the structural determinants required for HP1α localization in interphase and the regulation for HP1α dynamics have remained elusive. Here we show that centromeric localization of HP1α depends on histone H3 Lys-9 trimethyltransferase SUV39H1 activity in interphase but not in mitotic cells. Surprisingly, HP1α liberates from chromosome arms in early mitosis. To test the role of this dissociation, we engineered an HP1α construct that persistently localizes to chromosome arms. Interestingly, persistent localization of HP1α to chromosome arms perturbs accurate kinetochore-microtubule attachment due to an aberrant distribution of chromosome passenger complex and Sgo1 from centromeres to chromosome arms that prevents resolution of sister chromatids. Further analyses showed that Mis14 and perhaps other PXVXL-containing proteins are involved in directing localization of HP1α to the centromere in mitosis. Taken together, our data suggest a model in which spatiotemporal dynamics of HP1α localization to centromere is governed by two distinct structural determinants. These findings reveal a previously unrecognized but essential link between HP1α-interacting molecular dynamics and chromosome plasticity in promoting accurate cell division.

During mitosis, the duplicated genomes must be precisely and equally segregated into two daughter cells in order to inherit parental characteristics. Mitotic chromosome segregation is orchestrated by the dynamic interaction of spindle microtubules with the kinetochore. The kinetochore is a super proteinaceous complex that assembles on the centromere of each chromosome. The kinetochore contains two functional domains; the outer layer mediates kinetochore-microtubule connection, and the inner layer connects to centromeric DNA (1).

Human heterochromatin protein HP1 (heterochromatin protein 1) has three isoforms: HP1α, HP1β, and HP1γ. All three isoforms have a conserved N-terminal chromodomain (CD), a non-conserved hinge region, and a C-terminal chromoshadow domain (CSD) (2, 3). HP1 is a multifunction protein, involved in many biological progresses, such as heterochromatin formation (4–6), transcriptional regulation (7, 8), DNA recombination and damage repair (9–11), RNA interference (12, 13), and sister chromad cohesion regulation (16–19). HP1α localizes to centromeres in interphase through its CD domain, whereas mitotic centromeric localization depends on the CSD domain (20). Previous studies have shown that HP1α detects di- or trimethylated lysine 9 (H3K9me2/3) on histone H3. The conserved methyltransferase SUV39H1 catalyzes the H3K9me2/3 in interphase (1). The association of HP1α-H3K9me2/3 is regulated by Aurora B or mid (21, 22), which...
facilitates the release of HP1α from chromosomal arms due to the phosphorylation of adjacent serine 10 of methyl-lysine 9 during mitosis.

After DNA duplication in S phase, the cohesin complex (composed of Scc1/Rad21, Scc3/SA2, Smc1, and Smc3) associates with the sister chromatids and prevents their dissociation before mitosis. The release of sister chromatid cohesion is regulated by two different pathways. In prophase, cohesion of chromosome arms is released after SA2 phosphorylation by Plk1. However, centromere cohesion is protected by the PP2A phosphatase complex, which is recruited by Sgo1 and antagonizes Plk1 function. In metaphase, the protease separase is activated and cleaves the SA2 cohesin subunit, subsequently releasing Plk1 function. However, centromere cohesion is protected by the PP2A phosphatase complex, which is recruited by Sgo1 and antagonizes Plk1 function. In metaphase, the protease separase is activated and cleaves the SA2 cohesin subunit, subsequently releasing Plk1 function. In metaphase, the protease separase is activated and cleaves the SA2 cohesin subunit, subsequently releasing Plk1 function.

The molecular mechanism underlying localization of HP1α and the precise role of HP1α dynamics in mitosis remain poorly understood. In this study, we have revealed that HP1α localization is regulated by different molecular mechanisms during interphase and the onset of mitosis. Using H2B-HP1α fusion protein analysis, we have demonstrated that HP1α dissociation from chromosome arms is required for the correct centromere loading of chromosome passenger complex (CPC), Sgo1, Mis14, and MCAK. In addition, we show that forced localization of HP1α to chromosome arms results in Sgo1-mediated stabilization of sister chromatid cohesion and enhances spindle elongation, suggesting that temporal control of HP1α dissociation from chromosome arms orchestrates dynamic sister chromatid cohesion and spindle geometry. Together, our study demonstrates that HP1α orchestrates a hierarchical interaction essential for accurate chromosome dynamics in mitosis.

**EXPERIMENTAL PROCEDURES**

**DNA Construction—**GFP-HP1α was obtained from Addgene, and pEGFP-Sgo1 and pEGFP-MCAK were described previously (27, 28). Their mutants (GFP HP1α mutants (V22M, I165E, W174A, and V22M/W174A) and GFP-Sgo1 V453E) were constructed using standard molecular biology protocols (29), and Sgo1 siRNA-resistant plasmids (GFP-Sgo1-WT and V453E) were constructed using the same protocol with primer 5′-GTG-AAAGAAGCCCAAGACATAATCCTCCAAACTCAGAAAAG-3′. To generate mCherry-H2B-HP1α and mCherry-H2B-HP1α W174A mutant plasmids, the pmCherry-C2-H2B was initially generated, and H2B was subcloned from PCR by BglII and HindIII, and then cloned into pEGFP-C2-H2B plasmid. To generate mCherry-tagged HP1α, PCR-amplified HP1α was digested by HindIII and BamHI and then cloned into pmCherry-C2-H2B plasmid. To construct the GFP-Hecl1-MCAK or GFP-Hecl1C plasmids, Hec1(261–642) truncation was cloned by PCR with primer 5′-ACCATGGGCTTGCCTTG-3′ and 5′-ATCAAGCTGGAAGTCCAGAAAAG-AATGTTAC-3′, digested by BglII and HindIII, and subsequently cloned into pmCherry-C2. HP1α and its W174A mutant were subcloned from their GFP-tagged plasmids, digested by BglII and HindIII, and then cloned into pmCherry-C2-H2B plasmid. To generate mCherry-tagged HP1α, PCR-amplified HP1α was digested by HindIII and BamHI and then cloned into pmCherry-C2 vector. To construct the GFP-Hecl1C-MCAK or GFP-Hecl1C plasmids, Hec1(261–642) truncation was cloned by PCR with primer 5′-AAGCCTGGAGAGAGATGCTTTTAAGCTGGAATC-3′ and 5′-GCG-GAAGGTTCTCCTAGAAGACCTTATTAG-3′, digested by XhoI and HindIII, and then subcloned into pEGFP-C2-MCAK or pEGFP-C2 vector. All constructs were verified by DNA sequencing.

**Cell Culture, Transfection, and Synchronization—**HeLa and 293T cells from the American Type Culture Collection (Manassas, VA) were maintained as subconfluent monolayers in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (FBS; HyClone) and 100 units/ml penicillin plus 100 μg/ml streptomycin (Invitrogen) at 37 °C with 8% CO2. LAP-hMps1 HeLa stable cell lines were maintained in 0.1 μg/μl G418. HeLa cells were transfected by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s manual and synchronized at G1/S with 2.5 mM thymidine for 16 h, washed with phosphate-buffered saline (PBS) three times, and then cultured in thymidine-free medium for appropriate time intervals.

**siRNA, Drugs, and Antibodies—**Knockdown siRNAs against SUV39H1 (5′-ACCUCUUUGACCCUGACUA-3′), HP1α (5′-CCUGAGAAAAACUUGAUUTT-3′), HP1γ (5′-UAAUUC-CUGAAGUGGAAGTT-3′), and Sgo1 (5′-CAGUAGAAACC-UUGUCGAAAG-3′) were utilized in this study, and siRNA duplexes for knockdown of Haspin and a scramble control were provided by Qiagen. Where indicated, 2.5 mM thymidine, 100 ng/ml nocodazole, 2 μM chaetocin, 10 μM MG132, 2.5 μM ZM447439, and 100 nM BI2536 were added to the respective treatments.

Immunoblots and immunofluorescence experiments were performed with the following antibodies: anti-α-tubulin mouse antibody (DM1A, Sigma-Aldrich, T9026; 1:5,000), anti-centromere antibody (ACA, Immunovision; 1:400), anti-GFP mouse antibody (1:2,000) and anti-Aurora B mouse antibody (Aim-1, BD Biosciences; 1:500), anti-CDK1-E mouse antibody (Santa Cruz Biotechnology, Inc.; 1:1,000), anti-Hec1 mouse antibody (Abcam; 1:1,000), anti-INCCP-E rabbit antibody (Cell Signaling, 2786S; 1:50), anti-γ-tubulin mouse antibody (Sigma-Aldrich; 1:1,000), anti-HKP1α rabbit antibody (Cell Signaling; 1:1,000) and anti-SUV39H1 mouse antibody (Santa Cruz Biotechnology; 1:1,000), anti-Haspin rabbit antibody (Abcam; 1:1,000), anti-Sgo1 rabbit antibody (Abnova; 1:1,000), anti-H3S10ph rabbit antibody (Cell Signaling; 1:100), and anti-cyclin B mouse antibody (BD Biosciences; 1:400). Anti-MCAK rabbit antibody (1:100) was as described previously (30, 31).

**Immunofluorescence, Chromosome Spread, and Live Cell Imaging—**For immunofluorescence staining, cells were seeded onto sterile, acid-treated, 12-mm coverslips in 24-well plates (Corning Inc.). At 24–36 h after the aforementioned transfection, HeLa cells were rinsed for 1 min with PHEM buffer (100 mM PIPES, 20 mM HEPES, pH 6.9, 5 mM EGTA, 2 mM MgCl2, and 4 mM glycerol) and permeabilized with PHEM plus 0.1% Triton X-100, as described previously (32). Extracted cells were fixed in freshly prepared 3.7% paraformaldehyde in PHEM and rinsed three times in PBS. The cells were blocked with 0.05% Tween 20 in PBS (TPBS) with 1% bovine serum albumin (Sigma-Aldrich). These cells were incubated with the various primary antibodies in a humidified chamber for 1 h and then washed three times in TPBS. Primary antibodies were visualized with FITC-conjugated goat anti-mouse, -rabbit, or -human IgG; rhodamine-conjugated goat anti-human IgG; or Cy5-conjugated goat anti-mouse IgG. DNA was stained with DAPI.
(Sigma-Aldrich). For microtubule staining, HeLa cells were fixed by 3.7% paraformaldehyde in PTEM buffer (100 mM PIPES, pH 6.8, 10 mM EGTA, 1 mM MgCl2, 0.2% Triton X-100) for 10 min, washed three times in PBS, and blocked as described above.

In general, to produce the chromosome spreads, mitotic cells obtained by selective detachment were incubated in PTEM buffer (0.1 mM PIPES, 2 mM EGTA, 1 mM MgSO4, pH 6.8) for 10 min. After attachment to the glass coverslips by centrifugation at 1,000 rpm for 5 min, the chromosomal spreads were fixed with 4% paraformaldehyde in PHEM for 10 min and blocked with 1% BSA.

HeLa cells expressing different kinds of plasmids entered mitosis at 7 h after thymidine release. Transfected cells grown on glass-based dishes (MatTek) were replaced by CO2-independent medium (Invitrogen) supplemented with 10% FBS and observed using the DeltaVision RT system (Applied Precision) at 37 °C. The mCherry images were taken at 5 min intervals with an exposure time of 0.1 s. Images were analyzed with Softworx software (Applied Precision).

**Image Acquisition and Processing. Fluorescence Intensity Quantification**—Immunofluorescence images were collected on an inverted microscope (Olympus IX-70) with a ×60, numerical aperture 1.42 Plan Apo N objective. Step sections (0.25 μm) were acquired to generate three-dimensional image stacks. Olympus acquisition parameters, including exposure, focus, and illumination, were controlled by Softworx (Applied Precision). The three-dimensional image stacks were deconvolved and projected; subsequent analysis and processing of the images were performed by using Softworx, Photoshop, and Illustrator (Adobe). All statistical analysis was performed with GraphPad Prism V5 (GraphPad Software, Inc.).

Quantification of the levels of kinetochore-associated proteins was described previously (33). Briefly, the average pixel intensities within a 7 × 7-pixel square positioned over a single kinetochore were measured, and the background pixel intensities of a 7 × 7-pixel square positioned in a region of cytoplasm lacking kinetochores were subtracted. Maximal pixel intensities within a 7-pixel square positioned over a single kinetochore were measured, and the background pixel intensities at each kinetochore pair were then normalized against ACA pixel values to account for any variations in staining or image acquisition. All of the fluorescence intensity measurement was quantified by ImageJ (National Institutes of Health).

**RESULTS**

**Identification of Structural Determinants for HP1α Localization to Heterochromatin in Interphase and Mitosis**—HP1α consists mainly of two functional parts: the N-terminal CD, which is responsible for binding to H3K9me2/3, and the C-terminal CSD, which allows homo- or heterodimerization (Fig. 1A). Mounting evidence demonstrates three characterized functional sites on HP1α: Val-22, Ile-165, and Trp-174. It has been demonstrated that the V22M mutant prevents its binding to H3K9me2/3, the I165E mutation perturbs its dimerization capacity, and the W174A mutation disrupts its association with proteins containing the PXVXL motif (Fig. 1B) (34). However, it is not known whether those three sites function synergistically or independently to orchestrate the molecular dynamics of HP1α during different phases within the cell cycle.

To validate if HP1α localization to chromatin in interphase is totally dependent on the SUV39H1 pathway (35), aliquots of HeLa cells were transiently transfected to express GFP-HP1α, followed by suppression of SUV39H1 activity either by siRNA-mediated knockdown or chemical inhibition using protocols that we established previously (33). As shown in Fig. 1C, transfection of siSUV39H1 resulted in an efficient repression of the endogenous SUV39H1 at 48 h post-transfection. Both the nuclear localization and the centromere localization of HP1α are evident in interphase cells treated with solvent DMSO. However, HP1α failed to localize to centromere in chaetocin-treated cells or cells exposed to SUV39H1 siRNA (Fig. 1D). This result confirmed that the HP1α centromeric localization in interphase depends on SUV39H1 activity.

To further pinpoint the key residues responsible for HP1α localization, GFP-tagged HP1αV22M, HP1αI165E, and HP1αW174A mutants were generated, and their expression levels in HeLa cells were examined by Western blotting analyses (Fig. 1E). Our immunofluorescence analyses showed that HP1αV22M failed to localize to the centromeres in interphase cells (Fig. 1F, panel 2), which confirms that HP1α localization relies on H3K9me2/3. The HP1αI165E mutant was also unable to localize to centromeres (Fig. 1F, panel 3), suggesting that dimerization of HP1α is essential for a stable localization to the centromeres in interphase. Interestingly, the HP1αW174A mutant remained localized to the centromere region (Fig. 1F, panel 4).

To assess whether HP1α localization in mitosis depends on H3K9me2/3, HP1α localization was determined in the presence of chaetocin or SUV39H1 siRNA. Aliquots of HeLa cells were transiently transfected to express GFP-HP1α and followed with siRNA-mediated knockdown of SUV39H1 or chaetocin treatment as described previously (33). The transfected and treated cells were then subjected to an immunofluorescence study. As shown in Fig. 1G, HP1α localization to the centromeres was virtually unaltered by chaetocin treatment (panel 2), suggesting that centromeric localization of HP1α was not dependent on SUV39H1 activity in mitotic cells. In addition, HP1α localization to the centromeres was not abolished by depletion of SUV39H1 with siRNA (Fig. 1G, panel 3). Thus, we conclude that SUV39H1-elicited H3K9me2/3 is not the structural determinant for HP1α localization to the centromere in mitotic cells.

We next sought to examine the respective roles of individual structural determinants in HP1α distribution in mitosis. To this end, aliquots of HeLa cells were transiently transfected to express wild type and HP1α point mutants, including GFP-HP1αV22M, GFP-HP1αI165E, GFP-HP1αW174A, and GFP-HP1αV22M/W174A. These engineered proteins were expressed at comparable levels as judged by Western blotting analyses (Fig. 1E). As shown in the first two panels in Fig. 1H, the HP1αV22M mutant retained centromeric localization (panel 2), which is comparable with that of wild type HP1α (panel 1). This outcome is consistent with Fig. 1G, in which centromeric HP1α loading is independent of H3K9me2/3 in mitotic cells. Interest-
ingly, GFP-HP1\textsubscript{I165E} failed to localize at centromeres, suggesting that HP1\textsubscript{I165E} dimerization is required for a stable localization to the centromeres in both interphase and mitotic cells.

Surprisingly, in mitotic cells, the distribution of GFP-HP1\textsubscript{I165E} to centromeres was compromised by the W174A mutant, in contrast to its centromere-rich distribution in interphase cells.

**FIGURE 1.** Identification of structural determinants for HP1α localization to the heterochromatin during cell cycle. A, schematic representation of the domain structure of HP1α. B, schematic structure of HP1α derivatives. Wild-type HP1α is a dimer and can bind to H3K9me2/3, the V22M mutant loses its ability to associate with H3K9me2/3, the I165E mutation abolishes dimer formation, and the W174A mutant does not bind to PXXVXL motif-containing proteins. C, efficiency of siRNA interference of SUV39H1. Immunoblotting analysis of HeLa cells treated with the SUV39H1 siRNA oligonucleotide (100 nM) (lane 2) or with a mock siRNA oligonucleotide (lane 1). D, representative images of centromeric HP1α localization observed in the presence of DMSO (1), chaetocin (2), or SUV39H1 siRNA (3) at interphase. HeLa cells transfected with GFP-HP1α were treated with DMSO or 2 μM chaetocin for 1 h or SUV39H1 siRNA for 48 h before fixation, and the fixed cells were then stained for centromere (ACA) and DNA (DAPI). Scale bars, 10 μm. E, the expression levels of GFP-HP1α and its mutants in HeLa cells. HeLa cells were transfected with GFP (lane 1), GFP-HP1α\textsubscript{WT} (lane 2), GFP-HP1α\textsubscript{V22M} (lane 3), GFP-HP1α\textsubscript{I165E} (lane 4), GFP-HP1α\textsubscript{W174A} (lane 5), or GFP-HP1α\textsubscript{V22M/W174A} (lane 6). At 24 h post-transfection, cells were harvested, boiled in the SDS-PAGE sample buffer, and analyzed by Western blotting with an anti-GFP antibody (top) or anti-tubulin antibody (as a loading control; bottom). F, representative images of centromeric localization of HP1α WT (1) and mutants (V22M (2), I165E (3), W174A (4)) at interphase. Scale bars, 10 μm. G, representative images of localization of HP1α WT (1) and mutants (V22M (2), I165E (3), W174A (4)) in mitosis. Scale bars, 10 μm. H, summary of the results for dynamic localization of HP1α and its mutants and the HP1α localization decision factor during interphase and the onset of mitosis.
As shown in panel 4 (Fig. 1H), GFP-HP1α\textsuperscript{W174A} exhibited a diffused distribution on entire chromosomes in mitotic cells. The diffuse HP1α chromosomal localization obscures whether there is residual HP1α localized to the centromeres and whether HP1α loading on chromosome arms is dependent on H3K9me2/3. To address these two concerns, we constructed a double mutant in which both Val-22 and Trp-174 were simultaneously mutated. As shown in Fig. 1H, GFP-HP1α\textsuperscript{V22M/W174A} failed to localize to the chromosome arms or to the centromeres (panel 5), suggesting that the Val-22 cooperates with Trp-174 in loading HP1α onto the chromosome. Thus, we conclude that both Val-22 and Trp-174 are required for accurate loading of HP1α onto chromosomes in mitosis.

The fact that mutation of the HP1α V22M mutant disrupted the centromeric localization of HP1α by further mutating Trp-174 led us to speculate that the loading of HP1α to the centromere is dependent on uncharacterized structural determinants containing the PXVXL motif instead of H3K9me2/3. To validate this hypothesis, we employed two chemical inhibitors to suppress SUV39H1 activity and Aurora B activity. As shown in data this hypothesis, we employed two chemical inhibitors to suppress SUV39H1 activity and Aurora B activity. As shown in Fig. 1H, GFP-HP1α\textsuperscript{V22M/W174A} failed to localize to the chromosome arms or to the centromeres (panel 5), suggesting that the Val-22 cooperates with Trp-174 in loading HP1α onto the chromosome. Thus, we conclude that both Val-22 and Trp-174 are required for accurate loading of HP1α onto chromosomes in mitosis.

Accuracy Kinetochore Assembly—It is well established that HP1α disassociates from chromatin during entry into mitosis to facilitate chromosome dynamics. However, the physiological significance of this temporal regulation of HP1α distribution is less characterized. If HP1α disassociation from chromosome arms is essential for accurate mitosis, then persistent expression of HP1α on chromosome arms will perturb chromosome segregation in mitosis. To test our hypothesis, we generated fusion constructs containing H2B fused with HP1α. As shown in Fig. 2A, aliquots of HeLa cells were transiently transfected to express mCherry-H2B-HP1α wild type and point mutants. The H2B-HP1α and H2B-HP1α\textsuperscript{W174A} proteins exhibit a characteristic H2B distribution pattern on chromosome arms. If HP1α determines the localization of kinetochore components, persistent localization of HP1α would detour the localization of those components from kinetochore to chromosome arms. To this end, we employed an immunofluorescence assay to score the localization of 21 kinetochore components with distinct localization within the substructures of the kinetochore. As shown in Fig. 2B, six typical inner centromere proteins (i.e. Aurora B, INCENP, Borealin, Survivin, Sgo1, and Mis14) exhibit various degrees of concentration to chromosome arms in H2B-HP1α-expressing cells. As predicted, the abundance of the aforementioned proteins in the centromere was significantly reduced due to their relocation from centromeres to chromosome arms in mCherry-H2B-HP1α-expressing cells (Fig. 2C; \( p < 0.001 \)). However, the W174A mutation blocks relocation of those proteins from the centromere to chromosome arms, demonstrating that the interaction of Trp-174 with the PXVXL motifs of those tested proteins determines their localization in mitotic cells. Although microtubule depolymerase MCAK was not associated with chromosome arms in mCherry-H2B-HP1α-expressing cells, its centromeric localization was significantly reduced (Fig. 2C; \( p < 0.001 \)). As a control, the localization of other outer kinetochore proteins (i.e. outer kinetochore components Hec1, KLNL, CENP-E, BubR1, Mad1, SKAP, Skα1, and Zwint1) and inner kinetochore component CENP-H/I/L/U/S/T) was not altered by the persistent localization of HP1α on the chromosome arms in H2B-HP1α-expressing cells (Fig. 2B),\textsuperscript{4} indicating that the overall structure of kinetochore was not grossly altered in H2B-HP1α-expressing cells. Thus, we conclude that the spatial location of HP1α selectively governs the dynamics and precise distribution of CPC, Sgo1, Mis14, and MCAK via Trp-174.

It has been shown that loading of centromeric CPC depends on its association with Sgo1 upon Borealin phosphorylation by CDK1 (38). To assess whether the translocation of CPC to chromosome arms in H2B-HP1α-expressing cells is a function of a direct interaction between HP1α and Borealin and whether such an interaction is regulated by CDK1 (39), aliquots of H2B-HP1α-expressing cells were treated with CDK1 inhibitor RO3306 and control vehicle DMSO. As shown in Fig. 2D (panel 3), Aurora B relocates to chromosome arms in the presence of CDK1 inhibition, presumably due to the inhibition of CDK1-elicited Borealin phosphorylation. Thus, the localization of HP1α, and not Sgo1, provides the primary determinant for the spatial distribution of CPC in mitosis.

We next asked whether Aurora B kinase activity regulates CPC distribution in the presence and absence of HP1α. To this end, aliquots of HeLa cells were transiently transfected to introduce HP1α siRNA. Forty-eight hours after the transfection, transfected cells were treated with 2.5 \( \mu \text{m} \) ZM447439 or an equal volume of vehicle DMSO for 1 h before fixation and probing for Aurora B and ACA. As shown in Fig. 2E, the localization of CPC to the chromosome arms is negatively regulated by Aurora B kinase activity because chemical inhibition by ZM447439 promotes Aurora B retention on whole chromosome (panel 2). In order to examine how endogenous HP1α promotes chromosome Aurora B loading, we employed siRNA-mediated suppression of endogenous HP1α protein level. As shown in Fig. 2E (panel 4), suppression of HP1α attenuated the chromosomal localization of Aurora B localization (bottom panel, arrow). Thus, we conclude that localization of CPC on chromosome arms of mitotic cells is a function of HP1α.

Because HP1α promotes the localization of CPC on chromosome arms, we next determined whether endogenous HP1α affects centromere-associated localization of CPC. To this end, endogenous HP1α was suppressed by siRNA. Because Haspin and Sgo1 are both known to promote centromeric CPC loading (40, 41), Haspin and Sgo RNA interference were also performed as positive controls. As shown in Fig. 2F, HP1α, Haspin or Sgo1 was efficiently suppressed by the corresponding siRNAs. Our quantitative immunofluorescence analyses show

\[ L. Chu, Y. Huo, X. Liu, P. Yao, K. Thomas, H. Jiang, T. Zhu, G. Zhang, M. Chaudhry, G. Adams, W. Thompson, Z. Dou, C. Jin, P. He, and X. Yao, unpublished observation. \]
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that the centromeric localization level of Aurora B was decreased to 57.1%, similar to that of Haspin-deficient cells (56.2%) and to that of Sgo1-deficient cells (60%) (Fig. 2, G and H, p < 0.001), indicating that HP1α is indeed required for centromeric CPC loading. Thus, we conclude that dynamic localization of HP1α is essential for accurate assembly of centromere/kinetochore.

Chromosomal Arm HP1 Regulates Sister Chromatid Separation by Recruiting Sgo1—It has been reported that Sgo1 localization to chromosome arms is determined by HP1α (27). A recent study from the Ishizaka group (19) has demonstrated that HP1α and HP1γ but not HP1β are required for cohesion of the chromosomal arms. To determine whether HP1α affects chromosomal arm cohesion through Sgo1 and CPC, we first examined whether HP1α forcibly localized to chromosomal arms inhibits segregation of sister chromatids. Cells expressing mCherry-H2B, mCherry-H2B-HP1α, or mCherry-H2B-HP1αW174A were synchronized at the G1/S phase. At 7 h after G1/S release, cells were treated with nocodazole for 3 h. Chromosome spreads were then prepared and examined under a fluorescence microscope. As shown in Fig. 3A, the squashed chromosomes could be divided into five groups: (i) intertwined (most sister chromatids are less condensed and intertwined); (ii) hypocondensed and unseparated (all sister chromatids are less condensed and unseparated); (iii) unseparated (most sister chromatids are unseparated); (iv) arm open (most sister chromatids are separated in chromosome arms but keep connected in the centromere); and (v) hypercondensed (all sister chromatids are highly condensed and unseparated). Chromosome squash from mCherry-H2B-expressing cells exhibits typical separated sister chromatids (mainly group iv and v phenotypes). Surprisingly, most squashed chromosomes from mCherry-H2B-HP1α-expressing cells exhibit well separated sister chromatids (mainly group i, ii, and iii phenotypes), suggesting that ectopic expression of chromosome arm-localized HP1α disrupted normal chromosome cohesion, leading to premature separation of sister chromatids during mitosis. As a control, the majority of sister chromatids in H2B-HP1αW174A-expressing cells were arm-opened but unseparated (mainly group ii, iii, and v phenotypes) (Fig. 3B).

We next sought to examine whether the perturbation of sister chromatid cohesion seen in mCherry-H2B-HP1α-expressing cells is due to an alteration of Sgo1 spatiotemporal dynamics. To this end, we examined the chromosome spreads from cells expressing Sgo1WT and HP1α binding-deficient Sgo1V453E (36) in the absence of endogenous Sgo1. As shown in Fig. 3, C and D, most sister chromatids were unseparated (56.5% of chromosome arms open and 40.5% of arms closed) in the control group. Suppression of endogenous Sgo1 resulted in premature separation of arms and centromeric cohesion of sister chromatids, which is consistent with previous reports (17). Re-expressing Sgo1WT prevented premature sister chromatid separation (44.5% of chromosome arms separated but 52.5% of arms unresolved). However, re-expressing Sgo1V453E was unable to prevent premature separation of sister chromatids (52.0% of chromosome arms open, 21.5% of arms completely separated, and 26.5% of arms closed). These results indicate that Sgo1 plays a functional role in safeguarding centromeric cohesion that depends on its association with HP1α. Thus, we conclude that chromosome arm-associated HP1α recruits Sgo1 and thereby protects the cohesion of sister chromatids.

Although it has been reported that Aurora B is involved in the cohesion of chromosome arms (26), the underlying molecular mechanisms are not well understood. To delineate the aforementioned mechanisms, chromosome spreads were prepared to determine whether Aurora B is involved in the HP1α-Sgo1 pathway. As shown in Fig. 3E, suppression of Sgo1 or combined HP1α+γ resulted in a defect in sister chromatid cohesion (Fig. 3, E–G), which is consistent with several previous reports (19, 26, 42). Inhibition of PLK1 or Aurora B protected chromosome arm cohesion and led to closed chromosome arms (Fig. 3, E–G; p < 0.01). In addition, treatment with BI2536 was able to sustain sister chromatid cohesion in the absence of Sgo1 or HP1α+γ (Fig. 3, E–G) because the inhibition of SA2 phosphorylation by PLK1 promotes cohesion (24). In contrast, inhibition of Aurora B by ZM447439 did not attenuate the loss of sister chromatid cohesion resulting from the suppression of Sgo1 or HP1α+γ (Fig. 3, F–H). Collectively, these data suggest that the mechanisms by which Aurora B and PLK1 orchestrate the separation of sister chromatid cohesion are different and that Aurora B-elicited regulation of sister chromatid separation requires HP1α-Sgo1 interaction.

HP1α Dissociation from Chromosome Is Essential for Faithful Spindle Length—After having examined the impact of persistent expression of HP1α on chromosome plasticity and centromere protein targeting, we then asked whether persistent expression of HP1α on chromosome arms affects spindle geometry. The spindle lengths were measured in cells express-

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**FIGURE 2. HP1α dissociation from chromosome arms is essential for accurate centromere/kinetochore assembly.** A, representative images of HeLa cells transduced with mCherry-H2B, mCherry-H2B-HP1α, or mCherry-H2B-HP1αW174A. Cells were fixed and stained with ACA and DAPI. Scale bars, 10 μm. B, representative images of kinetochore components localization under mCherry-H2B, mCherry-H2B-HP1α, or mCherry-H2B-HP1αW174A overexpression, respectively, visualized by staining with anti-Aurora B, anti-INCENP, anti-MCAK, anti-Hec1, or anti-CENP-E antibody or by GFP-Mis14, GFP-Sgo1, GFP-Survivin, GFP-Borealin, and GFP-KNL1 expression. Scale bars, 10 μm. C, representative quantification of selected kinetochore proteins 24 h after mCherry-H2B, mCherry-H2B-HP1α, and mCherry-H2B-HP1αW174A overexpression, as shown in B (in each case, n = >10 kinetochores of >10 cells; mean ± S.E. (error bars), ***, p < 0.001; **, p < 0.01; *, NS (not significant; p > 0.05). D, representative images of HeLa cells expressing mCherry-H2B (1) or mCherry-H2B-HP1α treated with DMSO (2) or RO3306 (3) for 1 h. After fixation, the cells were stained with anti-Aurora B antibody and DAPI. Scale bars, 10 μm. E, representative images of Aurora B fluorescence intensity with the treatment of DMSO (1), ZM447439 (2), HP1α siRNA (3), or both HP1α siRNA and ZM447439 (4). HeLa cells were transiently transfected to introduce HP1α siRNA for 48 h and were exposed to 2.5 μM ZM447439 for 1 h before fixation and then stained with anti-Aurora B antibody, ACA, and DAPI. Scale bars, 10 μm. F, characterization of knockdown efficiency of siRNA interference of HP1α, Haspin, or Sgo1. Immunoblotting analysis of HeLa cells treated with the three siRNA oligonucleotides (100 nM for 48 h, 50 nM for 48 h, or 50 nM for 36 h, respectively) or with a scrambled siRNA oligonucleotide is shown. Cell extracts were probed with anti-HP1α antibody (top) and anti-tubulin antibody as an internal control (bottom). G, HeLa cells were treated with control scrambled siRNA, Haspin siRNA, or Sgo1 siRNA for 24 h and with HP1α siRNA for 48 h before fixation and then stained with anti-Aurora B antibody, ACA, and DAPI. Scale bars, 10 μm. H, statistical analyses of relative Aurora B immunofluorescence intensity at kinetochores as shown in G (in each case, n = >10 kinetochores of >10 cells; mean ± S.E. ***, p < 0.001).
FIGURE 3. Chromosome arm-associated HP1 regulates sister chromatids separation by recruiting Sgo1. A, representative images of HeLa cell chromosome spreads expressing mCherry-H2B, mCherry-H2B-HP1α, or mCherry-H2B-HP1αW174A. At 7 h after release from thymidine block, the cells were treated with 100 ng/ml nocodazole for 3 h, and the chromosomes were then squashed onto coverslips followed by fixation. Chromosome spread phenotypes were divided into five groups: intertwined (i); hypo-condensed and unseparated (ii); unseparated (iii); arm open (iv); and hypercondensed (v). Scale bars, 10 μm. B, quantification of the percentage of the five kinds of phenotypes of chromosome spreads as shown in A. In each case, nearly 50 cells were classified. C, representative images of HeLa cell chromosome spreads treated with control scrambled siRNA or Sgo1 siRNA or co-transfected with both Sgo1 siRNA- and RNA-resistant GFP-Sgo1 or Sgo1 siRNA- and RNA-resistant GFP-Sgo1V453E. Cells were treated with Sgo1 siRNA for 36 h before chromosome spreading and fixation and then stained with DAPI. Box 1, chromosome arms in the open configuration; box 2, chromosomes that are completely separated; box 3, chromosome arms that are closed. Scale bars, 10 μm. D, quantification of the percentages of the three kinds of chromosome spread phenotypes, as shown in C. In each case, nearly 50 cells were classified from two different preparations. E, representative images of HeLa cell chromosomal spreads treated with DMSO, Sgo1 siRNA, HP1α+γ siRNA, BI2536, both Sgo1 siRNA and BI2536, both HP1α+γ siRNA and BI2536, ZM447439, both Sgo1 siRNA and ZM447439, or both HP1α+γ siRNA and ZM447439. HeLa cells were treated with Sgo1 siRNA or HP1α+γ siRNA for 24 or 48 h, respectively, and exposed to 2.5 μM ZM447439 or 100 nM BI2536 for 10 h before centrifugation to generate chromosome spreads. Aurora B kinase inhibitor ZM447439 and PLK1 kinase inhibitor BI2536 were added to the cells after thymidine release, and 10 h after this treatment, chromosomes were spread, fixed, and then stained with ACA and DAPI. Scale bars, 10 μm. F, schematic diagram representing the chromosomal arm cohesion phenotypes identified in E; a–d, configuration with arms open, arms closed, arms partially separated, or arms completely separated, respectively. G, quantification of the percentage of the four kinds of chromosomal arm cohesion phenotypes observed in chromosome spread assays, as shown in F. In each case, nearly 70 cells were classified from two separate experiments. Error bars, S.D.
FIGURE 4. HP1α dissociation from chromosome is essential for faithful spindle length. A, HeLa cells expressing mCherry-H2B, mCherry-H2B-HP1α, and mCherry-H2B-HP1αW174A were methanol-fixed and then stained with anti-α-tubulin antibody and DAPI. Scale bars, 10 μm. B, quantification of spindle length shown in A. In each case, nearly 30 cells were classified. C, HeLa cells expressing mCherry-H2B, mCherry-H2B-HP1α, and mCherry-H2B-HP1αW174A were treated with MG132 for 2 h. After fixation, the cells were stained with anti-pS10-H3, anti-cyclin B antibodies, and DAPI. Scale bars, 10 μm. D, schematic illustration of engineered constructs expressing GFP-Hec1C (amino acids 261–642), GFP-MCAK, and GFP-Hec1C-MCAK fusion proteins. E, characterization of fusion protein expression levels of GFP, GFP-Hec1C, GFP-MCAK, and GFP-Hec1C-MCAK in 293T cells. The 293T cells were transiently transfected to express GFP, GFP-Hec1C, GFP-MCAK, and GFP-Hec1C-MCAK proteins. At 24 h post-transfection, cells were harvested, boiled in the SDS-PAGE sample buffer, and analyzed by Western blotting with an anti-mCherry antibody (top) or anti-tubulin antibody (as a loading control; bottom). F, representative images of HeLa cells co-expressing both mCherry-H2B and GFP-MCAK, both mCherry-H2B-HP1α and GFP-MCAK, or both mCherry-H2B-HP1α and GFP-Hec1C-MCAK. Centromeres were stained with ACA. Scale bars, 10 μm. G, representative images of HeLa cells expressing GFP-MCAK or GFP-Hec1C-MCAK, cells were treated with DMSO or Aurora B kinase inhibitor ZM447439 for 1 h before fixation and stained with ACA and DAPI. Scale bars, 10 μm. H, representative images of HeLa cells co-expressing both mCherry-H2B-HP1α and GFP-Hec1C, both mCherry-H2B-HP1αW174A and GFP-Hec1C, both mCherry-H2B-HP1αW174A and GFP-Hec1C-MCAK, both mCherry-H2B-HP1α and GFP-Hec1C-MCAK, or both mCherry-H2B-HP1α and GFP-Hec1C-MCAK in the presence of ZM447439. Cells were fixed and stained with anti-α-tubulin antibody and DAPI. Scale bars, 10 μm. I, quantification of spindle length shown in H. In each case, nearly 30 cells were classified.
ing H2B-HP1α. As shown in Fig. 4, A and B, the spindle length in H2B-HP1α-expressing cells increased to 15.5 ± 4.8 μm compared with 12.0 ± 2.6 μm in cells expressing H2B. In contrast, spindle length was 12.3 ± 4.1 μm in cells expressing H2B-HP1α<sup>W174A</sup>, similar to that observed in control cells. The increased spindle length did not result from anaphase spindle elongation events because the cyclin B level remains unaltered (Fig. 4C).

Because MCAK regulates microtubule dynamics and spindle length (43) and forced expression of H2B-HP1α liberates the localization of MCAK from the kinetochore, we sought to examine directly whether the reduction of MCAK at the kinetochore accounts for an increased spindle length. To perform these studies, we engineered a fusion protein containing MCAK and the coiled-coil domain of Hec1 (amino acids 261–642, termed Hec1C, as illustrated in Fig. 4D). In these studies, we transfected aliquots of HeLa cells were transiently transfected to express mCherry-H2B, mCherry-Hec1C-MCAK, and H2B-HP1α<sup>W174A</sup>, followed by real-time imaging analyses. In cells expressing H2B-HP1α, most cells entered anaphase 50 min after nuclear envelope breakdown, similar to that observed for cells expressing H2B or HP1α<sup>W174A</sup> (Fig. 5, C and D), suggesting that forced expression of H2B-HP1α does not induce significant delay in mitotic progression or cause mitotic arrest. Careful examination of chromosome dynamics revealed that the fidelity of chromosomal segregation was seriously altered in mCherry-H2B-HP1α-expressing cells. As shown in Fig. 5, D and E, 39% of the H2B-HP1α-expressing cells segregated sister chromatids without achieving metaphase alignment, and 27% of the H2B-HP1α-expressing cells exhibited chromatid bridges in anaphase, suggesting that the spindle checkpoint has been compromised. Furthermore, 25% of the H2B-HP1α-expressing cells failed to complete cytokinesis (Fig. 5D, panel 3), suggesting that HP1α orchestrates a hierarchical interaction essential for accurate chromosome dynamics in mitosis. Consistent with this notion, HP1α<sup>W174A</sup> expression does not induce severe chromosome segregation defects (Fig. 5, D and E).

To further elucidate the chromosome alignment defects in H2B-HP1α-expressing cells, we carefully examined the kinetochore-microtubule attachment by immunofluorescence. As shown in Fig. 5F, overexpression of H2B-HP1α, but not of H2B or of H2B-HP1α<sup>W174A</sup>, perturbed chromosome alignment efficiency. Those non-aligned chromosomes exhibit an appearance reminiscent of merotelic attachment (enlarged box). To ascertain the precise kinetochore-microtubule attachment on chromosomes with forced localization of HP1α, aliquots of HeLa cells were transiently transfected to express mCherry-H2B, mCherry-Hec1C-MCAK, or mCherry-H2B-HP1α<sup>W174A</sup> for 24 h, followed by a chilling on ice for 10 min to destabilize non-kinetochore microtubules (32). The transfected and chilled cells were then fixed and stained for tubulin and centrochrome markers. As shown in Fig. 5G, kinetochore microtubules in H2B-expressing cells remain intact and connected to the centromere. The magnified montage shows that spindle microtubules are captured by centromeres (top). However, many chromosomes were scattered around the spindle pole with unstable kinetochore fibers (Fig. 5G, middle) relative to those of the control cells, suggesting that HP1α dissociation from the chromosome arm during the onset of mitosis is essential for orchestrating a stable kinetochore-microtubule attachment for subsequent chromosome alignment at the equator. Consistent with this notion, kinetochore microtubules remain intact and stable in H2B-HP1α<sup>W174A</sup>-expressing cells (Fig. 5G, bottom).

Our real-time imaging analyses show that forced expression of H2B-HP1α promotes premature anaphase, as shown by sister chromatids segregating without achieving metaphase alignment. In addition, chromatid bridges in anaphase-like cells were readily apparent in H2B-HP1α-expressing cells. To ascer-
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A

B

C

D

E

F

G

H
tain whether H2B-HP1α-expressing cells are able to enter anaphase, we carried out immunofluorescence microscopic analyses of fixed H2B-HP1α-expressing cells. Those fixed cells were stained with centromere marker ACA. As shown in Fig. 5H, sister chromatids are intertwined and unresolved in these H2B-HP1α-expressing cells. In contrast, sister chromatids are fully segregated in cells expressing H2B or H2B-HP1αW174A, indicating that spatiotemporal dynamics of HP1α is essential for accurate mitotic progression.

**DISCUSSION**

In this study, we reported that the localization of HP1α in interphase and mitosis is orchestrated by distinct structural determinants. The centromere localization of HP1α is determined by its binding to H3K9me2/3 in interphase but to pericentromeric HP1α, indicating that spatiotemporal dynamics of HP1α is essential for accurate mitotic progression.

**Spatiotemporal Dynamics of HP1α in Mitosis**

In this study, we reported that the localization of HP1α in interphase and mitosis is orchestrated by distinct structural determinants. The centromere localization of HP1α is determined by its binding to H3K9me2/3 in interphase but to pericentromeric HP1α, indicating that spatiotemporal dynamics of HP1α is essential for accurate mitotic progression. However, the centromeric localization of HP1α-dependent localization is essential for Aurora B kinase activity in the centromere and accurate chromosome segregation in mitosis. Further analyses showed that either PXVXL-containing proteins, such as Mis14, are involved in determining the localization of HP1α to the centromere in mitosis. Our data support a model in which spatiotemporal dynamics of HP1α localization to the centromere is governed by two distinct structural determinants.

During interphase, HP1α is broadly localized across a broader range of chromatin structures, including centromeres. However, its binding and localization to the chromosomal arm is eliminated in mitosis (21, 22). H3K9me2/3 of histone H3 determines HP1α localization to heterochromatin in interphase cells via the CD of HP1α. However, at the onset of mitosis, the majority of HP1α dissociates from chromosome arms and maintains a centromeric localization via PXVXL motif-containing proteins through its CSD. Previously, we reported that centromeric localization of HP1α during mitosis is not exclusively dependent on H3K9me2/3 but also requires proteins containing the PXVXL motifs, such as INCENP or Mis14 (36, 37). In addition, dimerization of HP1α is essential for its centromeric localization.

Although the dissociation of HP1α from chromosome arms during mitosis is well established (34, 48), its physiological functions still remained unclear. By expressing persistent chromosomal arm-localized H2B-HP1α, we observed that chromosomal arm HP1α disrupted the correct centromere/kinetochore localization of CPC, Sgo1, Mis14, and MCAK. These experimental data suggest that the physiological significance of HP1α release from chromosomal arms during mitosis is to promote accurate centromere/kinetochore assembly.

Aurora B negatively regulates not only the localization of HP1α on the chromosomal arms but also its own localization to the chromosomal arms. Our studies suggest that there is an Aurora B loop on chromosomal arms consisting of Aurora B-H3S10ph-HP1-Borealin/INCENP-Aurora B and that inhibiting Aurora B activity prevents CPC and HP1α from dissociating. On the other hand, similar to Haspin and Sgo1, HP1α also affects centromeric CPC loading (40, 41, 49). Although the mechanism for this is currently elusive, we suggest that HP1α affects centromeric CPC assembly through providing a physical bridge for CPC to transport from the chromosomal arms to the pericentromere and then to the centromere.

This study has demonstrated that chromosomal arm-localized HP1α is responsible for Sgo1 localization. In addition, we show that HP1α is involved in sister chromatid arm cohesion protection through an HP1-Sgo1 pathway. Aurora B is also involved in cohesion dissociation of chromosomal arms (26), but its role and mechanism of action are also unknown. Our studies, however, suggested that Aurora B may exert its cohesion disassociation function through the HP1-Sgo1 pathway and that this molecular mechanism is different from that of Plk1. This mechanism is likely because we have observed that sister chromatid cohesion remained intact even in the absence of Sgo1 or HP1α when PLK1 was inhibited or SA2 was unphosphorylated. In contrast, sister chromatid cohesion was no longer maintained in the absence of Sgo1 or HP1α+γ with low Aurora B activity. Thus, we reasoned that inhibition of Aurora B activity alone can protect sister chromatid cohesion due to an active H3K9me2/3-HP1-Sgo1 axis.

The forced chromosomal localization of HP1α also prevents kinetochore MCAK assembly. The reason for this could be decreased Aurora B levels because it has been shown that Aurora B activity promotes kinetochore MCAK localization (45, 46). On the other hand, the decreased MCAK may be a factor responsible for the elongated spindle because forced kinetochore localized MCAK was only able to partially rescue the elongated spindle induced by H2B-HP1α expression. Obviously, it is possible that some other depolymerizing mechanism may also be involved in this process. Our experimental results presented here are the first to demonstrate that the chromatin-binding protein can affect spindle geometry and length via a
hierarchical interaction at the kinetochore-chromosome interface. Aurora B activity is required for the effective kinetochore localization of Mps1 (47). Forced chromosomal localization of HP1α lowered centromeric CPC loading. As a consequence, this caused a decreased Mps1 kinetochore localization. In excellent agreement with the decreased Aurora B and Mps1 signal, we observed a high frequency of anaphase-lagging chromosomes (Fig. 5E), indicating compromised spindle assembly checkpoint function. In H2B-HP1α-expressing cells, mitotic progression was not disturbed; however, sister chromatid alignment and segregation defects were obvious. The alignment defects may be due to incorrect kinetochore-MT attachment, and segregation defects may be caused by uncondensed or intertwined sister chromatids.

In summary, we have demonstrated that the structural determinants responsible for HP1α localization to the centromere are different between interphase and the onset of mitosis and that accurate localization of HP1α is essential for faithful mitotic progression. Furthermore, its dissociation from the chromosomal arms is required for sister chromatid segregation, chromosome condensation, spindle length control, chromosomal alignment, and faithful segregation. However, a number of questions still need to be resolved, including the function of centromeric HP1α. Although studies by Kang et al. (36) suggest that it is dispensable, our results show that knocking down HP1α levels and pulling HP1α toward the chromosomes both inhibit the centromeric assembly of CPC. Currently, it is unclear how centromere-associated HP1α affects the upstream localization of CPC at the mitotic centromere. Further questions that still need to be addressed by future studies include the following. (i) How was MCAK centromeric localization ablated by H2B-HP1α expression? (ii) Are there any other microtubule depolymerases involved in the elongated spindle regulation induced by H2B-HP1α expression? (iii) How is chromosome condensation affected in H2B-HP1α-expressing cells? (iv) Despite the fact that we have shown that the H2B-HP1αW174A mutation abolishes the phenotypes associated with H2B-HP1α expression, what are the other proteins that regulate spindle geometry via interaction with HP1α Trp-174? (v) Chromosome arm-localized HP1α orchestrates the cohesion between sister chromatid arms via recruiting Sgo1; do other cohesion protection proteins, such as Pds5 or Sororin, contribute to this process? The answers to all of the aforementioned questions and molecular delineation of underlying mechanisms will better our understanding of HP1α functional roles in mitotic progression.

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