Usp16 regulates kinetochore localization of Plk1 to promote proper chromosome alignment in mitosis

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During the G2 to M phase transition, a portion of mitotic regulator Plk1 localizes to the kinetochores and regulates the initiation of kinetochore–microtubule attachments for proper chromosome alignment. Once kinetochore–microtubule attachment is achieved, this portion of Plk1 is removed from the kinetochores as a result of ubiquitination. However, the crucial molecular mechanism that promotes the localization and the maintenance of Plk1 on the kinetochores until metaphase is still unclear. We report that ubiquitin-specific peptidase 16 (Usp16) plays a key role during this process. Usp16 deubiquitinates Plk1, resulting in an enhanced interaction with kinetochore-localized proteins such as BubR1, and thereby retains Plk1 on the kinetochores to promote proper chromosome alignment in early mitosis. Down-regulation of Usp16 causes increased ubiquitination and decreased kinetochore localization of Plk1. Thus, our data unveil a unique mechanism by which Usp16 promotes the localization and maintenance of Plk1 on the kinetochores for proper chromosome alignment.

Introduction

Plk1 is an important mitotic regulator that plays a critical role in regulating chromosome alignment (Glover et al., 1998; Barr et al., 2004; Matsumura et al., 2007; Reindl et al., 2008). During the G2 to M phase transition, a portion of Plk1 localizes to the kinetochores, which is mediated by polo box domain (PBD)–mediated binding to kinetochore-localized interacting proteins (Elia et al., 2003; Baumann et al., 2007; Lee et al., 2008a,b), and regulates the initiation of kinetochore–microtubule attachments for proper chromosome alignment (Lampson and Kapoor, 2005; Kang et al., 2006; Nishino et al., 2006; Qi et al., 2006; Elowe et al., 2007; Li et al., 2010; Liu et al., 2012a,b; Mondal et al., 2012) to achieve accurate chromosome segregation into the daughter cells in mitosis (Khodjakov et al., 1999; Clarke and Bachant, 2008; Amaro et al., 2010; Maia et al., 2012). Once chromosomes are properly aligned and spindle checkpoint is satisfied, this portion of Plk1 is ubiquitinated by cullin 3 (CUL3)–based E3 ligase at K492 within PBD, leading to the removal of Plk1 from the kinetochores most likely because of weakened binding between Plk1 and its interacting proteins localized on the kinetochores (Beck and Peter, 2013; Beck et al., 2013). To prevent premature removal of Plk1 from the kinetochores and ensure the proper alignment of chromosomes, it is most likely that a yet-to-be-identified deubiquitination mechanism promotes the recruitment of Plk1 to, and its retention on, the kinetochores by antagonizing the function of the CUL3-based E3 ligase in early mitosis. In this study, we show that ubiquitin-specific peptidase 16 (Usp16) is a novel substrate for Plk1, and sequential phosphorylation by CDK1 and Plk1 activates Usp16, which, in turn, deubiquitinates Plk1 and promotes the recruitment of Plk1 to, and its retention on, the kinetochores for proper chromosome alignment.

Results and discussion

Usp16 interacts with and deubiquitinates Plk1

It has been reported that Plk1 interacts with several deubiquitylases based on mass spectrometry (MS) analysis (Lowery et al., 2007). To identify any deubiquitylases that may deubiquitinate Plk1 in early mitosis, we performed reciprocal coimmunoprecipitation (coIP) assays and found that Usp16 specifically interacted with Plk1 in nocodazole-arrested prometaphase HeLa cells (Fig. 1, A and B). This interaction was verified by coIP of endogenous Plk1 and GFP-tagged Usp16 expressed in HeLa cells (Fig. 1 C). To determine whether this interaction is PBD mediated, bacteria-expressed GST-PBD or GST-PBD with

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Abbreviations used in this paper: CCD, charge-coupled device; IP, immunoprecipitation; MS, mass spectrometry; PBD, polo box domain; WT, wild type.

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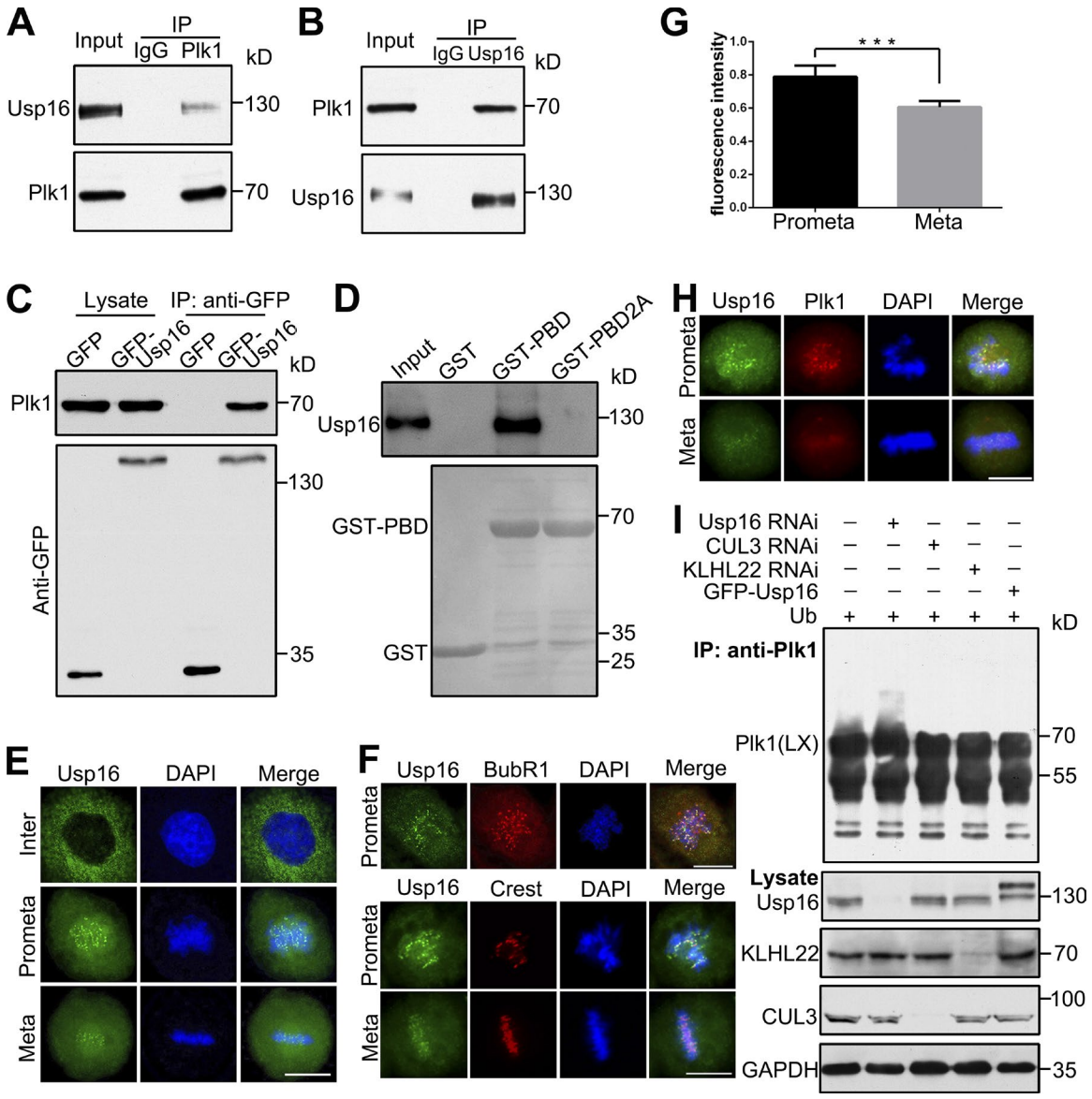

Figure 1. **Usp16 interacts with and deubiquitinates Plk1.** (A) Immunoblot of Plk1 or IgG mock IP complex from HeLa cell lysates. (B) Immunoblot of Usp16 or IgG mock IP complex from HeLa cell lysates. (C) Immunoblot of cell lysates and GFP IP complex. Cells were transfected with cDNAs coding for either GFP or GFP-Usp16. (D) Mitotic cell lysates were incubated with GST, GST-Plk1-PBD, or GST-Plk1-PBD-2A (H538A/K540A) before being blotted with Usp16. (Bottom) Coomassie blue staining. (E) The localization of Usp16 in interphase and mitotic HeLa cells. (F) Immunostaining shows that Usp16 colocalizes with BubR1 on the kinetochores but not with Crest on the centromere. (G) Ratios of the fluorescence intensity of Usp16 and Crest shown in F from three independent experiments with $n = 100–150$. Error bars indicate the SEM. ***, $P < 0.001$. (H) Plk1 and Usp16 colocalize in mitotic cells. (I) Immunoblot of Plk1 precipitated from mitotic HeLa cells with or without the knockdown of KLHL22, CUL3, Usp16, or overexpression of GFP-Usp16. LX, long exposure. Bars, 10 µm.

H538A/K540A (2A) mutations that disrupt the protein-interacting capability of the PBD (Elia et al., 2003) was incubated with prometaphase HeLa cell lysates. Examination of the GST pull-down complexes showed that Usp16 specifically interacted with the wild-type (WT) PBD but not the PBD2A mutant (Fig. 1 D), indicating that the interaction between Plk1 and Usp16 is PBD dependent. Because the subcellular location of Plk1 undergoes dramatic changes during the cell cycle, we wanted to know the localization of Usp16 in both interphase and M phase. For this purpose, we performed immunostaining and observed that most Usp16 were cytoplasmic in interphase but were on the kinetochores from prometaphase to the end of mitosis. However, its accumulation on the kinetochores was reduced after prometaphase (Fig. 1, E–G). It was also found that Usp16 colocalized with Plk1 on kinetochores at prometaphase, and the colocalization was reduced at metaphase (Fig. 1 H).

Because Usp16 interacts with Plk1, we suspected that it might deubiquitinate Plk1. To test this hypothesis, we analyzed the complex immunoprecipitated from prometaphase HeLa cell lysates with anti-Plk1 antibody and detected multiple bands in a ladder pattern above the main Plk1 band, suggesting that Plk1 might be ubiquitinated in prometaphase (Fig. 1 I). When Usp16 was knocked down by siRNA, we found that the ladder pattern bands above the main Plk1 band were enhanced, confirming our previous speculation that Usp16 might deubiquitinate Plk1 (Fig. 1 I). Because CUL3-based ubiquitin ligase ubiquitinates

Plk1 at K492 in vitro, to find out whether this ubiquitination contributed to the formation of the ladder pattern bands, CUL3 and its adaptor KLHL22 were knocked down individually by siRNA. It was found that the ladder pattern bands disappeared in CUL3 or KLHL22 knockdown cells (Fig. 1 I), suggesting that Plk1 is ubiquitinated by CUL3-based ubiquitin ligase in vivo. Importantly, expression of GFP-tagged Usp16 in cells arrested at prometaphase abolished the ladder pattern Plk1 bands (Fig. 1 I). Collectively, these results suggest that Plk1 is ubiquitinated by CUL3-based ubiquitin ligase in vivo, and the ubiquitination of Plk1 could be reversed by Usp16, which interacts with Plk1 in a PBD-dependent manner in early mitosis.

Usp16 regulates the kinetochore localization of Plk1 to promote proper alignment and timely separation of chromosomes

As ubiquitination promotes the removal of Plk1 from the kinetochores (Beck and Peter, 2013; Beck et al., 2013), we suspected that down-regulation of Usp16 would result in increased Plk1 ubiquitination and promote the removal of Plk1 from the kinetochores. Indeed, after Usp16 knockdown, we observed a dramatic decrease of Plk1 staining on the kinetochores in prometaphase cells (Fig. 2, A and B). On the other hand, when the CUL3-based ubiquitin ligase was suppressed by either KLHL22 or CUL3 siRNA, we observed a significant increase of Plk1 staining on the kinetochores (Fig. 2, A and B), which is consistent with previously reported results (Beck and Peter, 2013; Beck et al., 2013). Furthermore, to investigate whether Usp16 counteracts CUL3-based ubiquitination, we simultaneously knocked down both Usp16 and KLHL22 and found that the staining of Plk1 on the kinetochore remained largely stable (Fig. 2, A and B), suggesting that the dynamic equilibrium of ubiquitination and deubiquitination regulates the kinetochore localization of Plk1. To eliminate the possibility that these changes of Plk1 accumulation on the kinetochores were caused by the change of total Plk1 amount, we compared the level of total cellular Plk1 protein in cells before and after Usp16 knockdown but did not detect any difference (Fig. S1, A and B). This result suggests that Usp16 regulates the kinetochore localization but not the stability of Plk1 in early mitotic cells. To further clarify that the localization of Plk1 on kinetochores was regulated by ubiquitination/deubiquitination, we studied the interaction between Plk1 and the kinetochore-localized protein BubR1. We found that, whereas the kinetochore localization of BubR1 was unchanged in both the control and Usp16 knockdown HeLa cells, knockdown of Usp16 in the cells reduced the amount of Plk1 localized on the kinetochores (Fig. 2 D) and the binding between Plk1 and BubR1 (Fig. 2 C). On the other hand, when Usp16 was overexpressed, the Plk1–BubR1 interaction was enhanced (Fig. S1, C and D). These results strongly support the hypothesis that Usp16-mediated deubiquitination promotes kinetochore localization of Plk1 by enhancing its binding to yet-to-be-identified kinetochore-localized proteins, possibly including Usp16 and BubR1; on the other hand, ubiquitination reduces the binding of Plk1 with kinetochore-localized proteins and removes Plk1 from the kinetochores.

Because the kinetochore localization of Plk1 is necessary for kinetochore–microtubule attachment and subsequent chromosome alignment, we speculated that suppression of Usp16, i.e., reducing the amount of Plk1 on the kinetochores, would delay the chromosome alignment and anaphase onset. To test this, we knocked down Usp16 with siRNA and examined the chromosome alignment. We observed chromosomal misalignment in 40% of Usp16 knockdown cells, whereas only 2% of control cells displayed the misalignment (Fig. 2, E and F). We also monitored the chromosome alignment and anaphase onset with time-lapse microscopy and found that Usp16 knockdown caused a significant delay in chromatid segregation (Fig. 2, G and H; and Videos 1 and 2). Together, these results demonstrate that Usp16 plays an important role in chromosome alignment by regulating the kinetochore localization of Plk1.

Cdk1 phosphorylates Usp16 and enhances its binding to Plk1

Because Usp16 is phosphorylated by CDK1 (Cai et al., 1999; Xu et al., 2013), we speculated that CDK1 might serve as a priming kinase regulating Plk1–Usp16 interaction in a way similar to other cases (Liu and Maller, 2005; Zhang et al., 2009). To investigate this, we first set out to identify CDK1 phosphorylation sites on Usp16. By incubating GST-tagged Usp16 fragments, aa 1–150, aa 150–600, or aa 600–end individually with CDK1, we identified aa 150–600 as the only fragment being phosphorylated in vitro (Fig. 3 A). Sequence analysis revealed that S189 and S552 were potential phosphorylation sites for CDK1 (Fig. S2 A). Further analyses by in vitro phosphorylation assay and MS of peptides derived from proteins isolated from HeLa cells showed that S552 was the CDK1 phosphorylation site (Fig. S2, B and E), which was also reported recently (Xu et al., 2013). Next, we treated HeLa cells with CDK1 inhibitor RO3306 and observed a significant reduction in Plk1–Usp16 interaction (Fig. 3, C and E). Moreover, we found that only the WT GFP-Usp16, but not GFP-Usp16 S552A, could be coimmunoprecipitated with Plk1 from HeLa cell lysates (Fig. 3, D and F). These data strongly suggest that the phosphorylation of S552 by CDK1 promotes Plk1–Usp16 interaction.

Interestingly, when Plk1 inhibitor BI2536 was applied after the cells entered mitosis, the upshift of Usp16 was abolished (Fig. 3 G), suggesting that Usp16 was also phosphorylated by Plk1 in vivo. Next, Usp16 was identified as a substrate for Plk1 in an in vitro phosphorylation assay (Fig. 3 H). To determine the sites phosphorylated by Plk1, we used the same three Usp16 fragments as substrates in an in vitro kinase assay and found that once again, only the fragment aa 150–600 was phosphorylated (Fig. 3 I). By sequence analysis, we found three (S330, S386, and S486) potential Plk1 phosphorylation sites on Usp16 (Fig. S2 C). In vitro phosphorylation of Usp16 with single (S330A, S386A, or S486A) or collective 3A (S330A/S386A/S486A) mutation showed that Plk1 phosphorylated Usp16 at all three sites (Fig. S2 D). MS of peptides derived from proteins isolated from HeLa cells revealed two phosphorylation sites, S330 and S386 (Fig. S2 F), but not S486, possibly because of a lower level of phosphorylation to S486 or a lack of detection sensitivity. In agreement with our in vitro results, we analyzed Usp16 in lysates of synchronized HeLa cells by Western blot starting from S phase and found that the Usp16 band was upshifted during G2 to M phase transition, which correlates with the activation of Plk1 (Fig. 3 J). Importantly, the combined use of CDK1 and Plk1 enhanced the phosphorylation of the peptide aa 150–600 (Fig. 3 B), which is consistent with our early finding that CDK1 is the priming kinase for Plk1 in Usp16 phosphorylation. Collectively, our results strongly suggest that CDK1 promotes the interaction between Plk1 and Usp16 and enhances the phosphorylation of Usp16 by Plk1.

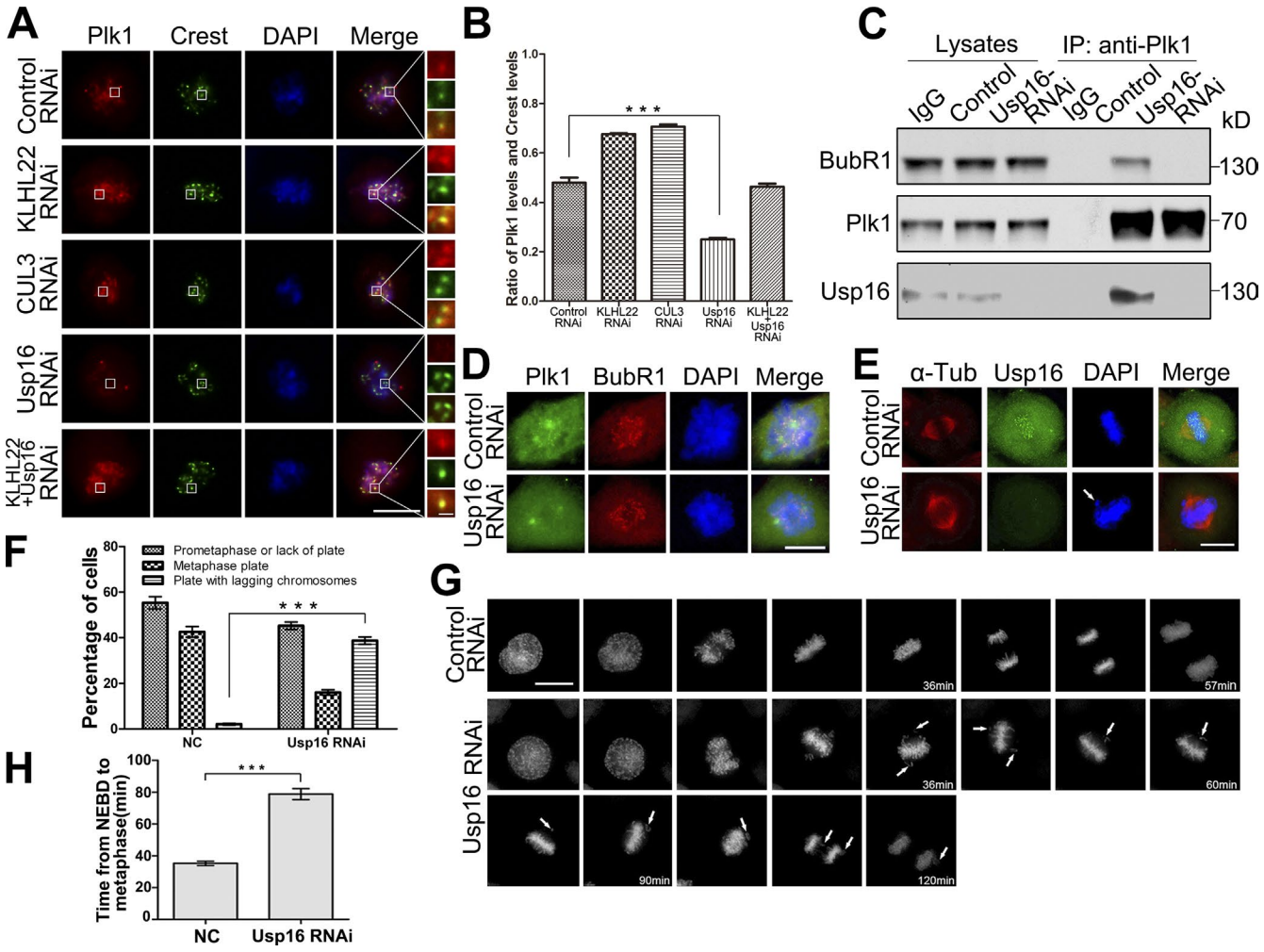

Figure 2. **Usp16 regulates the kinetochore localization of Plk1, leading to proper alignment and timely separation of chromosomes.** (A) Immunostaining of Plk1 in prometaphase HeLa cells treated with negative control siRNA or siRNA targeting KLHL22, CUL3, Usp16, or both Usp16 and KLHL22. Crest was used as a centromere marker. (B) The fluorescence intensity ratios of Plk1 and Crest shown in A, determined from three independent experiments with n = 100–150. (C) HeLa cells were transfected with control siRNA or siRNA targeting Usp16, and their lysates were blotted (left three lanes). Plk1 was immunoprecipitated with anti-Plk1 antibody or IgG in a mock IP. BubR1 coimmunoprecipitated with Plk1 was blotted (right three lanes). (D) Immunostaining of Plk1 and BubR1 in HeLa cells treated with either control siRNA or siRNA targeting Usp16. (E) Immunostaining of α-tubulin (α-Tub) and Usp16 in HeLa cells treated with control siRNA or siRNA targeting Usp16. The arrow points to misaligned chromosomes. (F) Percentage of cells with chromosome misalignment shown in E, determined from three independent experiments with n = 200–250. (G) Time-lapse microscopy of RFP-H2B–expressing HeLa cells treated with siRNA targeting Usp16 or control siRNA. Arrows point to misaligned or improperly separated chromosomes. (H) Time from nuclear envelope breakdown (NEBD) to metaphase in HeLa cells shown in G, determined from three independent experiments with n = 20–25. Error bars indicate the SEM. ***, $P < 0.001$. NC, negative control. Bars: (A, D, E, and G) 10 µm; (A, magnified images) 1 µm.

Plk1 phosphorylates and activates Usp16

Next, we set out to investigate the function of Usp16 phosphorylation by Plk1. We initially examined the localization of Usp16 in the presence or absence of Plk1 activity. As shown in Fig. S3 A, inhibition of Plk1 with BI2536 in mitotic HeLa cells did not cause any change on the kinetochore localization of Usp16. We also expressed GFP-fused WT Plk1 and PBD2A mutant and found that disruption of the kinetochore localization of Plk1 by the 2A mutations did not affect the kinetochore localization of Usp16 in prometaphase (Fig. S3 B). Next, to find out whether the activity of Usp16 was regulated by phosphorylation, the ubiquitination status of histone H2A, a physiological substrate of Usp16 (Joo et al., 2007), was examined. We found that the level of ubiquitinated histone H2A (ubH2A) was high in interphase and low in mitotic HeLa cells (Fig. S3, C–E), suggesting that Plk1 phosphorylates and activates Usp16. To prove this, purified recombinant *Xenopus laevis* Usp16 was incubated with total histones extracted from HeLa cells. The results showed that the amount of ubH2A was reduced in the presence of both Usp16 and *Xenopus* Plk1 compared with that in the presence of Usp16 alone and was reduced further if Usp16 was pretreated with CDK1, which supported our early result that CDK1 was a priming kinase for Plk1 (Fig. 4 A). To test this in vivo, we added BI2536 to HeLa cells and found that the level of ubH2A increased significantly, indicating that the activation of Usp16 was Plk1 dependent (Fig. 4, B and C). Moreover, the decrease of Usp16 activity as a result of the inhibition of Plk1 could be partially rescued by the expression of RNAi-resistant WT Usp16, but not the 3A mutant as judged by the ubH2A level (Fig. S3, F and G). These results strongly suggest that Plk1 phosphorylates and activates Usp16, but the exact molecular mechanism of the activation is not clear at the moment.

Because Usp16 deubiquitinates Plk1, we wondered whether Plk1-mediated Usp16 activation would also enhance

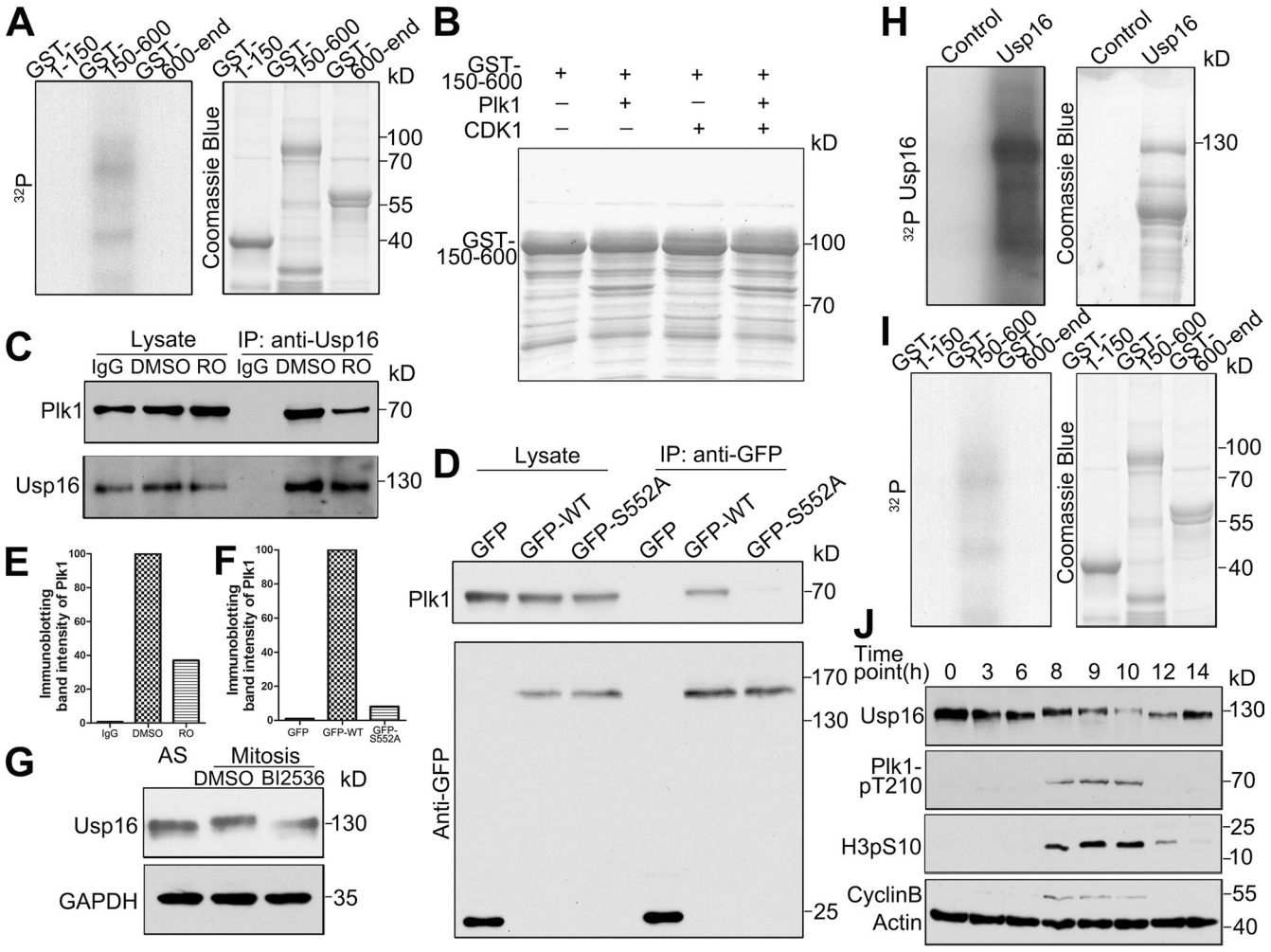

Figure 3. **Usp16 is phosphorylated by CDK1 and Plk1.** (A, left) Phosphorylation of GST-tagged Usp16 fragments by CDK1. (Right) Coomassie blue staining. (B) Coomassie blue staining of Usp16 fragment 150–600 phosphorylated by Plk1 and/or CDK1. (C) Immunoblot of Plk1 in cell lysates, Usp16 IP, or mock IP (IgG) complex. Mitosis-arrested HeLa cells were treated with DMSO or RO3306. (D, top) Immunoblot of Plk1 coimmunoprecipitated with GFP-tagged WT or S552A mutant Usp16. (Bottom) Immunoblot of GFP as a negative control. (E) The intensity of immunoblot bands of Plk1 shown in C, which was one representative experiment out of three repeats. (F) The intensity of immunoblot bands of Plk1 shown in D, which was one representative experiment out of three repeats. (G) Immunoblot of Usp16 from asynchronous (AS) or mitosis-arrested HeLa cells treated with either DMSO or BI2536. GAPDH was blotted as a loading control. (H, left) In vitro phosphorylation of Usp16 by Plk1. (Right) Coomassie blue staining. (I) In vitro phosphorylation of GST-tagged Usp16 fragments by Plk1 (left) and protein staining (right). (J) Immunoblot of Usp16, active Plk1 (pT210), and S10 phosphorylated histone H3 (H3pS10) in lysates of HeLa cells harvested at the indicated time points after being released from double-thymidine block. Both cyclin B and actin were blotted on the same membrane.

the deubiquitination of Plk1 itself. For this purpose, WT Usp16 and Usp16 mutants were ectopically expressed in HeLa cells and Plk1 ubiquitination was analyzed. We found that Plk1 could be efficiently deubiquitinated by the expression of WT or 3E mutant Usp16, but not the 3A mutant or enzyme-dead Usp16(C205S) (Fig. 4 D; Cai et al., 1999). These results suggest that Plk1 activates Usp16, which, in turn, deubiquitinates Plk1 itself.

Knowing that Usp16 regulates the localization of Plk1 on the kinetochores by deubiquitinating Plk1, we wanted to know whether Plk1 positively regulates its own kinetochore localization by phosphorylating and activating Usp16. To clarify this, we performed a rescue experiment by knocking down endogenous Usp16 followed by ectopically expressing RNAi-resistant WT Usp16 and its mutants in HeLa cells. It was found that the WT and 3E, but not 3A, Usp16 could restore the level of kinetochore-localized Plk1, which was initially reduced by Usp16 knockdown (Fig. 4, G and H). Furthermore, the WT and 3E (but not 3A and C205S mutants or the GFP tag) could rescue the chromosome misalignment caused by reduced kinetochore localization of Plk1 (Fig. 4, E and F). By time-lapse microscopy, we also demonstrated that the WT Usp16 and 3E, but not 3A, mutant could rescue the defects in chromosome alignment and separation caused by Usp16 knockdown (Fig. 4 I and Videos 3, 4, and 5).

In summary, we have identified an important molecular mechanism regulating the kinetochore localization of Plk1 for proper chromosome alignment in mitosis. It has been known that once spindle checkpoint is satisfied, the kinetochore-localized Plk1 is removed from the kinetochores by ubiquitination. Here, we showed that Usp16 antagonizes the activity of CUL3-based ubiquitin ligase by deubiquitinating Plk1, which not only promotes the localization of Plk1 to the kinetochores but also retains Plk1 there until metaphase. It is also possible that Usp16 may regulate the kinetochore localization of Plk1 independent

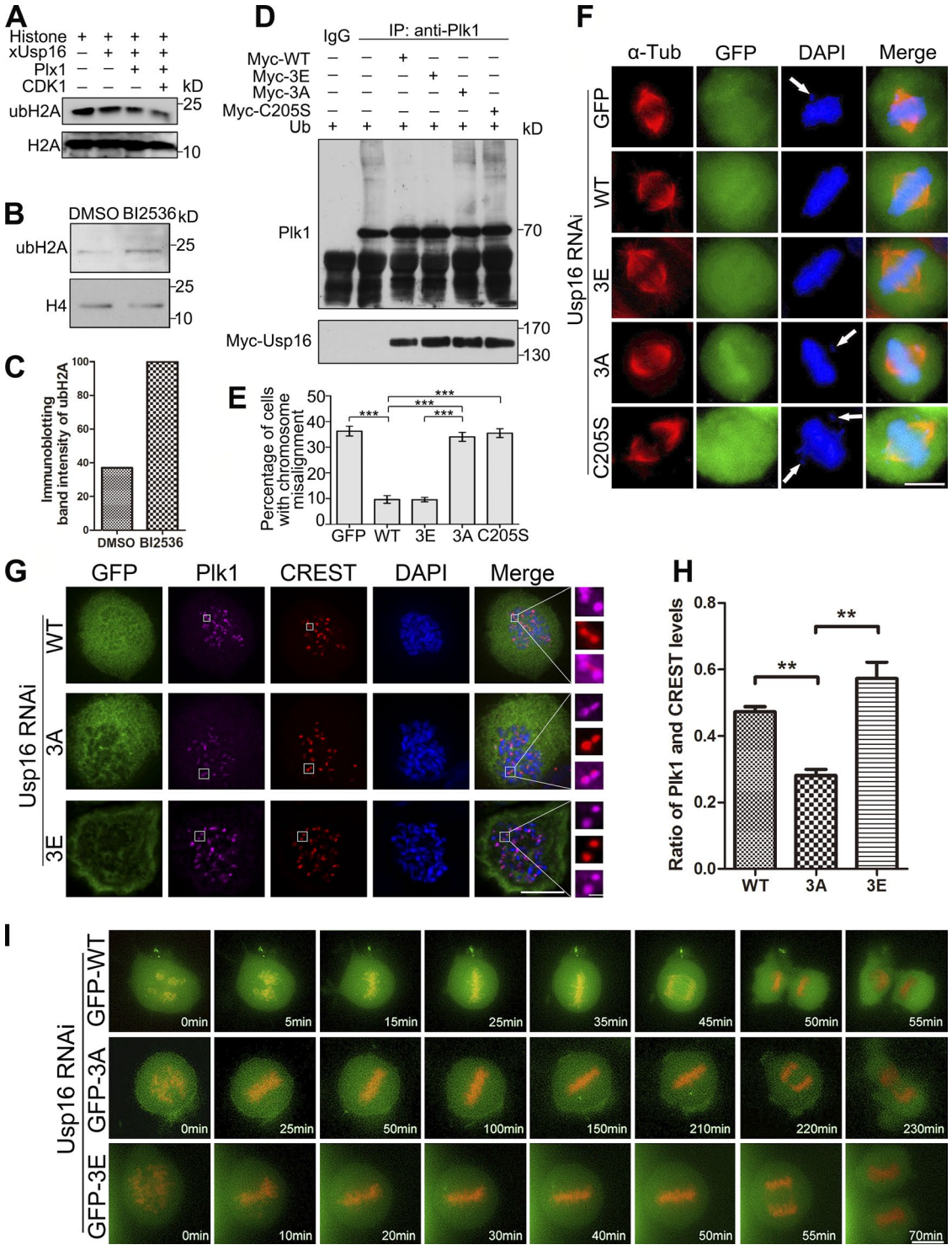

Figure 4. **The activity of Usp16 is enhanced by Plk1 phosphorylation.** (A) In vitro deubiquitination assay in the presence of *Xenopus* Usp16 (xUsp16), xUsp16 + *Xenopus* Plk1 (Plx1), or xUsp16 + Plx1 + CDK1 where xUsp16 was pretreated with CDK1. Total histones extracted from HeLa cells were used as deubiquitination substrates. Blots of ubH2A (top) and total histone H2A (loading control; bottom) are shown. (B, top) Immunoblot of ubH2A in lysates from asynchronous HeLa cells treated with DMSO or BI2536. (Bottom) Histone H4 was blotted as a loading control. (C) The intensity of immunoblot bands of ubH2A shown in B. The experiment was repeated three times. (D, top) Immunoblot of Plk1 in Plk1 IP complexes precipitated from mitosis-arrested HeLa cells expressing ubiquitin and Myc-tagged WT Usp16, 3E (S330E/S386E/S486E), 3A (S330A/S386A/S486A), or C205S mutant. (Bottom) Immunoblot of Myc-tagged Usp16. (E) Percentage of cells with chromosome misalignment shown in G, determined from three independent experiments with $n = 200–250$. ***, $P < 0.001$. (F) Cells with chromosome misalignment caused by Usp16 knockdown were rescued by expression of WT Usp16 and Usp16 3E, but not Usp16 3A and Usp16 C205S. All Usp16s were expressed from siRNA-resistant plasmids. GFP was transfected as a negative control. White arrows point to misaligned chromosomes. (G) Immunostaining of Plk1 in prometaphase HeLa cells with endogenous Usp16 depleted and the expression of RNAi-resistant WT, 3A, and 3E Usp16. Crest was used as a centromere marker. (H) The fluorescence intensity ratios of Plk1 and Crest shown in G, determined from three independent experiments with $n = 100–150$. **, $P < 0.01$. (I) Time-lapse microscopy of Usp16 knockdown HeLa cells expressing RFP-H2B and siRNA-resistant GFP-tagged Usp16 WT, GFP-Usp16 3A, or GFP-Usp16 3E. Error bars indicate the SEM. Bars: (F, G, and I) 10 µm; (G, magnified images) 1 µm.

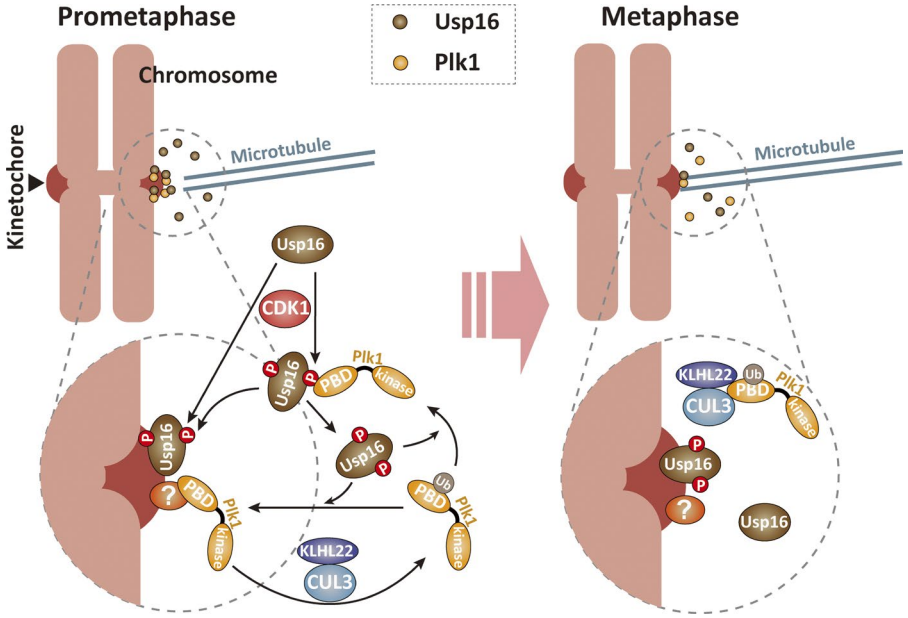

Figure 5. **The kinetochore localization of Plk1 is regulated by deubiquitination and ubiquitination.** The diagram shows that in early mitosis, cytosolic and possibly kinetochore-localized Usp16 is phosphorylated by CDK1 to generate a binding motif for PBD. A portion of preactivated Usp16 deubiquitinates Plk1 and promotes the binding of Plk1 to yet-to-be-identified kinetochore-localized proteins, which may include Usp16 as well, and the binding of Plk1 to cytosolic Usp16. Then, Plk1 further phosphorylates Usp16 to activate it. Usp16, in turn, keeps Plk1 in a deubiquitinated form and further enhances its binding to the kinetochore-localized proteins. By metaphase, CUL3 and substrate-specific adaptor KLHL22 become enriched at the kinetochores, which bind and ubiquitinate Plk1 within its PBD, resulting in the dissociation of Plk1 from its interacting proteins and the removal of Plk1 from the kinetochores. This allows the satisfaction of the spindle assembly checkpoint and subsequent chromosome separation. Question marks represent not-yet-identified kinetochore-localized proteins that may bind to Plk1. P, phosphorylation; Ub, ubiquitination.

of CUL3-based ubiquitination. Interestingly, the activity of Usp16 is positively regulated by a sequential phosphorylation of CDK1 and Plk1. Based on our present work and previous studies (Beck and Peter, 2013; Beck et al., 2013), we propose a model (Fig. 5) in which the equilibrium of deubiquitination and ubiquitination regulates the kinetochore localization of Plk1 from prometaphase to metaphase. The spatial and temporal regulation of Plk1 is required for initial kinetochore–microtubule attachment, proper chromosome alignment, and timely chromatid segregation. As aneuploidy is a result of separation of improperly aligned chromosomes in mitosis and is a hallmark associated with genetic diseases and cancer progression, our findings may contribute to the development of new therapeutic approaches for the treatment of such diseases.

Materials and methods

Molecular cloning and protein purification

Human Plk1 and Usp16 were cloned from a cDNA library by RT-PCR. In this study, cDNAs encoding full-length Usp16 WT/S552A/S189A/C205S/3A (S330A, S386A, and S486A)/3E (S330E, S386E, and S486E), RNAi-resistant Usp16-WT/3A/3E, Usp16 (1–150), Usp16 (150–600), Usp16 (600–end), full-length Plk1, Plk1 (326–end or PBD), and Plk1-PBD-H538A/K540A were subcloned into pEGFP-C2, pCMV-Myc, pET-28a, or pGEX-4T-1 vectors. All the His-tagged or GST-tagged Usp16 proteins were expressed in *Escherichia coli* and affinity purified using nickel–nitrilotriacetic acid agarose (for His-tagged proteins; QIAGEN) or glutathione–Sepharose 4B beads (for GST-tagged proteins; GE Healthcare) according to the manufacturers' protocols.

Cell culture, synchronization, and transfection

HeLa cells were cultured at 37°C in a 5% CO_2 incubator in DMEM (Gibco) with 10% FBS (HyClone Laboratories, Inc.), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). For the double thymidine block and release experiment, the cells were arrested for 18 h with 2.5-mM thymidine (Sigma-Aldrich) with a 10-h release interval. After release into fresh medium, cells were harvested at the indicated time points. Mitosis-arrested cells were obtained by adding 100 ng/ml nocodazole (Sigma-Aldrich) for 15 h after release from the thymidine block. Transient cDNA transfections and siRNA were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The following siRNA sequences were used: Usp16-1, 5′-GCCUAUGCCAAGGCAAGAA-3′; Usp16-2, 5′-CCUCCUGUUCUUACUCUUCAUUUAA-3′; Usp16-3, 5′-CCGGAAAUCUUAGAUUUGGCUCCUU-3′; KLHL22-1, 5′-GCAACAACGAUGCCGGAUA-3′; KLHL22-2, 5′-CCUAUAUCCUCAAAAACUU-3′; KLHL22-3, 5′-GGACUGGCUCUGUGAUAAA-3′; and CUL3, 5′-CAACACUUGGCAAGGAGAC-3′.

IP, GST fusion protein pull-down assay, and Western blotting

HeLa cells were washed with cold PBS and lysed in cell lysis buffer (20-mM Tris-HCl, pH 8.0, 150-mM NaCl, 2-mM EGTA, 0.5-mM EDTA, 0.5% NP-40, 5-mM NaF, 1-mM Na_3VO_4, 1-mM PMSF, and 500× protease inhibitor cocktail; Calbiochem) for 20 min on ice. For Usp16/Plk1 coIP assays, cell lysates were mixed with anti-Usp16 polyclonal antibodies (raised in the laboratory in rabbit using His-tagged Usp16 1–257 aa) or mouse anti-Plk1 antibodies (06-813; EMD Millipore) and 15 µl protein A–Sepharose beads (75% slurry) and incubated at 4°C for 1 h. After washing with lysis buffer, the beads were harvested and suspended in Laemmli sample buffer. For Plk1/BubR1 coIP assays, cell lysates were mixed with anti-Plk1 polyclonal antibodies (raised in the laboratory in rabbit using His-tagged Plk1 326–end). For GST pull-down assays, HeLa cell lysates were incubated with 5 µg of soluble GST or GST-fused proteins bound to 15 µl glutathione–Sepharose beads (75% slurry) at 4°C for 1 h. After washing with lysis buffer, the beads were harvested and suspended in Laemmli sample buffer.

After being resolved on SDS-PAGE gels, the proteins were transferred to nitrocellulose membranes that were then blocked in TTBS (20-mM Tris-HCl, pH 7.5, 500-mM NaCl, and 0.3% Tween 20) containing 3% nonfat milk at room temperature for 1 h. Then, they were probed with primary antibodies diluted in TTBS containing 3% nonfat milk at 4°C overnight. The following primary antibodies were used for immunoblotting: mouse anti-Plk1 (06-813; EMD Millipore), mouse anti-BubR1 (ab4037; Abcam), rabbit anti-Usp16 polyclonal antibodies, rabbit anti–Cullin 3 (ab108407; Abcam), and rabbit anti-KLHL22 (16214-1-AP; Proteintech). After washing, the membranes were incubated with HRP-conjugated secondary antibody at room temperature

for 1 h and washed with TTBS four times. The membranes were developed for visualization by enhanced chemiluminescence (Sigma-Aldrich) and x-ray film. The immunoblot band intensity was measured by ImageJ software (National Institutes of Health).

HeLa cells were synchronized by double thymidine (Sigma-Aldrich) or nocodazole (Sigma-Aldrich). Mitotic cells were collected by shake-off. The remaining cells were also harvested after trypsin treatment. An equal amount of proteins was subjected to immunoblotting with antibodies against mouse phospho-Plk1 (Thr210; 5472; Cell Signaling Technology), rabbit phospho-histone H3 (Ser10; 3377; Cell Signaling Technology), rabbit cyclin B (sc-25764; Santa Cruz Biotechnology, Inc.), rabbit actin (sc7210; Santa Cruz Biotechnology, Inc.), mouse ubH2A (05-678; EMD Millipore), rabbit H4 (04-858; EMD Millipore), and mouse α-tubulin (T6074; Sigma-Aldrich).

Immunofluorescence and microscopy quantifications

HeLa cells were grown on glass coverslips and fixed in cold methanol for 5 min or in PBS/4% PFA for 15 min and then permeabilized with 0.2% Triton X-100 for 5 min. Coverslips were incubated with primary antibodies at 4°C overnight, washed with PBS, and incubated with fluorescence-labeled secondary antibodies at room temperature for 1 h. The following antibodies were used: mouse anti-Plk1 (06-813; EMD Millipore), anti-Plk1 polyclonal antibodies (raised in the laboratory in rabbit using His-tagged Plk1 326–end), anti-Usp16 polyclonal antibodies (raised in the laboratory in rabbit using His-tagged Usp16 1–257 aa), mouse anti-ubH2A (05-678; EMD Millipore), mouse anti–α-tubulin (T6074; Sigma-Aldrich), mouse anti-BubR1 (ab4037; Abcam), human anticentromere antibody from CREST syndrome patients (15-234-0001; Antibodies Incorporated), Alexa Fluor 488 donkey anti–mouse, Alexa Fluor 594 donkey anti–rabbit, Alexa Fluor 488 donkey anti–rabbit, Alexa Fluor 594 donkey anti–mouse, Alexa Fluor 488 goat anti–human, and Alexa Fluor 546 goat anti–human (Invitrogen). After washing with PBS, the coverslips were mounted with mowiol (Sigma-Aldrich) containing 1 µg/ml DAPI (Sigma-Aldrich) for DNA staining. Images were analyzed under a 63×/1.4 NA oil objective of a microscope (Axiovert 200M; Carl Zeiss) and captured with a charge-coupled device (CCD) camera (MRM; Carl Zeiss) and the Axiovert image acquisition software. The RNAi rescue data were acquired with an imaging system (DeltaVision; Applied Precision) equipped with an inverted microscope (IX-71; Olympus) and a 100×/1.42 NA oil objective. The images were captured with a CCD camera (CoolSnap HQ2; Photometrics), and different z sections were deconvolved by softWoRx (Applied Precision). The fluorescence intensity was measured by ImageJ software.

Time-lapse microscopy

HeLa cells grown in a glass-bottomed dish were transfected with the indicated siRNA or cDNAs. Data were acquired with a DeltaVision live cell imaging system equipped with an inverted microscope (IX-71) and a 60×/1.42 NA oil objective. The images were captured with a CoolSnap HQ2 CCD camera, and different z sections were deconvolved by softWoRx.

In vitro and in vivo protein phosphorylation assays and MS

For in vitro protein phosphorylation analysis, 2 µg GST-Usp16 (150–600) were incubated with Cyclin B/Cdc2 (New England Biolabs, Inc.) and/or Plk1 (Cell Signaling Technology) in 50-mM Tris, pH 7.5, 10-mM $MgCl_2$, 2-mM EGTA, 5-mM DTT, and 100-mM ATP at 30°C for 30 min. The reactions were subjected to SDS-PAGE resolution, and the gel slices were processed for MS analysis. For in vitro protein phosphorylation autoradiography assays, 2 µg GST-tagged truncated or His-tagged full-length Usp16 proteins were incubated with either Plk1 (Cell Signaling Technology) or Cyclin B/Cdc2 (New England Biolabs, Inc.) in 50-mM Tris, pH 7.5, 10-mM $MgCl_2$, 2-mM EGTA, 5-mM DTT, 100-mM ATP, 0.25 mCi/ml γ-[^{32}P]ATP, and 1 µCi γ-[^{32}P]ATP (10 mCi/ml, 6,000 Ci/mmol; GE Healthcare) for 30 min at 30°C. The reactions were stopped by the addition of Laemmli sample buffer and were analyzed by SDS-PAGE and autoradiography. For in vivo protein phosphorylation assays, Usp16 (150–600) was fused with GFP-Hec1, and the fusion protein was transfected into HEK-293 cells. The cells were then synchronized by thymidine (Sigma-Aldrich) and nocodazole (Sigma-Aldrich) at prometaphase. The mitosis-arrested cells were shaken off and lysed in cell lysis buffer for 20 min on ice. The cell lysates were mixed with rabbit anti-GFP antibodies and 30 µl protein A–Sepharose beads (75% slurry) and incubated at 4°C for 1 h. After washing with lysis buffer, the beads were harvested and suspended in sample buffer. The reactions were subjected to SDS-PAGE resolution, and the gel slices were processed for phospho-MS analysis.

In vitro deubiquitination assay

In brief, purified recombinant *Xenopus* Usp16 was incubated with either buffer or cyclin B/Cdk1 (EMD Millipore) for 30 min at 30°C, supplemented with purified *Xenopus* Plk1 expressed and purified from Sf9 cells, and incubated at 30°C for another 30 min. 1 µg of total histones extracted from HeLa cells with the Histone Extraction kit (Epigentek) was added to the mixture, and the mixtures were incubated at 30°C for 30 min. The reaction was stopped by the addition of Laemmli sample buffer. Proteins were resolved in SDS-PAGE and probed with corresponding antibodies.

In vivo deubiquitination assay

In brief, HeLa cells were transfected with ubiquitin alone or cotransfected with ubiquitin and Myc-Usp16. The cells were treated with thymidine followed by nocodazole to be synchronized to prometaphase. After washing off the nocodazole, the cells were then synchronized with protease inhibitor MG132 to metaphase. The cell lysates were resolved in SDS-PAGE probed with anti-Plk1 and anti-Myc antibodies.

Online supplemental material

Fig. S1 shows that Usp16 does not regulate Plk1 stability but facilitates its localization to the kinetochores by enhancing the binding of Plk1 to kinetochore-localized BubR1. Fig. S2 shows the phosphorylation sites of Usp16 by CDK1 or Plk1. Fig. S3 shows that Plk1 activity is not required for the kinetochore localization of Usp16 but is required for Usp16 deubiquitination of histone H2A in mitosis. Video 1 shows control siRNA-treated HeLa cells expressing RFP-H2B. Video 2 shows Usp16 siRNA-treated HeLa cells expressing RFP-H2B. Video 3 shows Usp16 siRNA-treated HeLa cells expressing RFP-H2B and siRNA-resistant GFP-tagged Usp16 WT. Video 4 shows Usp16 siRNA-treated HeLa cells expressing RFP-H2B and siRNA-resistant GFP-tagged Usp16 3A. Video 5 shows Usp16 siRNA-treated HeLa cells expressing RFP-H2B and siRNA-resistant GFP-tagged Usp16 3E. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201502044/DC1.

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