PRC1 cooperates with CLASP1 to organize central spindle plasticity in mitosis

Jing Liu†, Zhikai Wang†, Kai Jiang†‡, Liangyu Zhang‡, Lingli Zhao‡, Shasha Hua†, Feng Yan†‡, Yong Yang‡, Dongmei Wang‡, Chuanhai Fu‡, Xia Ding‡, Zhen Guo†‡, & Xuebiao Yao†

†Anhui Key Laboratory for Cellular Dynamics & Chemical Biology, and Hefei National Laboratory for Physical Sciences at Nanoscale, Hefei 230027, China;‡Department of Physiology, Morehouse School of Medicine, Atlanta, GA 30310, USA;§Department of Medicine, Beijing University for Chinese Medicine, Beijing, China 100086

Running title: PRC1 cooperates with CLASP1

Address all correspondence to: Zhen Guo; Email: ustclcd@ustc.edu.cn; or Xuebiao Yao; Email: yaoxb@ustc.edu.cn

During cell division, chromosome segregation is governed by the interaction of spindle microtubules with the kinetochore. A dramatic remodeling of interpolar microtubules into an organized central spindle between the separating chromatids is required for the initiation and execution of cytokinesis. Central spindle organization requires mitotic kinesins, microtubule-bundling protein PRC1, and Aurora B kinase complex. However, the precise role of PRC1 in central spindle organization has remained elusive. Here we show that PRC1 recruits CLASP1 to the central spindle at early anaphase onset. CLASP1 belongs to a conserved microtubule-binding protein family that mediates the stabilization of overlapping microtubules of the central spindle. PRC1 physically interacts with CLASP1 and specifies its localization to the central spindle. Repression of CLASP1 leads to sister-chromatid bridges and depolymerization of spindle midzone microtubules. Disruption of PRC1-CLASP1 interaction by a membrane permeable peptide abrogates accurate chromosome segregation, resulting in sister chromatid bridges. These findings reveal a key role for the PRC1-CLASP1 interaction in achieving a stable anti-parallel microtubule organization essential for faithful chromosome segregation. We propose that PRC1 forms a link between stabilization of CLASP1 association with central spindle microtubules and anti-parallel microtubule elongation.

Key words: CLASP1, PRC1, Central spindle, metaphase-to-anaphase transition

To ensure that each daughter cell receives the full complement of the genome in each cell division, chromosomes move polewards and non-kinetochore fibers become bundled at the onset of anaphase, initiating assembly of the central spindle, a set of anti-parallel microtubules which serves to concentrate key regulators of cytokinesis (1-3). Chromosomal passengers are a group of evolutionarily conserved proteins that orchestrate chromosome segregation and central spindle plasticity (4,5). This protein complex containing Aurora B, survivin, INCENP, and borealin, is relocated from the kinetochore to the central spindle upon anaphase onset (5-9). Perturbation of their function results in defects in metaphase chromosome alignment, chromosome segregation, and cytokinesis (10).

Among central spindle maintenance components, only two have been reported to mediate the microtubule bundling in central spindle. One is centralspindlin, a hetero-tetramer containing CeMKLP1/ZEN-4 and RhoGAP/CYK-4 (11), while the other one is an evolutionarily conserved protein, PRC1 (also named Feo in fruit fry, Ase1 in yeast and MAP65 in Plant cells). PRC1 is a non-motor microtubule binding and bundling protein in human cells originally identified as a Cdc2 substrate essential for cytokinesis (12,13). Similar microtubule regulatory activities have been reported in yeast, fruit fly and plant cells. It is well known that overexpression of wild type PRC1 in HeLa cells can result in thick microtubule bundles in cells at interphase (13). Bundling activity of PRC1, as well as centralspindlin, is required for the organization of central spindle as well as for the successful progression of cytokinesis. PRC1 molecules accumulate on the midline of a central spindle with
the cell cycle progression to anaphase. As a non-motor microtubule binding protein, transportation of PRC1 to the midline is promoted by its association to kinesin, KIF4A and timing of this progression is controlled by the dephosphorylation of Thr481 on PRC1 when the cell exits metaphase by phosphatase Cdc14 (14). Our recent study shows that prevention of the phosphorylation of PRC1 at Thr470 causes an inhibition in PRC1 oligomerization in vitro and an aberrant organization of central spindle in vivo, suggesting that this phosphorylation-dependent PRC1 oligomerization ensures that central spindle assembly occurs at the appropriate time in the cell cycle (15).

Spatiotemporal regulation of microtubule organization and dynamics is responsible for the mitotic apparatus such as the central spindle. However, it has remained elusive as to how the central spindle microtubule organization and dynamics is regulated. There are a large variety of microtubule-associated proteins responsible for regulation of the dynamic behaviour of microtubules and microtubule-mediated transport. Among these, proteins that associate with the tips of microtubules are called +TIPs, for ‘plus-end tracking proteins’. These proteins have been shown to be important in different organisms and cellular systems (16). Using yeast two-hybrid assay, CLASPs were identified as interacting partners of the CLIPs and characterized as new +TIP proteins (17).

The microtubule-binding protein CLASP is emerging as an important microtubule regulator in the formation of the mitotic apparatus (18-22). CLASP is required for promoting plus-end growth of spindle microtubules in prometaphase (23). While the molecular mechanisms underlying its regulation of microtubule dynamics remain elusive, it is generally believed that CLASP orchestrates microtubule dynamics via its physical interacting with EB1, CLIP170 and microtubules (17,24).

To delineate the molecular function of PRC1 in central spindle organization and spatiotemporal regulation, we carried out a new search for PRC1-interacting proteins. Our studies show that PRC1 physically interacts with CLASP1 and the two proteins cooperate in the organization of the central spindle. Our studies provide a novel regulatory mechanism in which PRC1 complex operates central spindle organization in mitosis.

MATERIALS AND METHODS

Expression constructs

Human CLASP1 cDNA was obtained from the Kazuza DNA Research Institute. GFP-CLASP1 contained the whole open reading frame of KIAA0622, subcloned into Xhol-XhoI sites of the mammalian expression vector pEGFP-C1 (Clontech Inc., California, USA). All the deletions of CLASP1 were generated by PCR.

GST and 3×FLAG plasmids of full-length PRC1 and its various mutants were prepared as described previously (15).

Recombinant protein production

Purification of recombinant proteins was carried out as described previously (15). Briefly, 1 liter of LB media (10 g NaCl, 10 g tryptone and 5 g yeast extract, pH 7.4) was inoculated with bacteria transformed with GST-PRC1 and its mutants, respectively. When the optical density (A 600) of the bacteria culture reached between 0.6 and 0.7, the protein expression was initiated by addition of 0.5 mM isopropyl-D-thiogalactopyranoside for 3 hours. Bacteria were then collected by centrifugation and resuspended in PBS containing proteinase inhibitors (leupeptin, pepstatin, and chymostatin; 5 μg/ml) followed by sonication and clarification as described previously (15). The GST fusion protein in bacteria in the soluble fraction was purified using glutathione-agarose chromatography.

To generate TAT-GFP proteins, we recombinated pET-22b vector with an 11-animo acid TAT sequence followed by a GFP gene (25). TAT-GFP-his fusion proteins were expressed and purified as described previously (25).

Generation of TAT fusion protein for functional analysis

For introducing TAT-GFP fusion proteins to probe for the functional relevance of PRC1-CLASP1 interaction, HeLa cells were cultured to 50~60% confluency before experimentation. Just before introduction, the cells were washed with serum-free media and incubated with TAT-GFP fusion peptides at various concentrations. After incubation, the cells were washed with PBS and then examined directly under fluorescence microscopy.

To analyze the relative level of PRC1 and CLASP1 associated with the cytoskeleton in response to TAT-PRC1 peptide treatment,
transfected cells were rinsed with PHEM buffer (100 mM Pipes, 20 mM Hepes, pH 6.9, 5 mM EGTA, 2 mM MgCl2, and 4 M glycerol) twice followed by incubation in PHEM buffer containing a proteinase inhibitor mixture (pepsstatin-A, leupeptin, aprotinin, and chymostatin; final concentration 5 µg/ml for each inhibitor) plus 0.1% Triton X-100 for 2 min at room temperature to allow cytosolic proteins to be released into extracellular medium. The extracted cells were then harvested from the Petri dish and centrifuged at 1500 x g for 5 min. The resulting pellets, designated as insoluble materials, were solubilized in 1x SDS-PAGE sample buffer, whereas the supernatants, called the soluble fraction, were concentrated with 5% trichloroacetic acid followed by reconstitution in 1x SDS-PAGE sample buffer as described (26).

Cell cultures and transfection of plasmids and siRNAs

HeLa and 293T cells (American Type Culture Collection, Rockville, MD, USA) were cultivated as subconfluent monolayers in Dulbecco’s Modified Eagle’s Medium (Invitrogen) with 10% fetal bovine serum (HyClone, UT) and 100 units/ml penicillin plus 100 µg/ml streptomycin at 37 ºC with 10% CO2.

Lipofectamine transfection reagents (Invitrogen) were used for plasmid transfection. Cells were transfected with Lipofectamine 2000 pre-mixed with various plasmids (2 µg/ml) as described above. Stable clones were selected in the presence of 0.5-1 mg/ml G418 (Calbiochem). Synthetic siRNAs were transfected, using Oligofectamine (Invitrogen). SiRNA oligonucleotides were directed against the following target sites: CLASP1#B, 5’-GGATGATTTACAAGACTGG-3’ (24); PRC1, 5’-ATATGGGAGCTAATTGGGA-3’. In the trial experiments, different concentrations of siRNA oligonucleotides were used for different time intervals of treatment, and transfection efficiency was judged based on the uptake of FITC-conjugated oligonucleotides. In pilot experiments, HeLa cells were transfected with different concentration of siRNA oligonucleotides or control scramble oligonucleotide for different time intervals. Transfected cells were then collected and solubilized in SDS-PAGE sample buffer. The efficiency of this siRNA-mediated protein suppression was judged by Western blotting analyses of target proteins.

Antibodies

His-tagged full-length PRC1 protein, purified by Ni-agarose beads, was used for immunization of Balb/c mice. Three mice were boosted every three weeks until antibody titer reached 1:1,500 as judged by Western blotting. The splenocytes from immunized mice were fused with FO myeloma cells (American Type Culture Collection; Manassas, VA) according to standard protocol. Mono-specific antibodies against PRC1 were selected based on Western blotting and immunocytochemistry. The polyclonal antibodies against PRC1 were generated as described (27).

A rabbit anti-CLASP1 antibody is a kind gift from Dr. Fedor Severin (Moscow State University, Russia; reference # 22).

Pull-down assay

The GST-PRC1 (full-length and its mutants) fusion protein-bound Sepharose beads were incubated with 293T cell lysate containing GFP-tagged CLASP1 or deletion mutants for 4 h at 4 ºC. After the incubation, the beads were washed twice with PBS containing 0.5% Triton X-100 and twice with PBS and boiled in SDS-PAGE sample buffer. The bound proteins were then separated on 10% SDS-polyacrylamide gel. Separated proteins were then transferred onto nitrocellulose membrane for Western blotting with a monoclonal GFP antibody.

To test whether the recombinant TAT-GFP-PRC1486-620 peptide disrupts PRC1*CLASP1 interactions, GFP-CLASP1 bound GST-PRC1 affinity beads were incubated with 5 µM TAT-GFP-PRC1486-620 peptide for 30 min on a rotator at room temperature. The affinity beads were then collected and boiled in SDS-PAGE sample buffer. The proteins bound were subsequently subject to SDS-PAGE analysis as described previously.

Synchronization, immunoprecipitation and immunoblot analysis

293T or HeLa cells were cultured with DMEM supplemented with 10% fetal calf serum. To obtain the mitotic cells, cells were synchronized by treatment with thymidine for 16 h, washed with PBS three times, and incubated with fresh medium for 10–11 h. The cells or cell extracts at mitosis were subjected to immunofluorescence or immunoprecipitation analysis as described...
previously (15).

Briefly, for the immunoprecipitation assay, GFP-CLASP1 or its deletion mutants and 3×FLAG-PRC1 co-expressing 293T cells were solubilized in lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) on ice followed by centrifugation (14,000 rpm for 20 min at 4 ºC). 3×FLAG-PRC1 proteins were harvested with Ezview™ red Anti-FLAG M2 affinity beads (Sigma). These beads were washed and boiled in SDS-PAGE sample buffer and then subjected to immunoblot analysis with a monoclonal GFP antibody and FLAG antibody, respectively.

For immunoblotting, proteins were probed by appropriate primary antibodies. Immunoreactive signals were then detected with horseradish peroxidase-conjugated secondary antibodies using ECL kit (Pierce) and visualized by autoradiography on Kodak BioMAX MS film.

**Immunofluorescence microscopy**

After transfection or drug treatment, cells on acid-treated glass coverslips were washed with PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl2, pH 6.9). Permeabilization was carried out for 1 min in PHEM buffer containing 0.1% Triton X-100 at 37 ºC. Pre-extracted cells were fixed in 2% paraformaldehyde in PHEM buffer for 10 min. After washing three times with PBS, cells were blocked with 1% BSA (Sigma) in PBS containing 0.05% Triton X-100 for 30 min, and then incubated with primary antibodies for 1 h at room temperature then 1 h at 4 ºC, followed by secondary antibodies for 45 min. DNA was stained with DAPI (Sigma).

Deconvolution images were collected using a Deltavision wide-field deconvolution microscope system built on an Olympus IX-70 inverted microscope base. For imaging, a ×100 1.35 NA lens was used and optical sections were taken at intervals of 0.2 µm. Images were processed using DeltaVision Softworx software as described previously (27). Images for display were generated by projecting the sum of the optical sections using the maximum-intensity method. For presentation of details of the internal central spindle in Figure 6, projection images were constructed from a 1-µm section within the cell.

**Live cell imaging**

HeLa cells grown on coverslips stably expressed mCherry-tubulin. After appropriate treatment, the cells were observed with a DeltaVision system (Applied Precision, Issaquah, WA).

**Quantification of microtubule bundles in the central spindle**

To evaluate the functional relevance of PRC1-CLASP1 interaction in the central spindle organization, HeLa cells stably expressing mCherry-tubulin were synchronized and subjected to TAT-PRC1138-620 treatment before imaging. Optical sections taken from the middle of the z-stack were scored for the number of overlapped microtubule bundles in the TAT-PRC1138-620-treated cells. About 10 cells of each condition from 3 different preparations were scored and subjected to statistic analyses.

**Quantification of stable microtubules in the central spindle**

To evaluate the functional relevance of PRC1-CLASP1 interaction in stabilizing central spindle microtubule, HeLa cells stably expressing mCherry-EB3 were synchronized and subjected to TAT-PRC1138-620 treatment before imaging. Optical sections taken from the middle of the z-stack were scored for the number of mCherry-EB3-marked microtubule plus-ends in the TAT-PRC1138-620-treated cells. To quantify the number of EB3-marked microtubule plus-ends, we selected the region of overlapped central spindle microtubule (middle one third of the spindle axis; boxed area in Figure 6J). About 9 cells of each condition from 3 different preparations were scored and subjected to statistic analysis.

**RESULTS**

**CLASP1 interacts with PRC1 in vitro and in vivo**

Our early attempt to dissect central spindle revealed a complex of PRC1-CLASP1 (Fu and Yao, unpublished observation). The distribution of CLASP1 within the anti-parallel inter-digitating microtubules in anaphase is reminiscent of PRC1 distribution in anaphase cells (e.g., (15)). To examine whether CLASP1 interacts with PRC1 in vivo, we co-transfected 293T cells with GFP-CLASP1 and 3×FLAG-PRC1 and
immuno-isolated FLAG-PRC1 protein and its accessory proteins with an anti-FLAG M2 affinity gel. Immunoblotting with an anti-GFP antibody confirmed the presence of GFP-CLASP1 in FLAG-immunoprecipitates (Figure 1A, lane 6). However, CLIP170, a binding partner of CLASP1, was not recovered from the FLAG-immunoprecipitates, demonstrating the specificity of the CLASP1-PRC1 complex.

To validate if CLASP1 forms a cognate complex with PRC1, we carried out an anti-PRC1 immunoprecipitation assay. As shown in Figure 1C, anti-PRC1 antibodies pulled down PRC1 protein from HeLa cell lysates (lane 3). As predicted, CLASP1 protein was presented in the anti-PRC1 immunoprecipitates (lane 6). However, tubulin was not precipitated, validating the specific association of CLASP1 with PRC1. Thus, we conclude that PRC1 forms a complex with CLASP1 in vivo.

To test if CLASP1 physically interacts with PRC1, we performed an in vitro pull-down assay using GST-PRC1 as an affinity matrix to isolate CLASP1 and its deletion mutants from lysates of 293T cells transiently transfected to express GFP-CLASP1 wild type and deletion mutant proteins (Figure 1B). As shown in Figure 1D (lanes 6-8), the GST pull-down assay showed that GFP-CLASP1 and its two N-terminal deletions GFP-N1003 and GFP-N694 were specifically absorbed by GST-PRC1, suggesting that PRC1 interacts with CLASP1 via its N-terminus.

Our recent studies show that mitotic phosphorylation of PRC1 at Thr470 is required for PRC1 oligomerization and proper central spindle organization (15). To test whether mitotic phosphorylation of PRC1 at T470 and T481 modulates PRC1-CLASP1 interaction, we expressed phospho-mimicking and non-phosphorylatable PRC1 mutant proteins and examined for their co-localization with CLASP1 and tubulin. As shown in Figure 2B, GFP-CLASP11-1003 co-distributes with PRC1 to the spindle midzone in anaphase and to the midbody during telophase and cytokinesis (a-b). On other hand, GFP-CLASP11-694, previously reported to exhibit the microtubule binding activity (17), distributes along with the entire length of MTs, neither localizing to the central spindle nor to the midbody (Figure 2B; c-d). The GFP-CLASP11-1003 distribution relative to PRC1 in anaphase together with biochemical interaction established, such as those shown in Figure 1, suggests that PRC1 is required for the central spindle localization of CLASP1.

**PRC1-CLASP1 binding is necessary for CLASP1 distribution to the central spindle**

If PRC1 specifies the localization of CLASP1 to the central spindle, the region where CLASP1 binds to PRC1 should be localized to the central spindle. To test this hypothesis, we generated a series of CLASP1 deletion mutants tagged with GFP. HeLa cells were transiently transfected to express various CLASP1 deletion mutant proteins and examined for their co-localization with PRC1 and tubulin. As shown in Fig. 2B, GFP-CLASP11-1003 co-distributes with PRC1 to the spindle midzone in anaphase and to the midbody during telophase and cytokinesis (a-b). On other hand, GFP-CLASP11-694, previously reported to exhibit the microtubule binding activity (17), distributes along with the entire length of MTs, neither localizing to the central spindle nor to the midbody (Figure 2B; c-d). The GFP-CLASP11-1003 distribution relative to PRC1 in anaphase together with biochemical interaction established, such as those shown in Figure 1, suggests that PRC1 is required for the central spindle localization of CLASP1.

**PRC1 is essential for accurate localization of CLASP1 to the central spindle**

If PRC1 is required for the localization of CLASP1 co-distributes with PRC1 to the central spindle during mitosis

Given the physical interaction between CLASP1 and PRC1 established in the early studies (Figure 1), we sought to examine their spatiotemporal distribution profiles and interrelationship during cell division. To this end, HeLa cells were fixed and stained with antibodies against PRC1 and CLASP1. Both PRC1 and CLASP1 display a typical spindle-like distribution pattern in prometaphase and metaphase cells (Figure 2A; a, b). Compared to that of PRC1, CLASP1 localization to the spindle pole is apparent in metaphase cell (b). During metaphase-anaphase transition, the majority of PRC1 is relocated to the central spindle while the majority of CLASP1 remains associated with spindle and spindle pole (c). In anaphase B cells, the majority of CLASP1 relocates to the central spindle where it is superimposed onto that of PRC1 (d). From telophase to cytokinesis, both proteins are concentrated at the Flemming body (e-f). The double immunofluorescence study revealed that PRC1 is assembled onto the central spindle prior to the arrival of CLASP1.
CLASP1 to the central spindle, suppression of PRC1 would result in an aberrant localization of CLASP1 in anaphase. To test this hypothesis, HeLa cells were transfected with siRNA oligonucleotides to suppress PRC1. Suppression of PRC1 was successfully achieved by introducing siRNA oligonucleotide into target cells judged by Western blotting analysis (Figure 3A, top panel) without alteration of CLASP1 protein levels (middle panel). Quantitative Western blotting analyses show that treatment of 25 nM siRNA oligonucleotide resulted in a 93 ± 2% suppression of PRC1 protein. Immunofluorescence analysis validated the efficacy of the PRC1 protein repression as virtually no PRC1 protein was found in the siRNA-treated cells (Figure 3B; a, b, and c).

As shown in Figure 3B, the central spindle localization of PRC1 was not altered by the scramble siRNA oligonucleotide treatment (a', b', and c'). However, in these PRC1-repressed anaphase cells, anti-parallel microtubule organization was perturbed as spindle microtubules from two opposite poles are no longer overlapped (Figure 3B, a-b; arrowheads). The two half-spindles remained disconnected in those PRC1-repressed HeLa cells. Examination of DNA staining revealed an aberrant chromatid segregation (Figure 3B, a; arrows), consistent with previous studies (28). PRC1 suppression did not cause of gross change CLASP1 localization along the mitotic spindle (Figure 3C; a-c). However, repression of PRC1 abolished the overlapping of CLASP1 bundles from anti-parallel configuration (Figure 3C arrows; a-b). In addition, repression of PRC1 results in disappearance of CLASP1 in the Flemming body (Figure 3C; c) as the integral central spindle microtubules could not be formed in the absence of PRC1. Double labeling of CLASP1 and tubulin revealed that PRC1 is essential for formation of inter-digital microtubule bridge (Figure 3D; a-b). Thus, we concluded that PRC1 specifies the central spindle localization of CLASP1 during anaphase.

**CLASP1 depletion causes cell cycle delay and mitotic abnormalities**

The subcellular distribution of CLASP1 and its physical interaction with PRC1 prompted us to examine if CLASP1 functions in spindle integrity and chromosome dynamics. To this end, we suppressed the CLASP1 protein in HeLa cells by using siRNA oligonucleotide followed by phenotype-based characterization. Quantitative Western blotting analyses indicated that siRNA oligonucleotide suppressed the protein level of CLASP1 up to 70% at forty-eight hours post-transfection (Figure 4A, top panel). Control siRNA did not alter CLASP1, tubulin, or PRC1 protein level (Figure 4A).

For accurate analysis of the phenotype associated with the repression of CLASP1 activity, we examined the chromosome segregation and mitotic spindle plasticity in tubulin-marked HeLa cells treated with CLASP1 siRNA oligonucleotide under a fluorescence microscope. Knock-down of CLASP1 induced an anaphase chromatid bridge phenotype and disorganized central spindles (Figure 4B, a-c; arrows). We have scored 300 cells from 4 different preparations and our quantification shows that the percentage of cells containing misaligned chromosomes and premature anaphase chromatid bridges was significantly greater than those of control cells (Figure 4C, 4D). As shown in Figure 4C, the chromosome misalignment rate was significantly increased from 3 ± 1% of the control to 17 ± 3% of CLASP1-repressed cells (P<0.01). The percentage bearing anaphase bridge was increased from 2 ± 1% of the scramble control to 31 ± 5% in CLASP1-suppressed cells (P<0.01), supporting the notion that CLASP1 is essential for chromosome stability in mammalian cell division.

**PRC1-CLASP1 interaction is required for faithful mitosis**

The functional importance of CLASP1 in mitotic plasticity and PRC1-CLASP1 inter-relationship prompted us to examine the functional relevance of PRC1-CLASP1 interaction in mitosis. To pinpoint the region(s) of PRC1 which binds directly to CLASP1, we generated a series of deletion mutants of PRC1 (illustrated in Figure 5A) tagged with GST and used glutathione-bound GST PRC1 proteins as affinity matrices. Since our attempt to obtain recombinant full-length CLASP1 in bacteria was unsuccessful, we use cell lysates of 293T cells transiently transfected to express GFP-CLASP1 to incubate with GST-PRC1 affinity matrix. As shown in Figure 5B, GFP-CLASP1 binds to full-length PRC1 and the C-terminal PRC1438~620 judged by GFP antibody blotting analysis (Figure 5B; upper panel). Neither GST protein nor GST-PRC11~437 absorbed GFP-CLASP1, suggesting that the interaction between CLASP1...
and C-terminal 183 amino acids of PRC1 is specific. This binding assay also revealed weak CLASP1-binding activities in the N-terminal deletion mutants PRC1\(^{438-485}\) and PRC1\(^{486-620}\) (Figure 5B; lanes 5–6).

To explore the functional effect of CLASP1-PRC1 interaction in the mitotic process, we sought to introduce membrane permeable peptide containing PRC1\(^{438-620}\) to modulate endogenous PRC1-CLASP1 interaction. This is achieved by introducing an 11-amino-acid-peptide derived from TAT protein transduction domain (29,30) into GFP-PRC1\(^{438-620}\) fusion protein. The recombinant protein was histidine-tagged and purified to homogeneity using Ni-affinity beads (Figure 5C). As predicted, incubation of recombinant GFP-PRC1\(^{438-620}\) protein (5 µM) with GFP- CLASP1-bound GST-PRC1 affinity matrix disrupts the CLASP1-PRC1 association in vitro (Figure 5D; lane 3). Neither GFP nor TAT-GFP interferes with CLASP1-PRC1 interaction (Figure 5D; lanes 1 and 2), demonstrating the effect of PRC1\(^{438-620}\) in competing full-length PRC1 for CLASP1 association.

If recombinant TAT-GFP-PRC1\(^{438-620}\) fusion protein disrupts endogenous PRC1-CLASP1 interaction, addition of TAT-GFP-PRC1\(^{438-620}\) recombinant protein into HeLa cell culture would compete for endogenous PRC1 and liberate CLASP1 into the cytoplasmic soluble fraction. To determine whether there were any major changes in the behavior of endogenous PRC1 and CLASP1 in response to the addition of GFP-PRC1\(^{438-620}\), we measured the partitioning of endogenous PRC1 and CLASP1 into the Triton X-100-soluble fraction compared with the insoluble "cytoskeletal" fraction based on the Western blotting analyses. As summarized in Figure 5E, 71.7 ± 4.3% of endogenous PRC1 resides in the Triton X-100-insoluble fraction. Partitioning of CLASP1 is similar to endogenous PRC1; 69.3 ± 4.1% of endogenous CLASP1 is associated with the Triton X-100-insoluble fraction.

Our trial experiments show that the internalization of TAT-GFP and TAT-GFP-PRC1 proteins was seen in virtually all cells 30 mins after the addition of TAT-GFP fusion proteins in culture. Our quantitative blotting analyses show that addition of TAT-GFP-PRC1\(^{438-620}\) induced a dose-dependent liberation of CLASP1 into cytoplasmic “soluble” fraction (Supplemental Figure 1). Although TAT-GFP was internalized as effectively as TAT-GFP-PRC1\(^{438-620}\), TAT-GFP did not alter the partition of endogenous CLASP1 protein in cytoplasmic soluble and cytoskeletal insoluble fraction (Figure 5F). While 70.3 ± 4.5% of endogenous CLASP1 resides in the Triton X-100-insoluble fraction of TAT-GFP-treated cell culture, only 33.7 ± 7.7% of endogenous CLASP1 is associated with the Triton X-100-insoluble fraction in 500 nM TAT-GFP-PRC1\(^{438-620}\) treated cells (p<0.01), indicating that TAT-GFP-PRC1\(^{438-620}\) fusion protein disrupts endogenous PRC1-CLASP1 interaction in vivo. In contrast, there was no significant difference in the amount of EB1 and tubulin extracted from control or PRC1\(^{438-620}\) peptide-treated cells (data not shown).

**PRC1 cooperates with CLASP1 to orchestrate central spindle plasticity and chromosome stability**

Having demonstrated the ability of TAT-GFP-PRC1\(^{438-620}\) in perturbing endogenous PRC1-CLASP1 interaction, we sought to test whether the PRC1-CLASP1 interaction is specifically required for central spindle plasticity and dynamics. To overcome the potential pre-anaphase block due to the perturbation of PRC1-CLASP1 interaction, we adopted a protocol to synchronize cells at metaphase and then added TAT-PRC1 peptide to probe for functional relevance in anaphase onset and beyond (illustrated in Figure 6A). We began real-time imaging of metaphase-synchronized HeLa cells expressing mCherry-tubulin. TAT-GFP-PRC1\(^{438-620}\) was added into metaphase cells and time-lapse imaging started as the cells underwent anaphase-telophase transition. Our trial experiments show that treatment of S and G2 phase HeLa cells with TAT-GFP-PRC1\(^{438-620}\) (500 nM) did not alter cell cycle progression into mitosis or sister chromatid separation (Figure 6C) as compared to those treated with TAT-GFP control (Figure 6B). In general, it takes an average of 3103.3 ± 448.5 sec (n = 8 cells) for HeLa cells to transit from centrosomes separations to anaphase onset of sister chromatide separation, which is similar to that of TAT-GFP (500 nM) treated cells (average 2855.8 ± 388.7 sec; n = 8 cells; Figure 6E).

However, treatment of mitotic cells with TAT-GFP-PRC1\(^{438-620}\) (500 nM) protein perturbed central spindle assembly plasticity and prolonged the anaphase duration. On average, it takes about 700.6 ± 27.0 sec (n = 8 cells), for TAT-GFP-PRC1\(^{438-620}\)-treated HeLa cells to transit from the formation of anti-parallel overlapped
microtubule organization to midbody formation. However, it takes about $621.4 \pm 16.5$ sec ($n = 8$ cells; $p < 0.01$) for control HeLa cells treated with TAT-GFP to complete the aforementioned process (Figure 6F). Careful examination of time-lapse imaging revealed that the delay took place before the formation of contractile ring (Figure 6C; asterisks).

To investigate whether the delay in anaphase progress observed in TAT-GFP-PRC1 486-620 treated cells was due to the disturbance of endogenous PRC1 or that of CLASP1, we knocked down PRC1 and CLASP1 respectively. Down-regulation of CLASP1 had a detectable prolongation on anaphase duration (on average $1079.0 \pm 92.5$ sec versus $603.5 \pm 22.0$ sec in control cells). The duration from central spindle assembly to contraction ring formation has been extended more than 78.9%. However, suppression of PRC1 by siRNA did not change the anaphase duration (on average $641.0 \pm 27.8$ sec versus $603.5 \pm 22.0$ sec in control cells; Figure 6G). To test if synergism can be achieved when CLASP1 repression is combined with TAT-PRC1 438-620 peptide, synchronized HeLa cells were transfected with CLASP1 siRNA followed by incubation of TAT-PRC1 438-620 peptide as briefed above. As shown in Figure 6D, a combination of CLASP1 knock-down with perturbation of PRC1-CLASP1 interaction dramatically disrupted the number of overlapped central spindle microtubule bundles in addition to a prolonged anaphase onset, a phenotype seen in TAT-PRC1438-620-treated HeLa cells. Quantitative analyses show that perturbation of PRC1-CLASP1 interactions disrupted the central spindle plasticity as the number of overlapped anti-parallel microtubules is dramatically reduced (Figure 6I). The number of overlapped microtubules was reduced from an average of $10 \pm 2$ of control anaphase A cells to an average of $6 \pm 1$ of TAT-PRC1438-620-treated cells ($p<0.01$). A combination of siRNA-mediated repression of CLASP1 with TAT-PRC1438-620 treatment further reduced the number of overlapped microtubule bundles ($5 \pm 2$ in anaphase A; $p<0.01$). Similar profile was also observed in anaphase B cells in the presence of TAT-PRC1438-620 treatment.

To further evaluate the functional relevance of PRC1-CLASP1 interaction in stabilizing central spindle microtubule, we sought to examine the EB3 distribution in the anaphase central spindle microtubule in the presence of TAT-PRC1438-620 peptide treatment. To this end, HeLa cells stably expressing mCherry-EB3 were synchronized and subjected to TAT-PRC1438-620 treatment before imaging. Optical sections taken from the middle of the z-stack were scored for the number of mCherry-EB3-marked microtubule plus-ends in the TAT-PRC1 438-620-treated cells. EB3 is a plus-end tracking protein that marks the growth and stability of microtubule. To quantify the number of EB3-marked microtubule plus-ends, we selected the region of overlapped central spindle microtubule (boxed area in Figure 6J). The statistic analysis demonstrated that perturbation of PRC1-CLASP1 greatly reduced the stability of central spindle microtubules ($p<0.01$; compared to that of TAT-GFP-treated cells).

To elucidate the role of CLASP1-PRC1 interaction in the central spindle plasticity and chromosome stability, we carried out immunofluorescence study of mitotic cells treated TAT-GFP-PRC1438-620. As shown in Figure 6H, perturbation of PRC1-CLASP1 interaction resulted in chromosome lagging (b) and formation of a sister chromatid bridge (c), a characteristic phenotype associated with chromosome loss in cell division cycle. This phenotype is rarely seen in TAT-GFP-treated cells (Figure 6H; b”-d”). We have also included CLASP1 siRNA treatment and siRNA treatment plus TAT-PRC1 438-620 peptide treatment. The typical phenotype seen in PRC1-CLASP1 interaction disrupted cells is sister chromatid bridges. The quantification from three different preparations was presented in Figure 6K. Thus, we conclude that PRC1-CLASP1 interaction governs central spindle plasticity and dynamics that are essential for faithful chromosome segregation in mitosis.
DISCUSSION

Our studies reveal a key role for the PRC1-CLASP1 interaction in achieving the stable anti-parallel microtubule organization essential for faithful chromosome segregation. Our characterization demonstrates that the interaction between PRC1 and CLASP1 is essential and sufficient for the localization of CLASP1 to the central spindle. Given the disorganization of central spindle seen in CLASP1-repressed and CLASP1-PRC1 interaction perturbed cells, we reason that PRC1 specifies the central spindle localization of CLASP1 and CLASP1 is essential for central spindle plasticity and genomic stability in mitosis.

The spindle midzone, a highly organized network of anti-parallel inter-digitating microtubules between separating chromosomes, plays a crucial role in regulating the initiation and completion of cytokinesis. Cytokinesis is a highly orchestrated process that must coordinate precisely with other events of the cell cycle via coupling cytokinesis to Cdc2 inactivation. Down-regulation of Cdc2 activity initiates the accumulation of some proteins in the midline of central spindle. These proteins include MKLP1, MKLP2, MgcRacGAP, PRC1, etc. PRC1 is phosphorylated on Thr470 and Thr481 by Cdc2 in early mitosis. In fact, our early study showed that phosphorylation of PRC1 at Thr470 promotes the formation of PRC1 oligomers (15), which may promote the assembly of PRC1 protein onto overlapping microtubules at the central spindle (31). The fact that phosphorylation of Thr470 and Thr480 is not involved in PRC1-CLASP1 interaction suggests that central spindle plasticity is orchestrated by several interactive but perhaps parallel pathways. To this end, it would be of great interest to use super-resolution imaging microscopic analyses to delineate how spatiotemporal control of central spindle assembly is achieved at a finely tuned fashion. In addition, it would also be important to address whether other mitotic phosphorylation sites, other than Thr470 and Thr481, at the C-terminal PRC1 regulate the PRC1-CLASP1 interaction (32).

It has been shown that multiple microtubule-associated proteins act in concert to orchestrate cytokinesis. In anaphase cells, many of these proteins establish and/or associate with an anti-parallel central spindle microtubules. In addition to interacting with CLASP1 and KIF4 in setting up the central spindle, PRC1 also interacts with and recruits KIF14 to the central spindle (33). Further experimentation will be required to address how multiple PRC1 protein complexes and PRC1 oligomers coordinate in a spatiotemporal manner in establishing and/or maintaining central spindle organization and dynamics. In this regard, selective perturbation of PRC1 association with its binding partners will be essential for delineating the function of specific molecular interaction between PRC1 and its interaction proteins and the molecular pathway of PRC1-mediated central spindle organization. We propose that PRC1 forms a link between stabilization of CLASP1 association with central spindle microtubules and anti-parallel microtubule elongation.

It has been shown that human CLASP1 is an outer kinetochore component that regulates spindle microtubule dynamics (21). Recent studies show that improper microtubule-chromosome attachment (merotely) is a cause of chromosome mis-segregation in unstable cells and that increasing chromosome mis-segregation rates by elevating merotely during consecutive mitoses generates chromosome instability in otherwise stable, near-diploid cells (34). This chromosomal instability is commonly caused by persistent mal-oriented attachment of chromosomes to spindle microtubules. Chromosome segregation requires stable microtubule attachment at kinetochores, yet those attachments must be sufficiently dynamic to permit correction of mal-orientations. To explore how this balance is achieved, Compton and colleagues studied the permissible boundaries of attachment stability versus dynamics essential for genome stability. They identified two microtubule-depolymerizing kinesins that stimulate kinetochore-microtubule dynamics during distinct phases of mitosis to correct mal-orientations. These studies indicate that temporal control of microtubule attachment to chromosomes during mitosis is central to genome stability in human cells. Given the fact that repression of CLASP1 and perturbation of PRC1-CLASP1 interaction resulted in chromosome instability phenotypes such as chromatid bridges (Fig. 4B), it would be of great importance to ascertain if repression of CLASP1 alters the microtubule plus-end dynamics and/or the localization of plus-end tracking such as Kif2b. It would of great importance in the near future to elucidate the functional relevance of CLASP2 in central spindle
organization given the homologues seen between CLASP1 and CLASP2.

Taken together, we propose that CLASP1-PRC1 interaction establishes faithful chromatid segregation by specifying central spindle organization and orchestrating kinetochore-microtubule attachment at the kinetochore. It is likely that the kinetochore outer plate protein dynamics is highly coordinated with assembly of central spindle. The PRC1-CLASP1 interaction established here is a core of this dynamic interaction network that ensures the chromosome stability in the cell division cycle.
Acknowledgements

We thank members of our groups for insightful discussion during the course of this study. This work was supported by grants from Chinese 973 project (2002CB713700, 2007CB914503, and 2009CB861703), Chinese Academy of Science (KSCX1-YW-R65; KSCX2-YW-H-10), Chinese Natural Science Foundation (39925018 and 90508002 to XY; 30870990 to XD), China National Key Projects for Infectious Disease (2008ZX10002-021), a Georgia Cancer Coalition Breast Cancer Research grant, Anhui Province Chemical Biology Key Project grant (08040102005), an Atlanta Clinical & Translation Science Institute Research grant, and the National Institutes of Health (DK-56292, CA89019, CA92080) to X.Y. XY is a Georgia Cancer Coalition Eminent Scholar.
References

1. Cleveland, D. W., Mao, Y., and Sullivan, K. F. (2003) Cell 112, 407-421
2. Glotzer, M. (2001) Annual review of cell and developmental biology 17, 351-386
3. Scholey, J. M., Brust-Mascher, I., and Mogilner, A. (2003) Nature 422, 746-752
4. Adams, R. R., Carmena, M., and Earnshaw, W. C. (2001) Trends in cell biology 11, 49-54
5. Earnshaw, W. C., and Bernat, R. L. (1991) Chromosoma 100, 139-146
6. Gassmann, R., Carvalho, A., Henzing, A. J., Ruchaud, S., Hudson, D. F., Honda, R., Nigg, E. A., Gerloff, D. L., and Earnshaw, W. C. (2004) The Journal of cell biology 166, 179-191
7. Li, F., Ambrosini, G., Chu, E. Y., Plescia, J., Tognin, S., Marchisio, P. C., and Altieri, D. C. (1998) Nature 396, 580-584
8. Terada, Y., Tatsuka, M., Suzuki, F., Yasuda, Y., Fujita, S., and Otsu, M. (1998) The EMBO journal 17, 667-676
9. Wheatley, S. P., Carvalho, A., Vagnarelli, P., and Earnshaw, W. C. (2001) Curr Biol 11, 886-890
10. Vagnarelli, P., and Earnshaw, W. C. (2004) Chromosoma 113, 211-222
11. Mishima, M., Kaitna, S., and Glotzer, M. (2002) Dev Cell 2, 41-54
12. Jiang, W., Jimenez, G., Wells, N. J., Hope, T. J., Wahl, G. M., Hunter, T., and Fukunaga, R. (1998) Mol Cell 2, 877-885
13. Mollinari, C., Kleman, J. P., Jiang, W., Schoehn, G., Hunter, T., and Margolis, R. L. (2002) The Journal of cell biology 157, 1175-1186
14. Zhu, C., and Jiang, W. (2005) Proc Natl Acad Sci U S A 102, 343-348
15. Fu, C., Yan, F., Wu, F., Wu, Q., Whittaker, J., Hu, H., Hu, R., and Yao, X. (2007) Cell Res 17, 449-457
16. Galjart, N. (2005) Nat Rev Mol Cell Biol 6, 487-498
17. Akhmanova, A., Hoogenraad, C. C., Drabek, K., Stepanova, T., Dortland, B., Verkerk, T., Vermeulen, W., Burgering, B. M., De Zeeuw, C. I., Grosveld, F., and Galjart, N. (2001) Cell 104, 923-935
18. Mimori-Kiyosue, Y., Grigoriev, I., Sasaki, H., Matsui, C., Akhmanova, A., Tsukita, S., and Vorobjev, I. (2006) Genes Cells 11, 845-857
19. Hannak, E., and Heald, R. (2006) The Journal of cell biology 172, 19-25
20. Maiato, H., Sampaio, P., Lemos, C. L., Findlay, J., Carmena, M., Earnshaw, W. C., and Sunkel, C. E. (2002) The Journal of cell biology 157, 749-760
21. Maiato, H., Fairley, E. A., Rieder, C. L., Swedlow, J. R., Sunkel, C. E., and Earnshaw, W. C. (2003) Cell 113, 891-904
22. Goechzy, P., Echeverri, C., Oegema, K., Coulson, A., Jones, S. J., Copley, R. R., Duperon, J., Oegema, J., Brehm, M., Cassin, E., Hannak, E., Kirkham, M., Pichler, S., Flohrs, K., Goessen, A., Leidel, S., Alleaume, A. M., Martin, C., Ozlu, N., Bork, P., and Hyman, A. A. (2000) Nature 408, 331-336
23. Maiato, H., Khodjakov, A., and Rieder, C. L. (2005) Nat Cell Biol 7, 42-47
24. Mimori-Kiyosue, Y., Grigoriev, I., Lansbergen, G., Sasaki, H., Matsui, C., Severin, F., Galjart, N., Grosveld, F., Vorobjev, I., Tsukita, S., and Akhmanova, A. (2005) The Journal of cell biology 168, 141-153
25. Zhou, R., Guo, Z., Watson, C., Chen, E., Kong, R., Wang, W., and Yao, X. (2003) Mol Biol Cell 14,
26. Yao, X., Thibodeau, A., and Forte, J. G. (1993) *Am J Physiol* **265**, C36-46
27. Yao, X., Abrieu, A., Zheng, Y., Sullivan, K. F., and Cleveland, D. W. (2000) *Nat Cell Biol* **2**, 484-491
28. Mollinari, C., Kleman, J. P., Saoudi, Y., Jablonski, S. A., Perard, J., Yen, T. J., and Margolis, R. L. (2005) *Mol Biol Cell* **16**, 1043-1055
29. Green, M., and Loewenstein, P. M. (1988) *Cell* **55**, 1179-1188
30. Zhou, G. L., Zhuo, Y., King, C. C., Fryer, B. H., Bokoch, G. M., and Field, J. (2003) *Mol Cell Biol* **23**, 8058-8069
31. Kapitein, L. C., Janson, M. E., van den Wildenberg, S. M., Hoogenraad, C. C., Schmidt, C. F., and Peterman, E. J. (2008) *Curr Biol* **18**, 1713-1717
32. Smertenko, A. P., Chang, H. Y., Sonobe, S., Fenyk, S. I., Weingartner, M., Bogre, L., and Hussey, P. J. (2006) *J Cell Sci* **119**, 3227-3237
33. Gruneberg, U., Neef, R., Li, X., Chan, E. H., Chalamalasetty, R. B., Nigg, E. A., and Barr, F. A. (2006) *J Cell Biol* **172**, 363-372
34. Thompson, S. L., and Compton, D. A. (2008) *The Journal of cell biology* **180**, 665-672
Figure Legends

Figure 1. CLASP1 interacts with PRC1 in vitro and in vivo

A. Co-immunoprecipitates with anti-FLAG antibody. 293T cells were co-transfected with 3×FLAG-PRC1 and pEGFP-CLIP-170 or pEGFP-CLASP1. A control was performed using cell lysates from GFP-transfected 293T. Cell extracts were subjected to immunoprecipitation using anti-FLAG antibody, fractionated by SDS-PAGE and immunoblotted with anti-GFP antibody. Immunoprecipitates contained both 3×FLAG-PRC1 and pEGFP-CLASP1 (lane 6). Lanes 3 and 5 represent the supernatants after incubating with anti-FLAG antibody. The common band in the immunoprecipitates with anti-GFP antibody is antibody-derived protein. In the experiment shown in B, western blotting verifies co-immunoprecipitation of PRC1 and CLASP1.

B. Schematic representation of CLASP1 structure and a series of deletion mutant constructs used in immunofluorescence assay. Compared with CLASP1α isoform, CLASP1 lacks 249aa on the amino end. N’-terminal region of CLASP1 contains the domain that is responsible for the interaction of CLASP1 with microtubules. The C’-terminal of CLASP1 interacts with CLIPs, the Golgi apparatus or the cell cortex.

C. Co-immunoprecipitation of PRC1 and CLASP1 from mitotic cells. Extracts from nocodazole arrested mitotic cells were incubated with antibodies against PRC1 (lanes 1-2) and CLASP1 (lanes 4-5), and immunoprecipitates (IP) were resolved by SDS-PAGE. Lane 3, immunoprecipitation from PRC1 IP; Lane 6, immunoprecipitation from CLASP1 IP. Western blotting (WB) verified co-immunoprecipitation of CLASP1 (upper panel; CLASP1 blot) and PRC1 (middle panel). Purified rabbit IgGs were included as for the control immunoprecipitation experiment in which neither CLASP1 nor PRC1 was precipitated (lane 9). Note that tubulin was not pulled down by either PRC1 antibody (lower panel; lane 3) or CLASP1 antibody (lower panel; lane 6).

D. GST pull-down assay with GST-PRC1. Full-length GST-PRC1 recombinant proteins purified on glutathione-agarose beads were used as an affinity matrix for absorbing GFP-tagged CLASP1 and its deletions as described under “Materials and Methods.” GST protein-bound agarose beads (GST) were used as a control. 293T cell extracts were used. Proteins, retained by glutathione-agarose beads decorated with GST or with wild type GST-PRC1 were analyzed by western blotting. After washing, proteins bound to agarose beads were fractionated on a SDS-polyacrylamide gel followed by transferring onto a nitrocellulose membrane. Western blotting is shown with anti-GFP antibodies for the GFP fusions. GFP antibody reacts with full-length CLASP1 (lane 6), N1003 (lane 7) and N694 (lane 8) fusion proteins from wild type PRC1 pull-down. These results suggest that CLASP1 and two N’-terminal deletions can bind to PRC1 in vitro.

E. GST pull-down assay with the indicated GST fusions. GST tagged full-length and mutants of PRC1 proteins purified on glutathione-agarose beads were used as affinity matrix for absorbing GFP-tagged CLASP1. Western blotting analyses with an anti-GFP antibody are shown for the GFP proteins and anti-GST antibody for the GST fusions. GFP antibody reacts with a 170 kDa protein band of full-length CLASP1 fusion proteins from GST-PRC1 (lane 3), C-terminal (lane 5), phospho-mimicking mutants (lane 7 and lane 9) and non-phosphorylatable mutants (lane 6 and lane 8) of PRC1 pull-downs, but not GST pull-down (lane 10), indicating that both full-length and C-terminal deletion mutant PRC1 can absorb CLASP1 protein.

F. A schematic illustration showing pEGFP-tagged CLASP1, and a series of deletion mutant constructs used in the co-immunoprecipitation assays and pull-down assays. PRC1 binding was determined on the basis of GST pull-down data as +, detectable; -, undetectable.
Figure 2. PRC1 determines the central spindle localization of CLASP1

A. Co-localization of endogenous CLASP1 and PRC1 on the mitotic spindle, spindle midzone and midbody during mitosis is shown. In prometaphase and metaphase cells, both CLASP1 (red) and PRC1 (green) localize with the entire mitotic spindle (a-b). Then both proteins are concentrated as a series of narrow bars at the late mitosis spindle midzone (c-e). As cells progress through cytokinesis, CLASP1 and PRC1 are concentrated to the midbody, where the two proteins persist even after the completion of cytokinesis (f). Scale bars: 10 μm.

B. The co-localization of various exogenous GFP-CLASP1 proteins and PRC1 during late mitosis. For each construct, anaphase (a, c,) and cytokinesis (b, d,) cells are shown, stained for PRC1 (red) and α-tubulin (blue) to verify the position of the mitotic spindle. Removal of the C-terminal of CLASP1 (CLASP11~1003) does not affect its ability to localize on central spindle in anaphase (a) nor midbody during telophase and cytokinesis (b). Deletion of the central region of CLASP1 (e.g., CLASP11~694) failed to localize to the central spindle in anaphase (c), and the midbody during telophase and cytokinesis (d). Scale bars: 10 μm.
Figure 3. Repression of PRC1 eliminates CLASP1 localization to central spindle

A. Efficiency of RNA interference of PRC1. HeLa cells were transfected with the PRC1 siRNA oligonucleotide (25 nM) or a mock siRNA oligonucleotide and subjected to SDS-PAGE and immunoblotting analyses. Cell extracts were probed with anti-PRC1 antibody (top panel), anti-CLASP1 antibody (middle panel) and anti-tubulin antibody as internal controls (lower panel).

B. HeLa cells grown on coverslips were transfected with PRC1 siRNA (a-c) or scramble siRNA control (a’-c’). 24 h after transfection, cells were fixed and stained with anti-PRC1 antibody (green), anti-tubulin antibody (red) and DAPI (blue). PRC1-deficient cells exhibited a typical phenotype with segregated sister chromatids and disjoined two spindle halves (a-b, arrows, arrow heads). Scale bars: 10 μm.

C. HeLa cells, stably expressing EGFP-CLASP1, grown on coverslips were transfected with 25 nM PRC1 siRNA oligonucleotide. 24 h after transfection, cells were fixed and stained with anti-PRC1 antibody (red) and DAPI (blue). Compared with the controls (a’-c’), repression of PRC1 protein by siRNA treatment resulted in little distribution of CLASP1 to the central spindle and the midbody (a-c), indicating that PRC1 is required for CLASP1 distribution to the central spindle (b and b’; arrows) and the midbody during anaphase and telophase. Scale bars: 10 μm.

D. HeLa cells stably expressing EGFP-CLASP1 were transfected with 25 nM PRC1 siRNA oligonucleotide. 24 h after transfection, cells were fixed and stained with anti-tubulin antibodies (red) and DAPI (blue). Repression of PRC1 failed to form inter-digital microtubule bundles (b and b’; arrows). Scale bars: 10 μm.
Figure 4. CLASP1 is essential for faithful chromosome segregation and mitotic spindle assembly

A. Efficiency of RNA interference of CLASP1. Immunoblot analysis of HeLa cells treated with the CLASP1 siRNA oligonucleotide (25 nM) or with a mock siRNA oligonucleotide is shown. Cell extracts were probed with anti-CLASP1 antibody (top panel), anti-PRC1 antibody (2nd panel) and anti-tubulin antibody as internal controls (3rd panel).

B. Repression of CLASP1 resulted in mitotic abnormalities. HeLa cells, stably expressing mCherry-tubulin (red), grown on coverslips were transfected with 25 nM CLASP1 siRNA. 36~48 h after transfection, cells were fixed and stained for DNA (DAPI, blue) and CLASP1 (green). As shown in C and D, compared with control cells (a'-c'), CLASP1-depleted cells exhibit anaphase chromatide separation errors (a-b, arrows) and had difficulty in chromosome alignment (a, arrow). The cells also entered in anaphase with abnormal spindle (b-c) and a chromatin bridge (c, arrows). Scale bars: 10 μm.

C. Statistical analyses of cells exhibiting mitotic abnormalities, such as misaligned chromosomes and multi-polar spindle, in CLASP1-repressed metaphase cells (black bar) and control preparation (open bar). An average of 100 metaphase-like cells from three separate experiments was counted for HeLa cells which expressed mCherry-tubulin stably and were treated with CLASP1 siRNA oligonucleotide (36~48 h post-transfection). Cells were then examined under a fluorescence microscope and associated phenotypes were categorized and quantified as the percentage of total mitotic cells.

D. Statistical analyses of cells exhibiting chromatide bridges or multi-polar spindles in CLASP1-deficient anaphase cells (black bar) and control preparation (open bar). An average of 100 anaphase-like cells from three separate experiments was counted after being transfected with CLASP1 siRNA and scramble oligonucleotides (36~48 h post-transfection). Cells were then examined under a fluorescence microscope and associated phenotypes were categorized and quantified as the percentage of total mitotic cells.
Figure 5. PRC1<sub>438-620</sub> involves in association of PRC1 with CLASP1 in vitro and in vivo

A. Schematic diagram of the functional domains of PRC1. Phosphorylation sites (asterisks) correspond to residues Thr<sup>470</sup>, Thr<sup>481</sup>, Thr<sup>578</sup> and Thr<sup>616</sup>.

B. PRC1 binds to CLASP1 via its C-terminal region. Both full-length and deletion mutant GST-PRC1 proteins were expressed and purified using glutathione agarose beads. Purified GST-PRC1 proteins on sepharose beads were used as an affinity matrix for absorbing GFP-CLASP1. Retention of GFP-CLASP1 proteins on GST-PRC1 affinity beads was validated by immunoblotting using anti-GFP antibodies. Western blotting analyses revealed that the C-terminal domains of PRC1 (PRC1<sub>438-620</sub>) is responsible for its CLASP1-binding activity (lane 3, lane 5, lane 6 and lane 7).

C. Expression and purification of TAT-GFP fusion proteins in E. coli. Molecular size of protein is indicated on the left.

D. Addition of PRC1<sub>438-620</sub> recombinant protein disrupts CLASP1-PRC1 association. Purified GST-PRC1 protein on glutathione-sepharose beads were used as an affinity matrix for absorbing GFP-CLASP1. After extensive washes, aliquots of CLASP1-bound GST-PRC1 sepharose beads were incubated with 5 µM purified PRC1<sub>438-620</sub> protein on a rotator for 30 min at room temperature. After extensive washes, the sepharose beads were boiled in SDS-PAGE sample buffer and bound proteins were fractionated by SDS-PAGE followed by visualizing in Coomassie blue stain. Note that incubation of TAT-GFP-PRC1<sub>438-620</sub> peptide with CLASP1-bound GST-PRC1 sepharose beads disrupts PRC1-CLASP1 interaction.

E. The HeLa cells synchronized by mitotic shake-off were extracted with 0.1% Triton X-100 solution and separated into soluble (hatch bars) and insoluble (black bars) fractions as described under "Materials and Methods." Equivalent amounts of protein from the soluble and insoluble fractions were applied to SDS-PAGE. Following separation on 6–16% gradient SDS-PAGE and transblotting onto nitrocellulose membrane, the blots were probed with an anti-PRC1 antibody and an anti-CLASP1 antibody followed by visualization using an ECL kit (Pierce). The signals were quantified using a PhosphorImager with values expressed as a percentage of total (soluble + insoluble). The error bars represent S.E.; n = 3 preparations.

F. The PRC1-derived peptide TAT-GFP-PRC1<sub>438-620</sub> increases the extractable CLASP1 proteins. HeLa cells synchronized by mitotic shake-off were treated with the TAT-GFP-PRC1<sub>438-620</sub> peptide followed by a Triton X-100 extraction. The cellular proteins were separated into a soluble and insoluble fraction as described previously (24). Samples were run on SDS-PAGE, blotted to nitrocellulose, and immunoprobed for CLASP1. The signals were quantified using a PhosphorImager with values expressed as percentage of total (soluble + insoluble). Error bars represent SE; n = 4. Significant difference is found with respect to the controls (*p < 0.01).
Figure 6. PRC1 collaborates with CLASP1 for faithful mitosis

A. Schematic illustration of experimental strategy for assessing the role of PRC1-CLASP1 interaction in central spindle assembly and regulation. Monastrol-arrested prometaphase cells were used to induce mitotic progression by washout of monastrol and release into metaphase by inclusion of 20 μM MG132. To test for the functional relevance of PRC1-CLASP1 interaction, 500 nM TAT-GFP-PRC1438~620 peptide and its control peptide TAT-GFP were included for real-time imaging of mitotic progression.

B. Live-cell imaging of mitotic progression of a HeLa cell stably expressing mCherry-tubulin. The monastrol-arrested cell was subjected to monastrol wash-out and incubated with 20 μM MG132 and 500 nM TAT-GFP for 60 min before anaphase onset by release into MG132-free but TAT-GFP-containing media. Imaging time begins after the addition of TAT peptides. A representative cell was shown from prometaphase to anaphase onset until telophase. Scale bars: 10 μm.

C. Live-cell imaging of mitotic progression of a HeLa cell treated TAT-GFP-PRC1438~620 peptide (500 nM). In contrast, in TAT-GFP-treated cells, the duration of anaphase was prolonged for 2 min. Scale bars: 10 μm.

D. Live-cell imaging of mitotic progression of a HeLa cell treated CLASP1 siRNA for 24 hours before monastral synchronization and TAT-GFP-PRC1438~620 peptide was added (500 nM). Scale bars: 10 μm.

E-F. Quantitative analyses of mitotic progression in the PRC1-CLASP1 perturbed cells. Pre-anaphase progression (from prometaphase to anaphase onset) was analyzed and presented in E, which exhibits no statistically significant difference between TAT-GFP-treated and TAT-GFP-PRC peptide treated sample (p > 0.05). Post-anaphase progression (from anaphase onset to telophase) was analyzed and presented in E and F. In general, it takes 621.4 ± 16.5 sec (n = 8) for TAT-GFP-treated cells to complete the aforementioned process while it takes 700.6 ± 27.0 sec (n = 8) for TAT-GFP-PRC1438~620-treated cells to complete the same process. This difference bears statistical significance (p < 0.01).

G. Quantification of the duration of mitosis from anaphase onset to midbody formation in PRC1-suppressed cells and CLASP1-repressed cells. On average, control cells transfected with scramble oligonucleotides take 603.5 ± 22.0 sec (n = 4) to complete the aforementioned process while PRC1-depleted cells take 641.0 ± 27.8 sec and CLASP1-depleted cells require 1079.0 ± 92.5 sec to complete the same process (n = 4).

H. Perturbation of PRC1-CLASP1 interaction abrogates chromosome stability in mitosis. Asynchronized HeLa cells were treated with 500 nM recombinant TAT-GFP (a-d) or TAT-GFP-PRC1438~620 (a-d) for 30 min. After treatment, cells were extensively washed, fixed, and stained with an anti-tubulin antibody (red) and DAPI (blue). Recombinant GFP-his protein was added as a control (a-d'). Note that perturbation of PRC1-CLASP1 interaction by treatment of TAT-GFP-PRC1438~620 resulted in chromosome lagging (b) and formation of a sister chromatid bridge (c), a characteristic phenotype of chromosome instability. This phenotype is absent in TAT-GFP-treated and GFP-treated cells (b'-d' and b-d'). Scale bars: 10 μm.

I. Quantification of the overlapped microtubule bundles at the central spindle as function of PRC1-CLASP1 interaction. The monastrol-arrested cells stably expressing mCherry-tubulin were subjected to monastrol wash-out and incubated with 20 μM MG132 and 500 nM TAT-GFP for 60 min before anaphase onset by release into MG132-free but TAT-GFP or TAT-GFP-PRC1438~620 peptide containing media as illustrated in Figures 6B-D. Optical sections taken from the middle of the z-stack were scored for the number of overlapped microtubule bundles in the TAT-PRC1438~620-treated cells and expressed as mean ± SE, n=12 from 3 different preparations (P<0.01 compared to TAT-GFP treatment). Representative images from anaphase A and anaphase B were shown on the left while statistic analyses were listed on the right.

J. Quantification of the stable microtubule plus-ends at the central spindle as function of PRC1-CLASP1 interaction. The monastrol-arrested HeLa cells stably expressing mCherry-EB3 were subjected to monastrol wash-out and incubated with 20 μM MG132 and 500 nM TAT-GFP for 60 min before anaphase onset by release into MG132-free but TAT-GFP or TAT-GFP-PRC1438~620 peptide containing media. Optical sections taken from the middle of the z-stack were scored for the number of mCherry-EB3-marked microtubule plus-ends in the TAT-PRC1438~620-treated cells. The number of EB3-marked microtubule plus-ends within the middle one third of the spindle axis (boxed area) were scored and expressed as mean ± SE, n=9 from 3 different preparations (P<0.01 compared to TAT-GFP treatment).

K. Quantification of anaphase defects in PRC1-CLASP1 interaction-disrupted cells. The monastrol-arrested cells stably expressing mCherry-tubulin were subjected to monastrol wash-out and incubated with 20 μM MG132 and 500 nM TAT-GFP for 60 min before anaphase onset by release into MG132-free but TAT-GFP or TAT-GFP-PRC1438~620 peptide containing media as illustrated in Figures 6B-D. Aliquots of CLASP1-repressed and CLASP1-repressed and TAT-PRC1438~620-treated cells were also included for control. Note that synergistic effect was seen in CLASP1-repressed and TAT-PRC1438~620-treated cells. For quantification, the anaphase defected cells were
expressed as a percentage of mitotic cells (mean±SE, n=100 from 3 different preparations; (P<0.01 compared to TAT-GFP treatment).
Figure 1

A

Immunoprep.

| input       | S | P | S | P |
|-------------|---|---|---|---|
| +           | + |   |   |   |
| -           |   | + |   |   |

GFP-CLIP170
GFP-CLASP1

GFP blot

B

CLASP1 (KIAA0622)

MT-binding domain

CLIP binding domain

N1003

N694

695–1003

997C
C

**Immuno-precipitation**

| Input (5%) | Non-binding | PRC1 I.p. | Input (5%) | Non-binding | CLASP1 I.p. | Input (5%) | Non-binding | Control Ig G I.p. |
|------------|-------------|-----------|------------|-------------|-------------|------------|-------------|------------------|
|            |             |           |            |             |             |            |             |                  |

CLASP1

PRC1

tubulin

D

**Affinity matrix**

| GST-PRC1 | Input | GST-PRC1 Pull-down | GST Pull-down |
|----------|-------|---------------------|----------------|
| GFP-CLASP1 | 1 | 6 | 11 |
| GFP-N1003 | 2 | 7 | 12 |
| GFP-N694 | 3 | 8 | 13 |
| GFP-695-1003 | 4 | 9 | 14 |
| GFP-997C | 5 | 10 | 15 |

GFP blot
**E**

**Affinity matrix**

**Input**

GST-PRC1

**PRC1 bound**

GFP-CLASP1

**GFP blot**

**GST blot**

1 2 3 4 5 6 7 8 9 10

GST-PRC1 WT GST-N203 GST-304C GST-470EA GST-470E GST-481A GST-481E GST

**F**

**EGFP fusion**

**PRC1 binding**

**Cellular localization**

MT, Brute, Midzone, Masctoid, Kinocilium

| EGFP fusion | PRC1 binding | MT | Brute | Midzone | Masctoid | Kinocilium |
|-------------|--------------|----|-------|---------|----------|------------|
| CLASP1      | +            | +  | +     | +       | +        | 0          |
| N1003       | +            | +  | +     | +       | -        | 0          |
| N694        | -            | -  | -     | -       | -        | +          |
| 695–1003    | -            | -  | -     | -       | -        | +          |
| 997C        | -            | -  | -     | -       | -        | +          |
Figure 2

A

Prometaphase
Metaphase
Anaphase A
Anaphase B
Telophase
Cytokinesis
Figure 3

A

Scramble PRC1 siRNA

|          | PRC1 | CLASP1 | tubulin |
|----------|------|--------|---------|
| Scramble |      |        |         |
| PRC1 siRNA |      |        |         |

B

PRC1 siRNA

PRC1 | tubulin | DAPI | Merge

Scramble

a | a' |    |    |

PRC1 siRNA

b |    |    |    |

Scramble

c |    |    |    |

PRC1 siRNA

c' |    |    |    |

Scramble

c' |    |    |    |
D

PRC1 siRNA

GFP-CLASP1 tubulin DAPI Merge

Scramble

PRC1 siRNA

Scramble

PRC1 siRNA

Scramble

PRC1 siRNA

Scramble
Figure 4
A

|          | Scramble | CLASP1 siRNA |
|----------|----------|--------------|
| 24 h     |          |              |
| 24 h     |          |              |
| 48 h     |          |              |

CLASP1

PRC1
tubulin

1 2 3

B

C

D

Metaphase cells

Anaphase cells

Scramble CLASP1 si

Normal Misaligned chromosomes Multipolar spindle

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

Scramble CLASP1 si

Normal Chromatin bridges Multipolar spindle

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
Figure 5

A

Central spindle targeting µt-binding phosphorylation sites

B

input GFP-CLASP1 GFP

GFP blot

Affinity matrix

GST-PRC1
C

D

Affinity Matrix:
GST-PRC1

Input:
GFP-CLASP1
cell lysates

Modulator:
PRC1438-620

E

F

Protein partition (% of total)

Insoluble

Soluble

CLASP1

PRC1

Insoluble

Soluble

CLASP1 (% of total)

+TAT-GFP
+TAT-GFP-
PRC1438-620
+TAT-GFP-
PRC1438-620
+TAT-GFP-
PRC1438-620

5 nM
50 nM
500 nM
Figure 6

A

G2 cells

Monopolar prometaphase

50 μM monastrol
20 μM MG132

2 hours

Monastrol washout

+ MG132
+ TAT-PRC1 peptide

Metaphase

-G132
+ TAT-PRC1 peptide

Anaphase A

Anaphase B

G1

by guest on July 10, 2020
http://www.jbc.org/Downloaded from
C

+TAT-GFP-PRC1438-620

mCherry-tubulin

00:06:19 00:34:52 00:43:37 00:44:07 00:44:37
00:45:07 00:45:37 00:46:07 00:46:37 00:47:07
00:47:37 00:48:07 00:48:37 00:49:07 00:49:37
00:50:07 00:50:37 00:51:07 00:51:37 00:52:07
00:52:37 00:53:07 00:53:37 00:54:07 00:54:37
00:55:37 01:23:02
D CLASP1 siRNA+ TAT-GFP-PRC1438~620
I  

|                | Anaphase A | Anaphase B |
|----------------|------------|------------|
| TAT-GFP        |            |            |
| TAT-GFP-PRC1438~620 |            |            |
| CLASP1 siRNA + TAT-GFP-PRC1438~620 |            |            |

Number of overlapped MT bundles

|                | Anaphase A | Anaphase B |
|----------------|------------|------------|
| TAT-GFP        | 10±2       | 11±2       |
| TAT-GFP-PRC1438~620 | 6±1       | 7±2       |
| CLASP1 siRNA + TAT-GFP-PRC1438~620 | 5±2       | 5±2       |

J

EB3 spots in boxed area

K

|                | aberrant anaphase (% mitotic cells) |
|----------------|------------------------------------|
| Control        |                                    |
| TAT-GFP        |                                    |
| TAT-PRC1438~620|                                    |
| CLASP1 siRNA   |                                    |
| TAT-PRC1438~620 + CLASP1 siRNA | 100 |
PRC1 cooperates with CLASP1 to organize central spindle plasticity in mitosis
Jing Liu, Zhikai Wang, Kai Jiang, Liangyu Zhang, Lingli Zhao, Shasha Hua, Feng Yan,
Yong Yang, Dongmei Wang, Chuanhai Fu, Xia Ding, Zhen Guo and Xuebiao Yao
J. Biol. Chem. published online June 26, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M109.009670

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts