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Report

PHB2 Protects Sister-Chromatid Cohesion in Mitosis

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Summary

Cohesion between sister chromatids is essential for proper chromosome segregation in mitosis. In vertebrate mitotic cells, most cohesin is removed from the chromosome arms [1–4], but centromeric cohesin is protected by shugoshin until the onset of anaphase [5]. However, the mechanism of this protection of centromeric cohesion is not well understood. Here, we demonstrate that prohibitin 2 (PHB2) is involved in the regulation of sister-chromatid cohesion during mitosis in HeLa cells. PHB2 is an evolutionarily conserved protein in eukaryotes and has multiple functions, such as transcriptional regulation and cell viability and development [6–8]. However, its functions in mitosis have not yet been determined. We show that depletion of PHB2 by RNA interference (RNAi) causes premature sister-chromatid separation and defects in chromosome congression accompanied by mitotic arrest by spindle-checkpoint activation. In the absence of PHB2, cohesin is dissociated from centromeres during early mitosis, although the centromeric localization of shugoshin is preserved. Thus, our findings suggest that, in addition to the shugoshin, PHB2 is also required to protect the centromeric cohesion from phosphorylation by Plk1 during early mitosis and that its function is essential for proper mitotic progression.

Results and Discussion

PHB2 Is Required for Proper Mitotic Progression, Chromosome Congression, and Mitotic-Spindle Formation

PHB2 was previously identified in isolated human metaphase chromosomes [9, 10]. HeLa cells transiently expressing PHB2 fused with enhanced green fluorescent protein (EGFP) was localized in the cytoplasm and chromosomes of prophase and prometaphase cells (Figure S1 in the Supplemental Data available online). To examine the function of PHB2 on chromosomes, we performed RNAi-mediated depletion of PHB2. We designed two siRNAs (PHB2 siRNA-1 and siRNA-2) with different sequences for the PHB2 RNAi experiments, and both of these successfully repressed the expression of PHB2 (Figure S2). PHB2 depletion resulted in the accumulation of mitotic cells (Figure 1A). The mitotic index was 13.7% ± 2.0% in PHB2 RNAi cells, greater than that of control cells (4.9% ± 1.0%). Among PHB2-depleted cells, 57.5% ± 10.3% of mitotic cells were in a prometaphase state, whereas in control cells, 14.0% ± 4.0% of mitotic cells were in a prometaphase state (Figure 1B). In the absence of PHB2, aberrant chromosome behavior and mitotic delay were frequently observed by time-lapse observations of GFP-histone H1.2 (Figure S3). Figure 1C shows typical images of chromosomes observed in control and RNAi-treated cells after 4% paraformaldehyde (PFA) fixation. In PHB2-depleted cells, chromosome condensation occurred normally, but the chromosome alignment at the metaphase plate was significantly impaired (Figure 1D). However, the expression of RNAi-refractory PHB2 could reduce the percentage of chromosomes showing aberrations in PHB2 RNAi cells (Figure 1E). This indicates that the congression defects in mitotic chromosomes were a direct effect of PHB2 repression by RNAi. At the kinetochore of these aberrant chromosomes, intense signals of mitotic-spindle-checkpoint proteins, Bub1, BubR1, and Mad2, were detected (Figure S4A). Furthermore, inactivation of spindle checkpoint by RNAi of Aurora B or Mad2 rescued the mitotic arrest in PHB2-depleted cells (Figure S4B). Thus, the mitotic delay observed in PHB2-depleted cells was due to spindle-checkpoint activity. These findings demonstrate for the first time that PHB2 has some function in chromosome congression and mitotic progression.

We also observed defects in mitotic-spindle formation (Figure S5) and localization of the outer kinetochore proteins, CENP-E, CENP-F, and Hec1 (Figure S6). When HeLa cells were incubated in ice-cold media before fixation in order to depolymerize all microtubules except the stable kinetochore fibers, the kinetochore fibers in PHB2-depleted cells were significantly diminished compared with control cells (Figure S5E). Accurate outer kinetochore formation is required for stable microtubule formation [11, 12], and we therefore consider that decreases in CENP-E, CENP-F, and Hec1 at the outer kinetochores would cause destabilization of the mitotic spindle in PHB2-depleted cells. Thus, our findings indicate that PHB2 is required to form a stable mitotic spindle.

PHB2 Is Involved in Protection of Sister-Chromatid Cohesion

To estimate the effects of PHB2 depletion on chromosome structure, we prepared metaphase-chromosome

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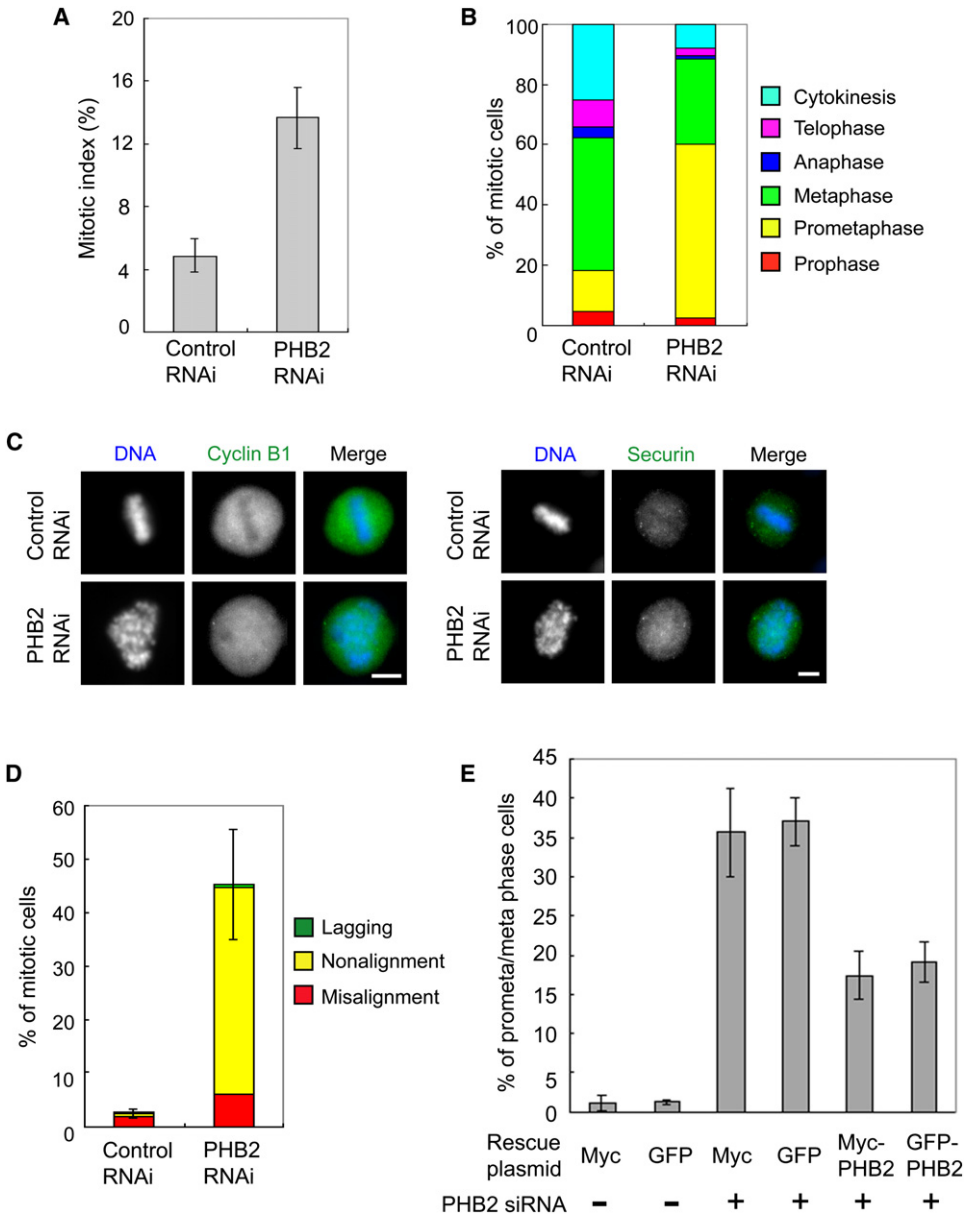

Figure 1. Localization of PHB2 during Mitosis and RNAi Phenotype of PHB2-Depleted Cells

(A) The mitotic indexes of control and PHB2 RNAi cells were calculated after immunostaining of the cells with an α-tubulin antibody. n = 5; a total of >1000 cells were counted in each experiment.

(B) Mitotic cells were classified into six mitotic stages according to their chromosome and mitotic-spindle morphologies after DAPI staining and tubulin staining, respectively.

(C) Control metaphase cells and PHB2-depleted cells showing aberrant chromosomes were stained with Cyclin B1 and securin antibodies (green) after 4% PFA fixation. Scale bars represent 5 μm.

(D) Chromosome showing aberrations were categorized into three groups, nonalignment, misalignment, and lagging. Unaligned chromosomes (more than ten chromosomes were not aligned at the metaphase plate or chromosomes were scattered throughout the cytoplasm) were most often observed in PHB2-depleted cells (38.7% of mitotic cells). Misaligned chromosomes (one to ten chromosomes were not aligned at the metaphase plate, although the majority of the chromosomes were aligned) were observed in 6.2% of PHB2-depleted cells. These chromosome defects were rarely observed in control cells (misalignment, 2.1%; nonalignment, 0.2%). n = 5; >1000 cells were counted in each experiment.

(E) The percentage of chromosomes showing congression defects in prometaphase and metaphase cells. HeLa cells were transfected with RNAi-refractory Myc- or GFP-PHB2 and were subsequently transfected with PHB2 siRNA. The percentage of chromosomes showing congression defects was decreased by the expression of Myc- or GFP-PHB2 in PHB2 RNAi cells (~18%), compared with cells expressing Myc or GFP (~36%). n = 5, >100 cells were counted in each experiment.

spreads from control and PHB2 RNAi cells after 3 hr of colcemid treatment (Figure 2A). Among the control cells, 79% of prometaphase cells showed open-arm chromosomes, and the mild and complete separation morphologies were rarely observed (Figures 2B and 2C). In contrast, among PHB2-depleted cells, sister chromatids were often separated along the whole chromosome length (mild separation, 6.7%; complete

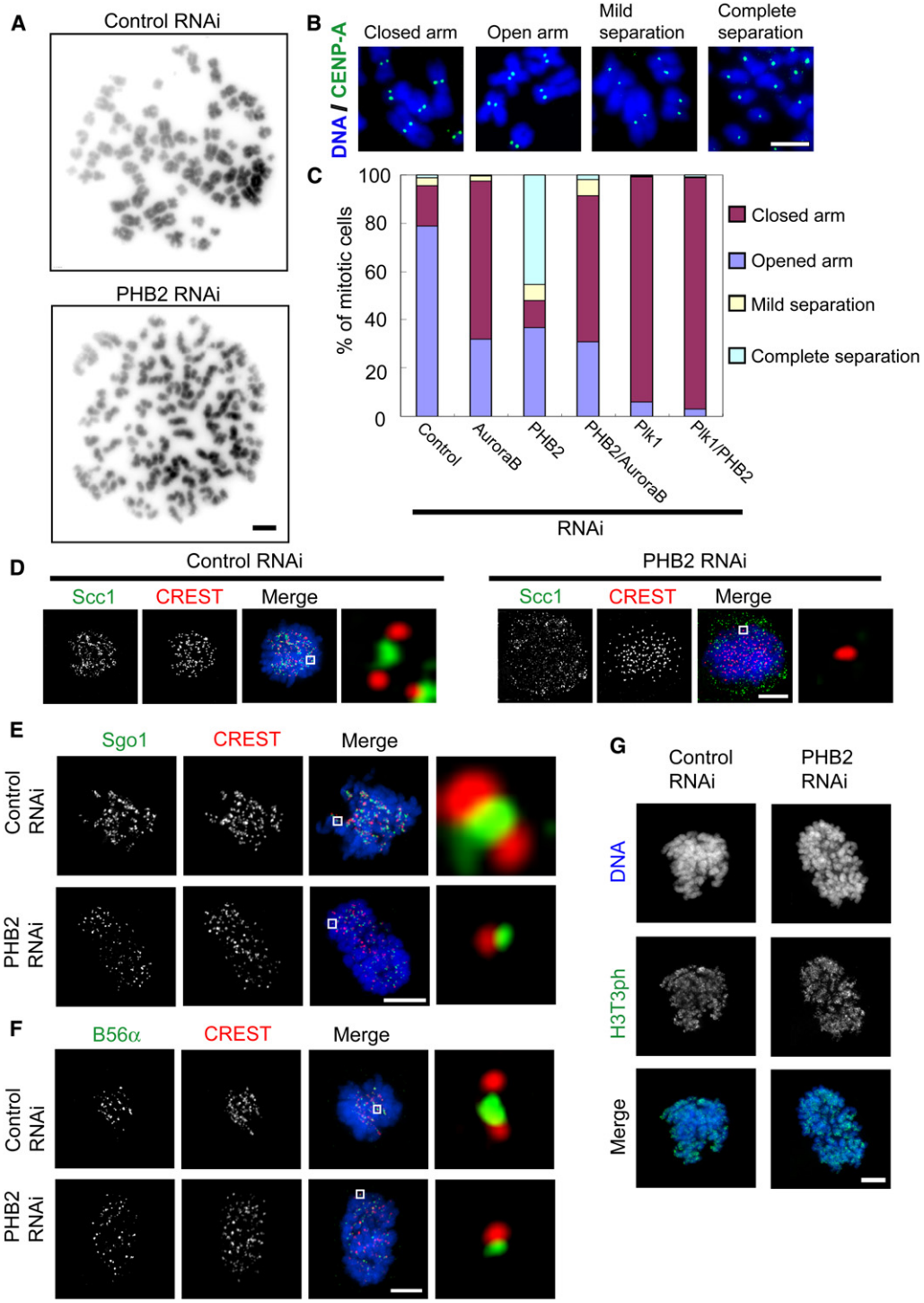

Figure 2. Defects in Sister-Chromatid Cohesion in PHB2-Depleted Cells

(A) Chromosome morphologies in control and PHB2 RNAi cells. The cells were synchronized at prometaphase by treatment with 0.1 μg/ml of colcemid for 3 hr and then spread on a glass slide with a cytospin. Chromosomes were visualized by DAPI staining. The scale bar represents 5 μm.

(B) The chromosome morphologies were categorized into four groups: closed arm, sister chromatids were cohesed along their entire length; open arm, sister chromatids were cohesed at the centromere but not at the chromosome arms; mild separation, sister chromatids were partially separated and their cohesion was maintained at the centromere or chromosome arms, and primary constriction was not observed at the centromere; and complete separation, sister chromatids were fully separated and hypercondensed. DNA was stained with DAPI (blue), and the kinetochores were visualized by CENP-A staining (green). The scale bar represents 5 μm.

(C) Frequencies of chromosome-spread morphologies observed in RNAi cells.

(D) Immunostaining for one of the cohesin complex subunits, Scc1 (green), reveals that the centromeric localization of cohesin has disappeared in PHB2-depleted cells. The kinetochores were stained with CREST (red). DNA was counterstained with DAPI (blue). The scale bar represents 5 μm.

separation, 45.4%). We also performed a FISH experiment to detect defects in chromatid cohesion during S or G2 phase (Figure S7) [13]. However, there were no significant differences in the interchromatid distance between control and PHB2 RNAi-treated cells, demonstrating that depletion of PHB2 induces no defects in chromatid cohesion until the onset of mitosis. This indicates that the defects in chromatid cohesion observed in PHB2-depleted cells were caused during prophase or prometaphase. Furthermore, the expression of RNAi-refractory PHB2 could rescue the cohesion defects in sister chromatids (Figure S2C). This indicates that PHB2 is actually required for chromatid cohesion and that the phenotypes observed in PHB2 RNAi cells were not derived from an offtarget effect of PHB2 RNAi. These results indicate that depletion of PHB2 causes loss of sister-chromatid cohesion at the centromere. However, we could not detect centromere-specific localization of PHB2, although it localized on chromosomes of the prophase cell (Figure S1B). PHB2 also localized in the cytoplasm during mitosis (Figure S1A). Therefore, we could not exclude the possibility that cytoplasmic PHB2 facilitates centromeric protection.

In more complex eukaryotes, sister-chromatid separation is achieved by a two-step dissociation of cohesin from the chromosomes [2]. First, cohesin is predominantly dissociated from chromosomes except for the centromeric region by phosphorylation of their Scc3 subunits by the protein kinase Plk1 during prophase and prometaphase [1, 4]. Second, the residual cohesin at the centromere is removed from chromosomes by proteolytic cleavage of the cohesin subunit Scc1, which is mediated by a protease called separase at the onset of anaphase [3]. The expression levels of Cyclin B and securin in PHB2-depleted cells were the same as those in control metaphase cells (Figure 1C). Degradation of Cyclin B1 and securin is required for the onset of anaphase [14], and thus cells showing chromosome scattering were in prometaphase or metaphase. Furthermore, inhibition of APC/C activity with MG132 did not decrease the frequencies of the chromosome showing aberrations (Figure S8). These findings indicate that the defects in chromosome congression are actually generated before the onset of anaphase. Given our observations of the chromosome-spread morphologies, these aberrant chromosomes were generated by precocious sister-chromatid separation before separase activation at the onset of anaphase. Thus, we supposed that the centromeric cohesin was removed by Plk1 in an untimely manner in PHB2-depleted cells. Therefore, we then investigated the localization of the cohesin subunit Scc1 in control and PHB2-depleted cells (Figure 2D and Figure S9). Immunofluorescence experiments revealed that the cohesin localization at the centromere was impaired by PHB2 depletion. Cohesin signals were detected between sister centromeres in control cells, whereas no intense cohesin signals were detected at the centromere in PHB2-depleted cells. These findings indicate that the precocious sister-chromatid separation is caused by loss of cohesin at the centromere before anaphase. Next, we performed double knockdown of PHB2 with Plk1 to examine whether the precocious chromatid separation observed in PHB2-depleted cells was caused by Plk1. Reductions in both Plk1 and PHB2 in the double knockdown cells were confirmed by immunoblotting (Figure S10A). In Plk1-depleted cells, closed-arm chromosomes were often observed (Figure 2C), as reported previously [15]. When double knockdown of PHB2 and Plk1 was performed, the precocious chromatid separation was dramatically diminished (Figure 2C). These results suggest that centromeric cohesin is inappropriately removed by kinase activity of Plk1 in PHB2-depleted cells.

PHB2 Regulates Chromatid Cohesion Independently of the Centromeric Localization of Sgo1 Complexes

Sgo1 complexes composed of Sgo1 and PP2A have been identified as protectors of centromeric cohesion because depletion of Sgo1 or PP2A results in loss of cohesin at the centromere and premature sister-chromatid separation [16, 17]. Thus, we investigated the localizations of Sgo1 and one of the PP2A subunits, B56α, after PHB2 RNAi. In control cells, signals for Sgo1 and B56α were detected between paired kinetochores visualized by CREST during prometaphase (Figures 2E and 2F). In PHB2-depleted cells, single intense CREST signals were detected from scattered chromosomes. This indicates that the sister chromatids were not paired, consistent with our observations for the chromosome spreads. However, signals for Sgo1 and B56α were detected adjacent to the CREST signals in separated chromatids. This result implies that centromeric localization of Sgo1 complexes is not sufficient to protect centromeric cohesion during early mitosis in the absence of PHB2.

The phenotype of PHB2 RNAi appears similar to the phenotype of depletion of Haspin, which is a kinase required for phosphorylation of histone H3 threonine-3 phosphorylation (H3T3ph) during mitosis [18]. Haspin RNAi also causes premature sister-chromatid separation without any defect in Sgo1 localization at the centromere; this separation is accompanied by a significant reduction in H3T3ph [19]. Thus, we examined whether PHB2 depletion influences Haspin activity on H3T3ph by using immunofluorescence. Contrary to Haspin RNAi, depletion of PHB2 did not reduce the level of H3T3ph compared with that in control cells (Figure 2G). This indicates that phosphorylation of H3T3 is not always sufficient for the binding of cohesin to chromosomes and that the functions of Haspin, except for phosphorylation of H3T3, might be important for chromatid cohesion, for example, phosphorylation of cohesin.

We further found that the premature chromatid separation was prevented by Aurora B depletion in PHB2-depleted cells. Depletion of Aurora B induced delocalization of Sgo1 from the centromere, and it became

(E) Immunostaining for Sgo1 (green) in RNAi cells after 4% PFA fixation. The kinetochores and DNA were also stained with CREST (red) and DAPI (blue), respectively. The scale bar represents 5 μm.
(F) Immunostaining of one of the PP2A subunits, B56α (green), in RNAi cells after methanol fixation. The scale bar represents 5 μm. The farthest-right panel in (D), (E), and (F) shows the enlarged image of insets in the merged image.
(G) HeLa cells were stained with H3T3Ph antibody (green) after RNAi treatment. The scale bar represents 5 μm.

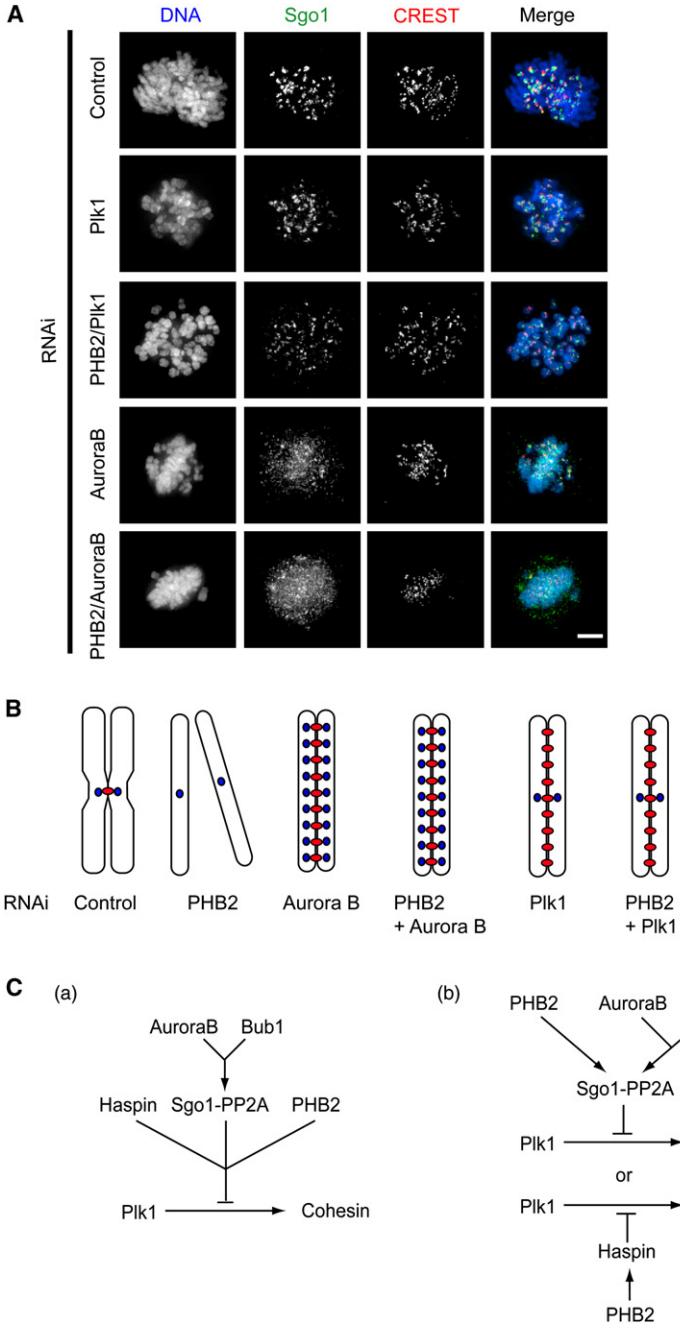

Figure 3. Model of the Regulation of Chromatid Cohesion during Early Mitosis

(A) Sgo1 localization after Plk1 and Aurora B depletion. The centromeric localization of Sgo1 (green) is preserved in Plk1-depleted cells. In contrast, the Sgo1 localization is diffuse from the centromere to the chromosome arms in Aurora B-depleted cells. The kinetochores and DNA were also stained with CREST (red) and DAPI (blue), respectively. The scale bar represents 5 μm.

(B) Summary of the localizations of Sgo1 (blue) and cohesin (red) on metaphase chromosomes in RNAi cells. In control cells, Sgo1 is concentrated at the centromere, and sister-chromatid cohesion is preserved. After PHB2 depletion, the sister-chromatid cohesion is disrupted, although the centromeric localization of Sgo1 is preserved. This chromatid separation is abrogated by repression of Aurora B or Plk1 expression. In Aurora B-depleted cells, Sgo1 is localized along the chromosome arms, and chromatid cohesion is preserved along the entire chromosome arms. In Plk1-depleted cells, the localization of Sgo1 at the centromere remains unchanged, but dissociation of cohesin from the chromosome arms is inhibited.

(C) There are at least two models for PHB2-mediated protection of chromatid cohesion. Arrows (→) denote positive regulation, and blocks (⊥) denote negative regulation. (C_a) shows that three molecules, Sgo1-PP2A complex, PHB2 and Haspin, are independently required to protect centromeric cohesin from phosphorylation by Plk1. Bub1 and Aurora B regulate the centromeric localization of Sgo1. (C_b) shows that PHB2 regulates the function of Sgo1-PP2A complexes or Haspin to protect chromatid cohesion. PHB2 might be required for the phosphatase activity of Sgo1-PP2A complexes for dephosphorylation of cohesion, or it might required for the kinase activity of Haspin for cohesion; these activities are required for retention of cohesin at the centromere.

distributed along the chromosome arms (Figure 3A), as recently reported [19]. In contrast, depletion of Plk1 did not affect the centromeric localization of Sgo1 (Figure 3A). These observations suggest that Aurora B is essential for centromere targeting of Sgo1 or maintenance of Sgo1 at the centromere. Consistent with this hypothesis, potential roles for passenger-protein complexes in regulating the centromeric localization of MEI-S332, a *Drosophila* homolog of Sgo1, through a direct interaction between INCENP and MEI-S332 and the kinase activity of Aurora B have been reported [20]. Thus, Sgo1 diffusing from the centromere to the chromosome arms after Aurora B depletion could protect cohesin from phosphorylation by Plk1 along the chromosome arms during early mitosis, such that chromatid cohesion could be maintained in the large majority of chromosome arms even in the absence of PHB2.

In summary, PHB2 has important roles in protection of centromeric cohesion during early mitosis. The localizations of Sgo1 and cohesin in RNAi cells were summarized in Figure 3B. In the absence of PHB2, centromeric cohesin was removed by Scc3 phosphorylation mediated by Plk1, whereas Sgo1 and PP2A were normally localized at the centromere. In fact, depletion of Plk1 could rescue the sister-chromatid separation in PHB2-depleted cells, and we therefore propose that PHB2 is required for protection against the centromeric removal of cohesin by phosphorylation via Plk1. Haspin is also required for the maintenance of chromatid cohesion. Therefore, our results suggest that there are at least

three different pathways that protect chromatid cohesion, namely the Sgo1-PP2A pathway, Haspin pathway, and PHB2 pathway (Figure 3C). The centromeric localization of Sgo1 is regulated by Aurora B and Bub1 [20–22]. Alternatively, PHB2 may be required for the activity of Sgo1-PP2A, Haspin, or other potential proteins required for the protection of centromeric cohesion.

Supplemental Data

Experimental Procedures and ten figures are available at http://www.current-biology.com/cgi/content/full/17/15/1356/DC1/.

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