PCM1 recruits Plk1 to the pericentriolar matrix to promote primary cilia disassembly before mitotic entry

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Accepted 11 December 2012
Journal of Cell Science 126, 1355–1365
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doi: 10.1242/jcs.114918

Summary

Primary cilia, which emanate from the cell surface, exhibit assembly and disassembly dynamics along the progression of the cell cycle. However, the mechanism that links ciliary dynamics and cell cycle regulation remains elusive. In the present study, we report that Polo-like kinase 1 (Plk1), one of the key cell cycle regulators, which regulate centrosome maturation, bipolar spindle assembly and cytokinesis, acts as a pivotal player that connects ciliary dynamics and cell cycle regulation. We found that the kinase activity of centrosome enriched Plk1 is required for primary cilia disassembly before mitotic entry, wherein Plk1 interacts with and activates histone deacetylase 6 (HDAC6) to promote ciliary deacetylation and resorption. Furthermore, we showed that pericentriolar material 1 (PCM1) acts upstream of Plk1 and recruits the kinase to pericentriolar matrix (PCM) in a dynein-dynactin complex-dependent manner. This process coincides with the primary cilia disassembly dynamics at the onset of mitosis, as depletion of PCM1 by shRNA dramatically disrupted the pericentriolar accumulation of Plk1. Notably, the interaction between PCM1 and Plk1 is phosphorylation dependent, and CDK1 functions as the priming kinase to facilitate the interaction. Our data suggest a mechanism whereby the recruitment of Plk1 to pericentriolar matrix by PCM1 plays a pivotal role in the regulation of primary cilia disassembly before mitotic entry. Thus, the regulation of ciliary dynamics and cell proliferation share some common regulators.

Key words: PCM1, Plk1, Primary cilium

Introduction

Primary cilium is an important cell organelle that assembles based on basal body and projects from cell surface, which plays critical roles in sensation and cellular signal transduction owning to the enrichment of receptors and channels on ciliary membrane (Berbari et al., 2009; Singla and Reiter, 2006). The defects of primary cilia function lead to numerous cilium-related human diseases, such as Bardet-Biedl Syndrome (BBS), retinal degeneration, polycystic kidney disease and other disorders (Ishikawa and Marshall, 2011). Primary cilia assemble when cells exit from cell cycle and enter quiescence, while the cilia of proliferating cells often exhibit dynamic probably due to the fact that cilia disassembly liberates centrioles to undergo duplication and separation for bipolar spindle assembly, although centriole duplication can also take place while the mother centriole still acts as the ciliary basal body (Fonte et al., 1971; Quarmby and Parker, 2005; Rieder, 1979; Seeley and Nachury, 2010; Uetake et al., 2007). Ciliogenesis is strikingly controlled, and numerous evidences have shown that intraflagellar transport (IFT), membrane trafficking and cilia associated proteins, such as BBSome, are involved (Follit et al., 2006; Ishikawa and Marshall, 2011; Jin et al., 2010; Nachury et al., 2007; Pedersen and Rosenbaum, 2008; Qin et al., 2005). Over the past decades, evidences have shown that the cilia assembly and disassembly dynamics accompany with the cell cycle. However, the detailed mechanisms underlying the relationship between cell proliferation and ciliary dynamics remain elusive, though both Pitchfork and HEF1 were revealed recently to be responsible for the cilia disassembly by regulating Aurora A activity, which subsequently phosphorylates and activates HDAC6 to regulate the primary cilia resorption (Kinzel et al., 2010; Pugacheva et al., 2007).

Centrosome maturation is a crucial step for the bipolar mitotic spindle assembly and this process has been characterized in principal by the features of the recruitment and re-organization of additional pericentriolar material, the phosphorylation of centrosomal proteins and the dramatic increase of the microtubule nucleation and anchoring capacity of the centrosome (Bornens, 2002; Schatten, 2008). A number of mitotic protein kinases, including Plk1, Aurora A and CDK1, are crucial for the maturation of centrosome by phosphorylating the centrosomal proteins. In addition, in the recruitment of additional pericentriolar materials, Nedd1 is required for maturation and microtubule nucleation of the centrosome by targeting γ-TuRC to centrosome at the G2/M phase transition, and this process needs Nedd1 to be sequentially phosphorylated by CDK1 and Plk1 (Haren et al., 2006; Lüders et al., 2006; Schatten, 2008; Zhang et al., 2009). Also, activated Plk1 could phosphorylate Ninein-like protein (Nlp) to promote its displacement from maturing centrosome, and the displacement is important for mitotic

spindle formation at the onset of mitosis (Casenghi et al., 2005; Casenghi et al., 2003). Plk1 exhibits diverse localizations and functions from mitotic entry to cytokinesis. Its localization capability depends on its C-terminal portion, termed as polo-box domain (PBD), which serves as a phosphorylation-dependent binding domain (Elia et al., 2003; Lowery et al., 2007; Lowery et al., 2004; Petronczki et al., 2008). Previous research indicated that hCenexin1 participates in the recruitment of Plk1 to centrosome and also contributes to primary cilia formation independent of Plk1 (Soung et al., 2006; Soung et al., 2009). However, unlike interphase centrosome, depletion of hCenexin1 only modestly delocalizes Plk1 from the mitotic centrosome, suggesting that other component(s) on maturing centrosome may also play an important role for the enrichment of Plk1 on centrosome during mitosis (Soung et al., 2006).

In this work, we identified that PCM1, a centriolar satellite protein that serves as a scaffold to target a number of proteins to centrosome during centrosome maturation (Balczon et al., 1994; Dammermann and Merdes, 2002; Ge et al., 2010; Hames et al., 2005; Kubo et al., 1999), is phosphorylated by CDK1, which in turn recruits Plk1 to the pericentriolar matrix before mitotic entry. The kinase activity of pericentriolar matrix-localized Plk1 is required for primary cilia resorption by interacting with and activating HDAC6, thereby inducing ciliary microtubules deacetylation and disassembly. Subsequently, disassembly of the cilia release the basal body/mother centriole into the state required for the bipolar mitotic spindle assembly. Our data provide a new insight on the relationship between primary cilia dynamics and cell cycle regulation.

Results

Plk1 kinase activity is required to promote primary cilia disassembly before mitotic entry

Previous studies indicated that primary cilia assembly and disassembly are related to cell cycle, and the ciliary resorption wave occurs before mitotic entry (Ishikawa and Marshall, 2011; Pugacheva et al., 2007), when Plk1 is phosphorylated and activated by Aurora A (Macůrek et al., 2008; Seki et al., 2008). Hence, we speculated that Plk1 might act as a potential linker orchestrating ciliary dynamics and cell cycle regulation to promote primary cilia disassembly before mitotic entry.

To verify this hypothesis, we stained acetylated α-tubulin as a marker of cilium and established a system to study ciliary dynamics in NIH3T3 cells. After 24 hours of serum starvation, more than 70% of NIH3T3 cells showed clearly visible cilia (supplementary material Fig. S1A). Two primary cilia disassembly waves occurred after 10% serum stimulation of these ciliated cells. One occurred about 2 hours after serum stimulation as reported previously (Pugacheva et al., 2007) and the other one happened about 18 hours after the stimulation (Fig. 1A). Strikingly, the second wave occurred by the time of the activation of Plk1 before mitotic entry (Fig. 1A; supplementary material Fig. S1B). Western blotting analysis showed that Plk1 and Aurora A were highly expressed from 16 hours to 22 hours after serum stimulation, overlapping the duration when the cells undergo G2 to M transition (Fig. 1A) (Ferris et al., 1998). We then tested the effect of Plk1 kinase activity on the primary cilia resorption by using Plk1 inhibitor BI2536 (100 nM) (Steegmaier et al., 2007). When NIH3T3 cells were released from serum starvation for 24 hours, as shown in Fig. 1A, the second primary cilia resorption wave occurred in G2 phase in the control cells. In contrast, the second primary cilia resorption wave was largely delayed in the cells treated with BI2536. The length of the cilia was longer in cells treated with BI2536 about 18 hours after serum stimulation than that in control cells as well (Fig. 1B). To confirm that the phenotype of defective ciliary resorption is due to Plk1 suppression, we immunostained NIH3T3 cells treated with RNA interference targeting Plk1 and found that the second resorption wave of primary cilia was disturbed too (Fig. 1C). Evidences have shown that Plk1 is phosphorylated and activated by Aurora A at T210 (Macůrek et al., 2008; Seki et al., 2008). Immunostaining of primary cilia in NIH3T3 cells overexpressing GFP, GFP–Plk1 wild type, kinase active (Plk1T210D) or kinase dead (Plk1K82R) mutants confirmed that the kinase active mutant Plk1T210D showed about 2 folds efficiency increase in promoting ciliary resorption compared with the wild type. However, the GFP control or the kinase dead mutant Plk1K82R had no such effect (supplementary material Fig. S1C).

Remarkably, in G0 phase cells, we could hardly detect the signal of centrosomal Plk1; whereas the cells without cilia showed preferentially the pericentriolar matrix localization of Plk1 that occurred in synchronization with the primary cilia disassembly before mitotic entry (Fig. 1D,F; supplementary material Fig. S1D). We speculated that the accumulation of pericentriolar Plk1 resulted in ciliary disassembly. This hypothesis was examined by ectopic expression of Plk1–pericentrin (Plk1–PCNTB) fusion proteins, including constitutively active Plk1 ($Plk1^{CA}$)–PCNTB and kinase dead Plk1 ($Plk1^{KD}$)–PCNTB in NIH3T3 (Fig. 1E) (Lee and Rhee, 2011). These fusion proteins could stably localize at pericentriolar matrix. As expected, cilia were significantly disassembled in the $Plk1^{CA}$–PCNTB-expressing cells even in G0 phase, while neither PCNTB alone nor $Plk1^{KD}$–PCNTB promote ciliary disassembly (Fig. 1E). Together, these data support the hypothesis that the pericentriolar matrix-localized active Plk1 kinase activity is responsible for the resorption of primary cilia before mitotic entry.

Plk1 promotes primary cilia resorption by activating HDAC6

HDAC6 is a tubulin deacetylase that functions in modulating cell spreading and motility, as well as primary cilia resorption (Hubbert et al., 2002; Pugacheva et al., 2007; Zhang et al., 2007). Multiple mechanisms have been found to be involved in regulating HDAC6 deacetylase activity. Pugacheva and colleagues revealed that Aurora A promotes primary cilia disassembly by phosphorylating and activating HDAC6 (Pugacheva et al., 2007). As one of the key regulators of cellular proliferation, Aurora A activates Plk1 before mitotic entry when the second wave of primary cilia disassembly occurs. We proposed that activated Plk1 may also participate in the primary cilia resorption process independent of Aurora A. To verify this hypothesis, we treated NIH3T3 cells released from serum starvation with 0.5 μM MLN8237 to inhibit Aurora A activity. Simultaneously, the cells released from serum starvation were transfected with GFP, GFP-fused wild type Plk1, Plk1T210D or T201A (mimic non-phosphorylation) mutant in the presence of the inhibitor for about 18 hours. We found that Plk1T210D, but not the wild type or T210A mutants, could efficiently promote the primary cilia resorption comparing with the GFP control (Fig. 2A; supplementary material Fig. S2). These data indicate that activated Plk1 promotes primary cilia disassembly even in the absence of Aurora A activity.

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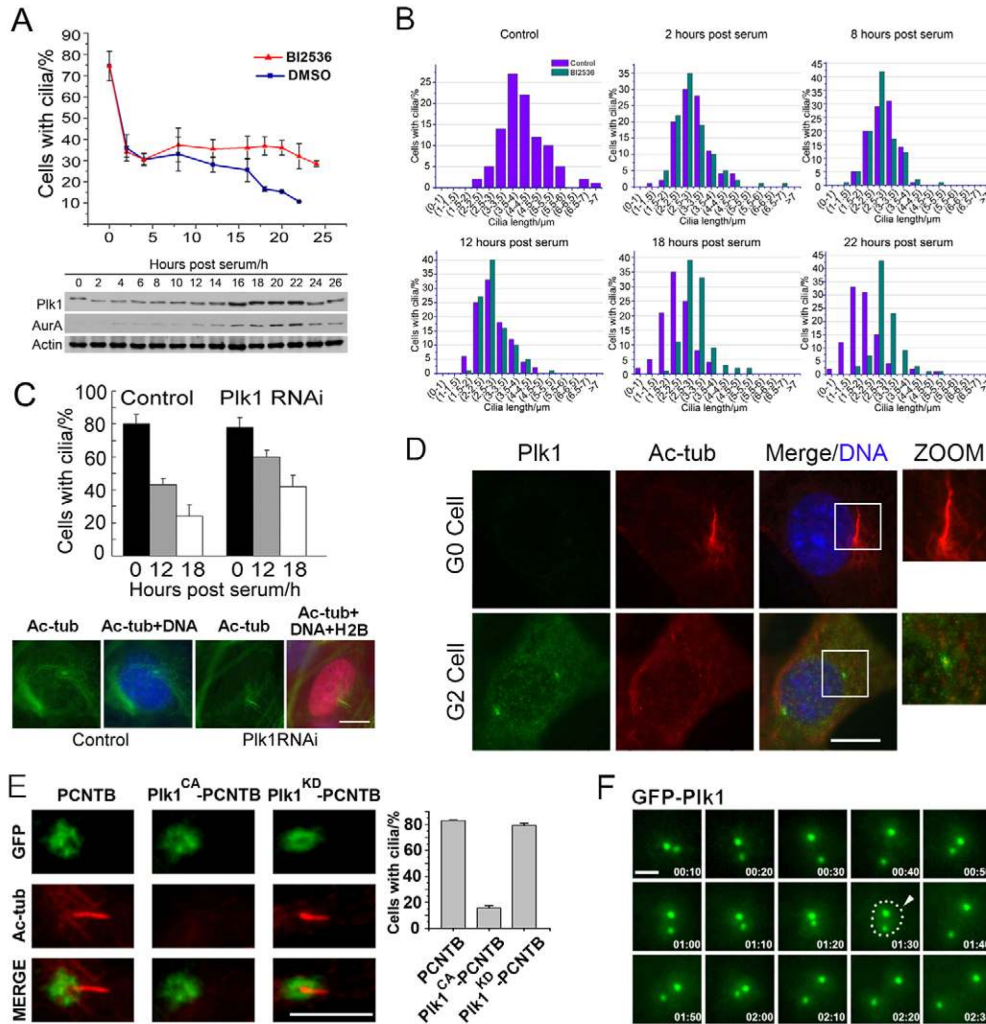

Fig. 1. Plk1 kinase activity promotes primary cilia resorption. (**A**) Time course showing the percentage of the NIH3T3 cells with cilia after release from serum starvation in the presence or absence (control) of the Plk1 inhibitor BI2536. Upper panel: in control cells, two ciliary resorption waves occurred at about 2 and 18 hours after release from serum starvation (blue). When Plk1 kinase activity was inhibited by BI2536, the second resorption wave was abolished (red). Averages of 200 cells were counted in two independent experiments. Error bars show s.d. Lower panel: western blotting analysis showing that Plk1 and Aurora A were highly expressed in the control cells at 18 hours after release. Actin was blotted as a loading control. (**B**) The cilia in BI2536-treated NIH3T3 cells were longer than those of control cells at about 18 hours after release from serum starvation. The histograms show the percentage of cells with particular-length cilia at some of the time points after release from serum starvation shown in A. Violet bars represent the control, and green bars represent cells treated with BI2536. (**C**) NIH3T3 cells were transfected with RFP–H2B and p-Super-Plk1 or p-Super as control, and simultaneously treated with serum starvation for 48 hours. After release at different time points, the cells were collected for immunofluorescence microscopy using anti-acetyl-tubulin antibody for cilia. The percentage of the cells with cilia were counted and are shown in the upper panel (histogram), and representative immunofluorescence images of cells released for 18 hours are shown in the lower panel. p-Super-Plk1 and RFP–H2B were co-transfected at a ratio of 20:1 (Chen et al., 2008). Green, acetyl-tubulin (Ac-tub); red, RFP–H2B; blue, DNA. Error bars show s.d. Scale bar: 10 μm. (**D**) Accumulation of pericentriolar Plk1 correlates with the ciliary disassembly. NIH3T3 cells were arrested in G0 phase by serum starvation for about 36 hours (upper panel), released for 18 hours to G2 phase (lower panel), and immunostained with antibodies against Plk1 and acetyl-tubulin (Ac-tub). The squares were zoomed into the right-hand panels to show the pericentriolar Plk1. Scale bar: 10 μm. (**E**) NIH3T3 cells were transfected with the indicated fusion protein, and then starved for 24 hours. The cells were counted to determine the percentage of cells with primary cilia (histogram on right). Representative immunofluorescence images are shown on the left. Averages of 200 cells were counted in two independent experiments. Error bars show s.d. Scale bar: 10 μm. (**F**) NIH3T3 cells were transfected with GFP–Plk1 and RFP–H2B, synchronized at G1/S phase by double-thymidine block, and released for 9 hours to G2 phase. Movie images of the cells were then captured. Selected magnified images at different time points were selected to show the dynamics of pericentriolar Plk1. The arrowhead shows the accumulation of pericentriolar Plk1 (area surrounded by white dotted line). See supplementary material Fig. S1D for images representing the full movie. Scale bar: 1 μm.

Both HDAC6 and Plk1 are required for primary cilia resorption, thus we presumed that HDAC6 could be a candidate substrate of Plk1. To assess whether Plk1 facilitates cilia disassembly by activating HDAC6, we immunostained NIH3T3 cells that were released from serum starvation for 0 hour and 18 hours with HDAC6 and acetyl-tubulin antibodies. We observed that HDAC6 localized on the centrioles or the basal body of the cilium, and also the centrosomes when primary cilia were already disassembled (Fig. 2B). Furthermore, HDAC6 co-localized with Plk1 on the centrosome 18 hours after released

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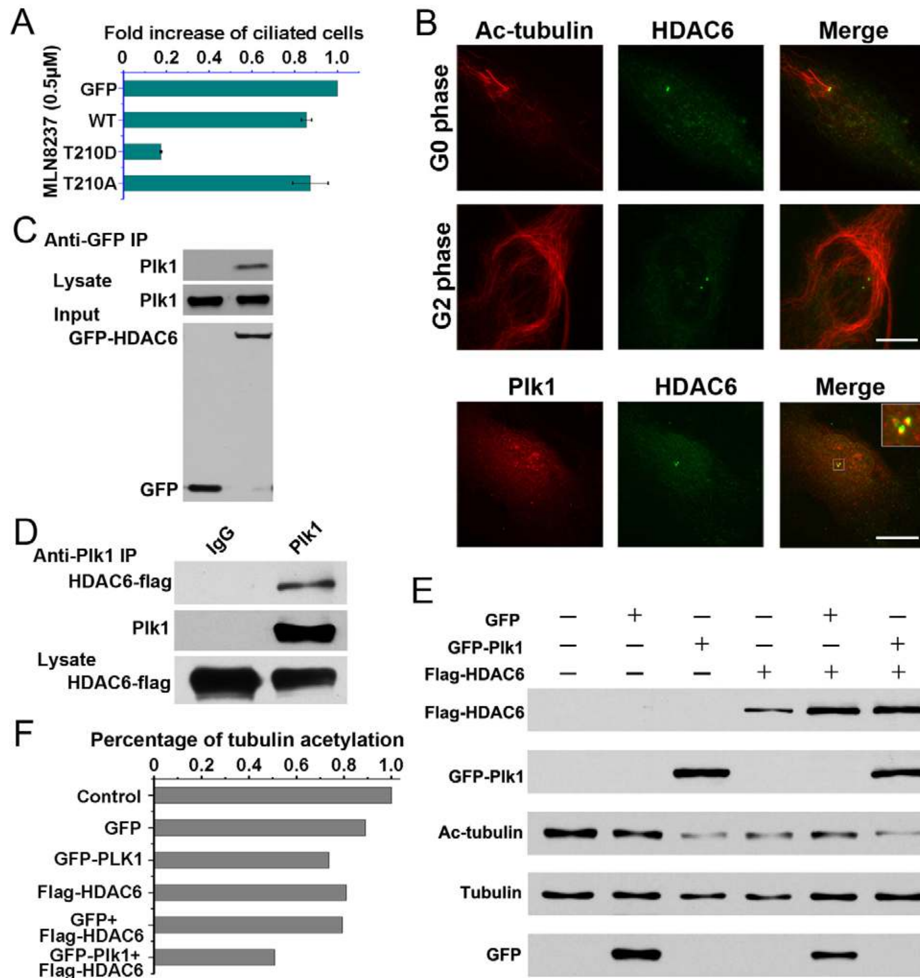

Fig. 2. Plk1 binds to and activates HDAC6 to promote tubulin deacetylation. (**A**) NIH3T3 cells overexpressing GFP, GFP-tagged wild type (WT) Plk1, phosphorylation-mimic Plk1 mutant T210D or nonphosphorylatable Plk1 mutant T210A were released from serum starvation, and treated with 0.5 μM MLN8237 to inhibit Aurora A activity. At 18 hours later, the cells were analyzed by immunofluorescence microscopy, and the percentage of ciliated cells was counted and showed as green bars. Error bars show s.d. (**B**) NIH3T3 cells released from serum starvation for 0 hours (G0) or 18 hours (G2) were co-immunostained with antibodies against HDAC6 and acetyl (Ac)-tubulin. The images show that HDAC6 localized on the basal body of the primary cilium in G0 cells and the centrosomes in G2 cells (top and middle panels). Cells were immunostained with antibodies against HDAC6 and Plk1. The images show that Plk1 co-localizes with HDAC6 on the centrosome (bottom panel). (**C**,**D**) Plk1 interacts with HDAC6. HEK293T cells were transfected with GFP- or Flag-tagged HDAC6 and subjected to immunoprecipitation (IP) assay. The IP complexes were analyzed by western blotting and probed with the indicated antibodies. (**E**) Plk1 stimulates deacetylase activity of HDAC6. HEK293T cells transfected with GFP, GFP–Plk1, Flag–HDAC6, Flag–HDAC6 plus GFP, or Flag–HDAC6 plus GFP–Plk1 were subjected to a tubulin deacetylase assay. The cells were lysed at room temperature for 15 minutes and the deacetylation level of tubulin was analyzed by western blotting. The Plk1 and HDAC6 combination could significantly promote tubulin deacetylation. α-Tubulin was probed as a loading control. (**F**) Quantification of the *in vitro* tubulin deacetylation assay from (**E**) by densitometry. The percentage of tubulin acetylation of the control was taken as 1.0 (i.e. 100%).

from serum starvation (Fig. 2B). Next, we transfected HEK293T cells with GFP- or Flag-tagged HDAC6 and performed immunoprecipitation (IP) assay. The lysate of cells overexpressing GFP–HDAC6 was immunoprecipitated by anti-GFP antibody and the IP complex was then probed with anti-Plk1 antibody. We found that Plk1 could interact with HDAC6 (Fig. 2C) and this interaction was further supported by the co-IP of Flag–HDAC6 and Plk1 (Fig. 2D). Intriguingly, by *in vitro* deacetylation assay (Destaing et al., 2005), we found that Plk1 could stimulate the deacetylase activity of HDAC6 thereby promoting tubulin deacetylation. To access tubulin deacetylation effect, HEK293T cells were transfected with either GFP alone, GFP–Plk1 alone, Flag–HDAC6 alone, Flag–HDAC6 and GFP together, or Flag–HDAC6 and GFP–Plk1 together, respectively. Then, the level of acetylated tubulin of the cells was analyzed by western blotting. Results showed that, compared with the control and GFP alone, GFP–Plk1 or Flag–HDAC6 alone, or GFP together with Flag–HDAC6 could slightly stimulate the deacetylation of tubulin. Remarkably, when both of GFP–Plk1 and Flag–HDAC6 were expressed in cells, the tubulin was dramatically deacetylated (Fig. 2E,F). Taken together, we concluded that the activated Plk1 interacts with HDAC6 to stimulate its deacetylation activity, resulting in the primary cilia resorption. During this process, Plk1 may be regulated by Aurora A and/or coordinates with Aurora-A to promote primary cilia disassembly.

Plk1 is recruited to pericentriolar matrix by PCM1

As described above, along with the resorption of primary cilia, Plk1 accumulated around pericentriolar matrix at G2 phase

before mitotic entry (Fig. 1F; supplementary material Fig. S1B,D). In accordance with previous reports (Kishi et al., 2009; Mahen et al., 2011), we also observed that, during G1 and S phases, a portion of Plk1 concentrated on the centrosome with one or two bright dot-staining style. During G2 to prophase, the centrosomal Plk1 could be divided into two subpopulations: the centriolar Plk1 and the pericentriolar material Plk1 (data not shown). Accordingly, GFP–Plk1 expression followed by time-lapse microscopy also showed that Plk1 accumulated on pericentriolar material during the period from G2 phase to NEBD (nuclear envelope breakdown), whereas the level of centriolar Plk1 kept steady (Fig. 1F; supplementary material Fig. S1D).

The spatial and temporal correlation between cilia disassembly and accumulation of pericentriolar localized Plk1 inspired us to look for the component(s) coupling these two processes. We performed an immunoprecipitation using anti-Plk1 antibody followed by mass spectrometry analysis and found that PCM1 is among the proteins co-immunoprecipitated with Plk1 (data not shown). Plk1 co-localizes with PCM1 around pericentriolar material in G2 phase and through immunoprecipitation assay, we confirmed that both PCM1 and Plk1 interact with each other in cells arrested in mitosis (Fig. 3A,B). Overexpression of C-terminus-deleted PCM1 causes aggregation of not only the mutant proteins but also a subset of its cargo proteins, such as centrin, pericentrin, as well as endogenous PCM1 (Dammermann and Merdes, 2002; Kubo and Tsukita, 2003). In this study, we expressed a C-terminus-deleted PCM1 (GFP–PCM1-ΔC) in the cells and confirmed that this truncated PCM1 was more likely to aggregate in cytoplasm than to localize on centrosome (supplementary material Fig. S3A,B). By co-immunostaining of Plk1 in GFP–PCM1-ΔC expressing cells, we found that the localization of Plk1 was also disrupted by overexpression of the truncated PCM1. Instead, the endogenous Plk1 co-localized with the aggregation of the granules (Fig. 3D). This result suggests that Plk1 interacts with the truncated PCM1. To determine which domain of Plk1 is required for the interaction with the truncated PCM1 in the cells, a number of truncated Plk1 proteins were generated and co-expressed with GFP–PCM1-ΔC (Fig. 3E,F). The results showed that the Plk1 PBD domain, but not the kinase domain, is responsible for the interaction in cytoplasmic aggregates. Considering previous work that demonstrated the need of PBD domain for localization of Plk1 on centrosome (Seong et al., 2002), a function-loss Plk1 PBD mutant PBD2A (H538A, K540A) was generated and co-expressed with GFP–PCM1-ΔC in HeLa cells. As suspected, PBD2A neither localized on centrosomes nor co-localized with GFP–PCM1-ΔC (Fig. 3G). Furthermore, the

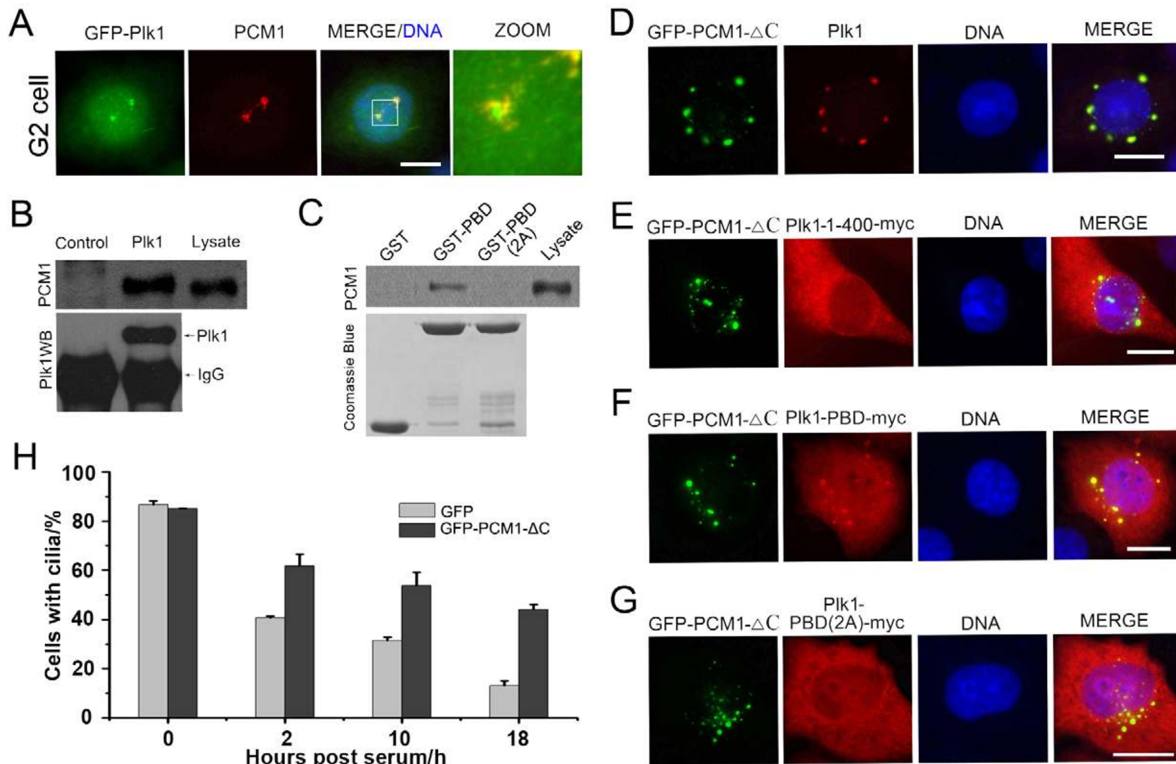

Fig. 3. Plk1 co-localizes and interacts with PCM1. (**A**) Plk1 co-localizes with PCM1 in the pericentriolar matrix of G2 cells. G2 phase HeLa cells expressing GFP–Plk1 were fixed with cold methanol and immunostained with anti-PCM1 antibody. Scale bar: 10 μm. (**B**) The cells arrested in G2/M phase were subjected to immunoprecipitation (IP) experiments with anti-Plk1 antibody and IgG control, and the IP complexes were then analyzed by western blotting with the indicated antibodies. (**C**) Plk1 PBD domain interacts with PCM1. Mitotic HeLa cell lysates were subjected to a GST pull-down assay with GST-, GST–PBD- or GST–PBD2A-coated glutathione–Sepharose 4B for 3 hours at 4℃. The beads were isolated and the proteins bound to the beads were separated by SDS-PAGE and blotted with anti-PCM1 antibody. (**D**–**G**) Cells were transfected with GFP–PCM1-ΔC alone or co-transfected with GFP–PCM1-ΔC and Myc-tagged Plk1 truncates Plk1 1–400, Plk1–PBD or Plk1 PBD (2A), and immunostained with antibodies against Plk1 or Myc. Only endogenous Plk1 (D) and its PBD domain (F) co-localize with GFP–PCM1-ΔC. Scale bars: 10 μm. (**H**) NIH3T3 cells were transfected with GFP or GFP–PCM1-ΔC, and then cultured without serum for 24 hours. Subsequently, we immunostained and counted the percentage of cells with cilia at different time points after serum re-stimulation. Two independent experiments were performed. Error bars show s.d.

mitotic cell lysates were subjected to a pull-down assay using GST–PBD or GST–PBD2A, and PCM1 was specifically co-precipitated with the former one (Fig. 3C). Together, these results indicate that Plk1 is recruited to pericentriolar material by interacting with PCM1 via its PBD.

Since pericentriolar material localized Plk1 is responsible for ciliary disassembly and overexpressed PCM1-ΔC disrupts both of PCM1 and Plk1 PCM localization, we detected the effect of PCM1-ΔC on ciliary resorption. NIH3T3 cells were transfected with GFP or GFP–PCM1-ΔC and cultured without serum for 24 hours. Then, the percentage of cells with cilium was counted at different time points after serum re-stimulation (Fig. 3H; supplementary material Fig. S3D). As expected, we found that, similar to Plk1 RNA interference, GFP–PCM1-ΔC, but not GFP control, could dramatically inhibit ciliary resorption. These data are consistent with the observation that PCM localized active Plk1 promotes primary ciliary resorption, and also suggest that the presence of PCM1 greatly contributes to cilia disassembly.

Phosphorylation of PCM1 by CDK1 is essential for its interaction with Plk1

According to the canonical model, Plk1 binds to its substrates with the assistance of priming kinase (Petronczki et al., 2008). Next, we set to seek for the priming kinase regulating the interaction between PCM1 and Plk1. Lysate of interphase and mitotic HeLa cells synchronized by nocodazole treatment were separated respectively, and immunoblotted with anti-PCM1 antibody. It was observed that the band of PCM1 in mitosis was clearly up-shifted (Fig. 4A). When the mitotic lysate was treated with λ-PPase, the up-shifted band disappeared and a lower band occurred, suggesting that PCM1 was phosphorylated (Fig. 4A). A number of protein kinase inhibitors were introduced into the mitotic cell lysate (data not shown) to determine which one is responsible for the phosphorylation of PCM1. We found that, when RO3306 (9 μM) or roscovitine (10 μM), the inhibitors for CDKs was present (De Azevedo et al., 1997; Vassilev et al., 2006), the lower band of PCM1 occurred again (Fig. 4B and data not shown), suggesting that CDK1 could be the kinase that

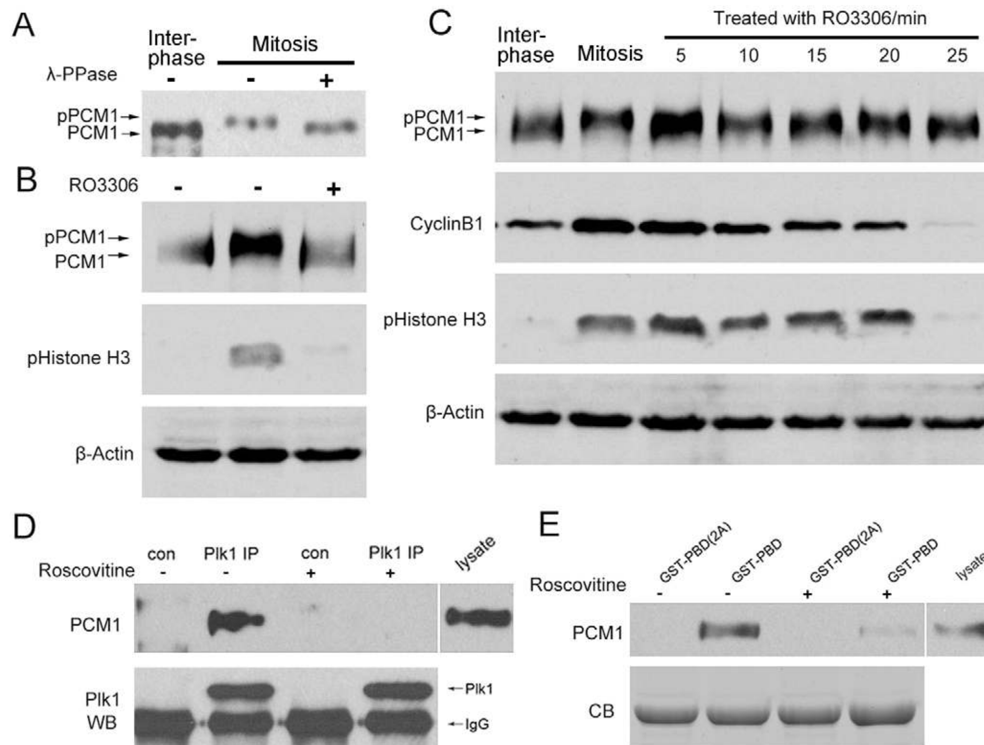

Fig. 4. Binding of PCM1 with Plk1 at mitosis is Cdk1 kinase activity dependent. (**A**) Interphase and mitotic HeLa cell lysates treated with λ-PPase (400 U for 30 minutes at 30℃) or untreated as a control were analyzed by western blotting with anti-PCM1 antibody. Compared with the interphase lysate, the positive band up-shifted in mitosis, and came down in the presence of λ-PPase. (**B**) Interphase and mitotic HeLa cell lysates were analyzed by western blotting with anti-PCM1 antibody. When treated with the CDK1 inhibitor RO3306, the up-shifted band that occurred during mitosis disappeared. Ser10-phosphorylated histone H3 was probed as an indicator of mitosis and β-Actin was probed as a total protein loading control. (**C**) Interphase, mitosis and mitosis-released HeLa cell lysates were analyzed by western blotting with anti-PCM1 antibody. The up-shifted band was shifted down after treatment with 9 μM RO3306 for 25 minutes, a time when the cells exited from metaphase. Cyclin B1 and Ser10-phosphorylated histone H3 were probed as the indicators of the cell cycle phases. β-Actin was probed as the total protein loading control. (**D**,**E**) Inhibition of the kinase activity of CDKs disrupts the PCM1–Plk1 interaction. Mitotic HeLa cell lysates treated with or without CDK kinase inhibitor roscovitine (10 μM) for 1 hour at 37℃ were subjected to immunoprecipitation assay by rabbit anti-Plk1 antibody or rabbit IgG as a control. The IP complexes were analyzed by western blotting with the indicated antibodies (D); or the lysates treated with or without roscovitine were subjected to a GST pull-down assay by incubating with GST-, GST–PBD- and GST–PBD2A-coated glutathione–Sepharose 4B at 4℃ for 3 hours. The beads were isolated and the proteins bound to the beads were analyzed by western blotting with anti-PCM1 antibody. PCM1 could be pulled down by wild type Plk1 PBD domain but not by nonphosphorylatable PBD 2A mutant, and the interaction of PCM1 with Plk1 PBD was disrupted by roscovitine. Coomassie Blue (CB) staining showed the total loading proteins of GST–PBD and GST–PBD2A (E). con, control.

Journal of Cell Science

phosphorylates PCM1 during mitosis. We chose roscovitine to investigate whether the phosphorylation of PCM1 would affect its interaction with Plk1, because RO3306 could dramatically decrease PCM1 phosphorylation level rapidly in about 25 minutes after the treatment (Fig. 4C). We found that the phosphorylated PCM1 was co-immunoprecipitated with Plk1; while in the presence of roscovitine, the interaction of Plk1 with PCM1 was clearly abolished (Fig. 4D). When GST–PBD fusion protein was used in the pull-down assay, similar to the loss-of-function mutant GST–PBD-2A, the capacity of the wild type GST–PBD to pull down PCM1 was largely reduced by the addition of roscovitine (Fig. 4E). These results prompted us to speculate that CDK1 may acts as the priming kinase regulating the PCM1–Plk1 interaction.

Knowing that the interaction between Plk1 and PCM1 may be in a PCM1 phosphorylation-dependent manner, we set to map the phosphorylation site on PCM1 as its phosphorylation is required for the interaction. For this purpose, three PCM1 fragments fused with GFP were constructed and expressed in HeLa cells (Fig. 5A). Fluorescence microscopy revealed that only the middle fragment (F2) of PCM1 co-localized with Plk1 and γ-tubulin at the spindle pole in mitosis, while the other two fragments were dispersed in the cytoplasm (Fig. 5B; supplementary material Fig. S4). We confirmed this with co-IP assay that only the middle fragment of PCM1 could interact with Plk1 (Fig. 5C). By analyzing the amino acid sequence in the middle fragment of PCM1, we noticed that T703, a conserved amino acid from *Gallus* to human, and surrounding residues resemble the binding motif SS/TP for Plk1, and may serve as a binding site for PBD (Fig. 5D). Furthermore, the T703 is also a conserved phosphorylation site of CDK1, suggesting that CDK1 may be the priming kinase that phosphorylates this site and promotes the binding of PCM1 to PBD (Fig. 5D). To verify this speculation, GST-tagged F2 and its non-phosphorylation mutant (T703A) expression vectors were constructed and the proteins were purified for *in vitro* kinase assay. Phosphorylation assay showed that the middle fragment of PCM1 (F2) can be phosphorylated by CDK1 *in vitro* (Fig. 5D). To determine if the phosphorylation of PCM1 at T703 is important for PCM1–Plk1 interaction, we subsequently performed protein binding assays. F2–GFP and F2 (T703A)–GFP mutant were transfected into HeLa cells and the proteins were immunoprecipitated by

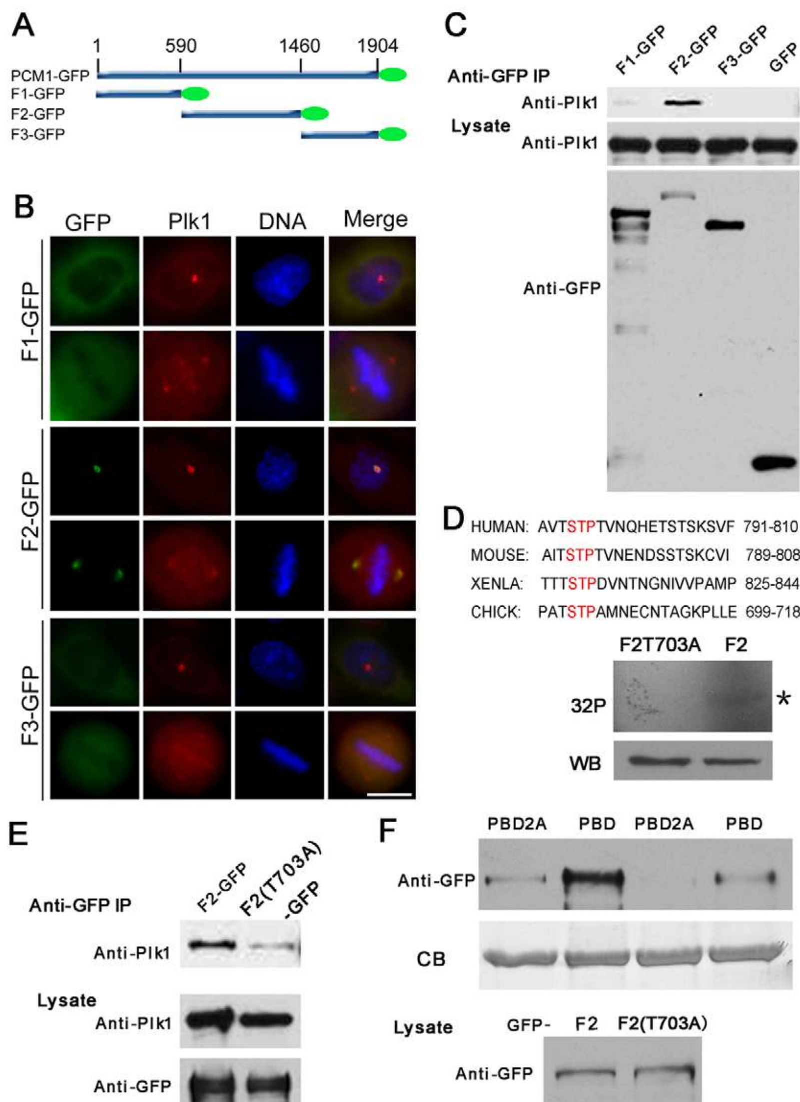

Fig. 5. Phosphorylation of PCM1 T703 by CDK1 is required for the interaction between PCM1 and Plk1. (**A**) Diagram of three truncated mutants of PCM1. F1, F2 and F3 represent amino acids 1–590, 591–1460 and 1461–end, respectively. The truncated mutants were cloned into pEGFP-N3 vector. (**B**) HeLa cells transfected with the truncated mutants showed in A were probed with anti-Plk1 antibody. Among these three PCM1 mutants, only F2 could co-localize with Plk1 on centrosomes, whereas the others dispersed into the cytoplasm. Scale bar: 10 μm. (**C**) HeLa cells transfected with GFP, F1–GFP, F2–GFP, or F3–GFP were lysed and immunoprecipitated (IP) by anti-GFP antibody. The IP proteins were analyzed by western blotting with anti-Plk1 antibody. (**D**) Sequence analysis showing the conserved SS/TP (threonine 703, or T703) motif among the protein sequences of PCM1F2 fragment in chicken, *Xenopus* (XENLA), mouse and human. The purified GST-tagged PCM1F2 and PCM1F2 T703A proteins were subjected to a phosphorylation assay with CDK1 kinase *in vitro* (middle panel). The loading of GST-tagged proteins in this assay was probed by anti-GST antibody (bottom panel). The asterisk marks phosphorylated PCM1F2. WB, western blot.
(**E**,**F**) Phosphorylation of PCM1 T703 is responsible for PCM1–Plk1 interaction. HeLa cell lysates expressing F2–GFP and F2 (T703A)–GFP were subjected to immunoprecipitation and GST pull-down assay. For immunoprecipitation, the lysates were immunoprecipitated by rabbit anti-GFP antibody. IP complexes were analyzed by western blotting with anti-Plk1 antibody (E). For GST pull-down assay, the lysates were incubated with GST–PBD- or GST–PBD2A-coated glutathione–Sepharose 4B. The beads were isolated and the proteins bound to the beads were analyzed by western blotting with anti-GFP antibody. Coomassie Blue (CB) staining showed the total loading of GST–PBD and GST–PBD2A proteins (F).

anti-GFP antibody. The IP complexes were then probed with anti-Plk1 antibody. Result showed that the amount of Plk1 associated with the non-phosphorylation mutant was dramatically reduced (Fig. 5E). To further verify our results, the lysates of the HeLa cells overexpressing F2 and F2 (T703A) were subjected to a pull-down assay by incubating with GST–PBD and GST–PBD2A separately. Similar to the result showed in Fig. 5E, the binding capacity of the F2 (T703A) mutants with the PBD was abolished comparing with the wild type F2 (Fig. 5F). Based on these data, we concluded that the phosphorylation of PCM1 at T703 by CDK1 creates a binding site for Plk1 PBD domain, which is responsible for the interaction between PCM1 and Plk1.

Recruitment of Plk1 in pericentriolar matrix depends on the entirety of PCM1

According to the report that the accumulation of PCM1 on centrosomes requires the entirety of microtubules and dynein-dynactin complex activity (Gurling et al., 2006; Kamiya et al., 2008; Kim et al., 2004), we treated the cells with nocodazole to disrupt microtubule network and followed the localization of both Plk1 and PCM1. We found that along with the impairment of PCM1 on centrosome, the accumulation of Plk1 on the centrosome was abolished (Fig. 6A; supplementary material Fig. S3C). Interestingly, we observed that, although both PCM1 and Plk1 did not become enriched in pericentriolar material in the presence of nocodazole, they could co-localize in random areas of the cytoplasm in a punctate staining manner (Fig. 6A). In addition, we disrupted dynein activity by overexpressing GFP-tagged p-50 dynamitin, which antagonizes dynactin function (Vaughan and Vallee, 1995), would also mislocalize Plk1 (Fig. 6B), coinciding with the dynein-dynactin complex dependent-recruitment of PCM1 to centrosome along microtubule (Dammermann and Merdes, 2002; Kamiya et al., 2008; Kim et al., 2004; Vaughan and Vallee, 1995).

Then, we constructed p-Super vector containing shRNA against PCM1 or irrelevant sequence that were co-transfected into HeLa cells with GFP–H2B, which functioned as a transfection indicator (Chen et al., 2008), to deplete PCM1 in the cells. The depletion efficiency was examined by western blotting and immunostaining (Fig. 6C). In the cells depleted of PCM1, the accumulation of Plk1 around pericentriolar material was blocked, while in the control cells in the same field, the localization of Plk1 was unaffected (Fig. 6D; supplementary material Fig. S5). To eliminate the potential off-target effects, we

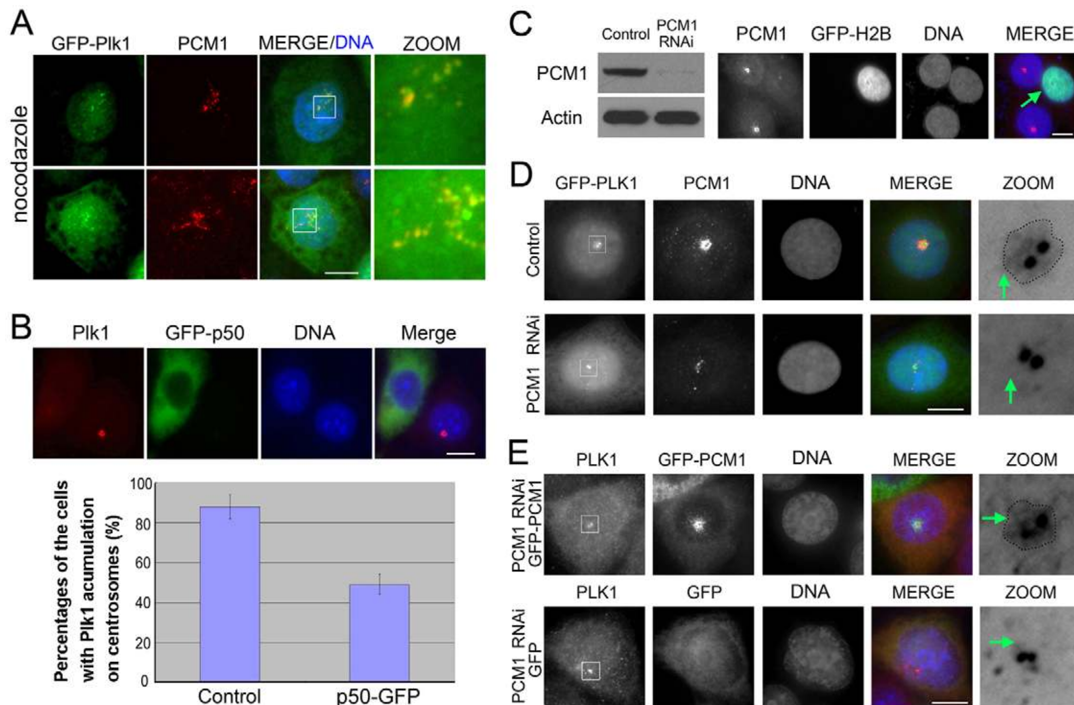

Fig. 6. PCM1 is responsible for pericentriolar accumulation of Plk1. (**A**) Microtubule disassembly disrupts the accumulation of Plk1 and PCM1 on centrosomes. HeLa cells expressing GFP–Plk1 were treated with 1 μM nocodazole for 4 hours, fixed and immunostained with anti-PCM1 antibody. (**B**) The accumulation of Plk1 on centrosomes requires dynein-dynactin complex activity. HeLa cells transfected with p50–GFP were fixed and immunostained with anti-Plk1 antibody (upper panel). Statistical analysis revealed the percentages of the cells with pericentriolar accumulated Plk1 with or without p50 overexpression (lower panel). (**C**) Efficiency of PCM1 RNAi knockdown in HeLa cells. Western blotting showed the efficiency of PCM1 interference. p-Super-PCM1 and GFP–H2B were co-transfected at a ratio of 20:1. Forty hours after transfection, the cells were fixed and immunostained with anti-PCM1 antibody. The immunofluorescence photograph with transfected and untransfected cells (MERGE) was chosen to show that the pericentriolar accumulation of PCM1 was completely disrupted by PCM1 RNAi (indicated by green arrow). (**D**) PCM1 RNAi abolishes pericentriolar accumulation of exogenous GFP–Plk1. HeLa cells expressing GFP–Plk1 were transfected with p-Super (control) or p-Super-PCM1 and immunostained with anti-PCM1 antibody. (**E**) Loss of pericentriolar accumulation of endogenous Plk1 by PCM1 RNAi can be rescued by overexpression of RNAi-resistant exogenous wild type GFP–PCM1 but not GFP. Cells were co-transfected with PCM1 RNAi and RNAi-resistant exogenous wild type GFP–PCM1 or GFP vectors at a ratio of 20:1 and immunostained with anti-Plk1 antibody. Compared with the expressed exogenous GFP control (lower panel), the expressed RNAi-resistant exogenous GFP–PCM1 rescued the defects of Plk1's pericentriolar accumulation (upper panel). Green arrows indicate the pericentriolar areas. Scale bars: 10 μm.

Journal of Cell Science

Journal of Cell Science

co-transfected shRNA against PCM1 with exogenous RNAi-resistant PCM1 and GFP control, respectively, and confirmed that, only exogenous PCM1 but not GFP control could rescue the pericentriolar localization of Plk1 (Fig. 6E). These data suggest that the accumulation of Plk1 on pericentriolar matrix depends on the entirety of PCM1.

Discussion

Primary cilia dynamics and cell cycle regulation seem to be independent yet correlated with each other. We were curious in finding the component(s) that links the two events. Recent work has identified that Aurora A, one of the key regulators of cell proliferation, participates in inducing primary cilia resorption by phosphorylating and activating HDAC6 to deacetylate polymerized tubulin (Hubbert et al., 2002; Pugacheva et al., 2007). We found here that Plk1 kinase activity is also responsible for the process of primary cilia disassembly, as overexpression of active Plk1 in NIH3T3 cells at G0 phase would dramatically reduce the length and percentage of primary cilia, and depletion of Plk1 or inhibition of its kinase activity delays ciliary resorption. Furthermore, Plk1 could bind to and activate HDAC6 to induce its deacetylase activity thereby promote primary cilia disassembly before mitotic entry. All of these evidences imply that Plk1 could negatively regulate the formation of primary cilia.

Plk1 is one of many proteins, including Aurora A, CDK1 and pericentrin, which relocate to and function on the centrosome during the centrosome maturation and the mitotic spindle assembly (Barr et al., 2004; De Luca et al., 2006; Lee and Rhee, 2011). Comparing with the abundant functional studies of centrosomal Plk1, the investigation on how this protein kinase is recruited to centrosome remains scarce; although it was reported that hCenexin1 recruits Plk1 to the centrosome in interphase cells (Soung et al., 2006). As to the function to promote primary cilia disassembly, how is Plk1 relocated onto pericentriolar matrix? We demonstrated here that Plk1 is recruited to the centriolar satellites by PCM1, which has been implicated in the recruitment of many centrosomal proteins such as ninein, centrin, pericentrin, Nek2, etc. (Dammermann and Merdes, 2002; Hames et al., 2005). Moreover, the interaction between PCM1 and Plk1 depends on the kinase activity of CDK1. To determine whether PCM1 is simply one of the substrates or a docking-site for Plk1, we explored RNAi to deplete PCM1 or the known Plk1 substrate Nedd1. As shown by us previously, Nedd1 depletion by RNAi did not affect the recruitment of Plk1 on the pericentriolar material (Zhang et al., 2009). On the contrary, depletion of PCM1 abolished Plk1 in this area. So, the accumulation of Plk1 on centrosome pericentriolar material is the consequence of the recruitment by PCM1 rather than the simple binding of Plk1 to its substrates. It is worthy noticing that PCM1 is not a permanent pericentriolar protein and itself is recruited to centrosome by DISC1-BBS4 protein complex transported by dynein-dynactin complex along with the microtubules (Gurling et al., 2006; Kamiya et al., 2008; Kim et al., 2004). Therefore, the recruitment of Plk1 to the pericentriolar material by PCM1 may be an intermediate step of the centrosome maturation and bipolar spindle assembly, which acts downstream of assembling the scaffold of pericentriolar material and upstream of the recruitment of γ-TuRC for nucleation of microtubules. Unlike Plk1, the depletion of PCM1 would lead to ciliary formation defect (data not shown). Previous work suggests that PCM1 is important in the formation of primary cilia by associating with CEP290 and BBSome during the ciliogenesis (Kim et al., 2008; Nachury et al., 2007), indicating that PCM1 may play dual role in regulating primary cilia dynamics: When cells exit cell cycle and enter quiescence, PCM1 maintains steady state for primary cilia assembly independent of Plk1; whereas in proliferating cells, PCM1 is needed to recruit Plk1 to induce ciliary resorption before mitotic entry. During the preparation of this manuscript, two reports were published online and showed that the kinase activity of Plk1 is required for primary cilia disassembly (Lee et al., 2012; Seeger-Nukpezah et al., 2012). Lee and co-workers showed that Plk1 interacts with Dishevelled 2 (Dvl2) and promotes cilia disassembly; and Schermer and co-workers showed that Plk1 induces the phosphorylation of nephrocystin-1. Taken together, our results suggest a mechanism regulating primary cilia disassembly before mitotic entry. During the process, Plk1 is recruited to pericentriolar matrix by CDK1 phosphorylated PCM1 and then activated by Aurora A. The activated pericentriolar Plk1 in turn induces primary cilia disassembly by interacting with and activating HDAC6.

Materials and Methods

Cell culture, transfection and synchronization

HeLa, HEK293T and NIH3T3 cells were cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin and 100 µg/ml streptomycin at 37℃ at 5% CO_2 atmosphere. For immunofluorescence, cells were planted on 22 mm No.1 glass coverslips one day before use, and grew overnight to 80%∼90% confluence. For transfection, the cells were grown on coverslips in 35-mm diameter culture dishes to about 80% confluence and transfected with the indicated plasmids utilizing Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. As the indicator of shRNA transfection, the RFP–H2B or GFP–H2B and targeting plasmids were co-transfected into cells with the ratio of 1:20 as described previously (Chen et al., 2008). With the treatment of double thymidine (2.5 mM, Sigma), cells were arrested in G1/S phase transition. To obtain mitosis-arrested cells, 100 ng/ml nocodazole (Sigma) were added into the medium after about 5 hours released from the thymidine block. The inhibitor B12536 was purchased from Axon Medchem, MLN8237 from Selleck, RO3306 from Calbiochem and roscovitine from Sigma.

Plasmid construction and antibody preparation

Human Plk1 gene and antibody were obtained as described previously (Zhang et al., 2009), and the Plk1K82R (kinase dead mutant), Plk1T210D (constitutively active form of Plk1) (Lee and Erikson, 1997), Plk1T210A, Plk12A (H538A/K540A), Plk1 (1–400), Plk1PBD (358-end) and Plk1PBD2A (358-end, H538A/K540A) were then cloned into p-EGFPC2, p-CMV-myc, pET28a or pGEX-4T-1 vectors. After purifying the His-tagged Plk1 protein, we immunized rabbit with the recombinant protein to obtain the polyclonal antibody against Plk1. The mouse polyclonal Plk1 antibody was purchased from Millipore. The gene and antibody of PCM1 were gifts from Dr Merdes (Dammermann and Merdes, 2002), and we also raised the polyclonal antibody against human PCM1 by immunizing mouse with PCM1 C-terminal fragment (1665–2024). Both antibodies were powerful tools for immunostaining and western blotting assay. The truncated and point mutation mutants of PCM1, including PCM1-ΔC, PCM1-F1, PCM1-F2, PCM1-F3 and PCM1-F2T703A, were subcloned in a way like the construction of wild type Plk1 expression construct. The full-length *PCNTB*, *Plk^{CA}-PCNTB*, *Plk1^{KD}-PCNTB* cDNA were gifts from Kunsoo Rhee (Department of Biological Sciences, Seoul National University, Seoul, South Korea). Mouse monoclonal antibodies against γ-tubulin, Myc, GFP, Flag, α-tubulin and acetyl-tubulin were purchased from Sigma. Rabbit anti-HDAC6 was from Abgent. Rabbit anti-cyclin B1 and PCM1 were from Santa Cruz and both rabbit anti-Histone H3 (p-10Ser) and anti-β-Actin antibodies were from Sigma. All animal experiments were performed according to approved guidelines.

Immunofluorescence microscopy

Cells on coverslips were briefly washed in PBS, and fixed with 4% paraformaldehyde for 15 minutes at room temperature. After being washed in PBS three times, cells were permeabilized by 0.2% Triton X-100 for 5 minutes on ice, and incubated with primary antibodies (diluted in PBS containing 3% BSA) at 4℃ overnight. The cells were then washed in PBS three times and incubated with secondary antibodies for 1 hour at room temperature. For primary cilia immunofluorescence, the cells were fixed and permeabilized with methanol at −20℃ directly before being incubated with the antibodies. Coverslips were

Journal of Cell Science

mounted with Mowiol added with 1 μg/ml DAPI for DNA staining, and analyzed with standard FITC and TRITC, rhodamine and DAPI filter sets on a ZEISS 200 M immunofluorescence microscope. Images were captured with cool CCD and Axvert image software.

GST fusion protein pull-down assay and immunoprecipitation

HeLa cells were harvested and lysed in 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×cocktail) for 30 minutes on ice and centrifuged at 15,000 g for 15 minutes to obtain the supernatant as described previously (Zhang et al., 2009). GST and GST fusion proteins were expressed in *E. coli* strain BL21 and purified using glutathione–Sepharose 4B beads (GE Healthcare) by following the manufacturer's protocol. For GST fusion protein pull-down assay, 5 μg of soluble GST or GST fused proteins and 15 μl glutathione–Sepharose 4B beads were incubated with HeLa cell lysates at 4℃ for 3 hours. The beads were washed five times with lysis buffer before being collected by brief centrifugation and then suspended in gel sample buffer. For immunoprecipitation assay, the supernatant was incubated with the indicated antibodies for 1 hour at 4℃. Fifteen microliters of protein A or G Sepharose (GE Healthcare) were added and the mixtures were rotated at 4℃ for 2 hours. After incubation, the beads were washed and harvested and suspended in gel sample buffer. Finally, results of both the assays were analyzed by western blotting.

In vitro protein phosphorylation assay and tubulin deacetylase assay

For *in vitro* protein phosphorylation assay, 2 μg of GST-tagged PCM1F2 and F2T703A proteins were incubated with 200 ng CDK1 (New England Biolabs) 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, Amersham) at 30℃ for 30 minutes. Reactions were stopped by adding gel sample buffer, and samples were analyzed by SDS-PAGE and subsequently autoradiography (Zhang et al., 2009).

For *in vitro* tubulin deacetylase assay, HEK293T cells were transfected with 1 μg Flag–HDAC6 and/or GFP–Plk1 plasmids, and the subsequent assay was done as described by Destaing and colleagues (Destaing et al., 2005).

Gel electrophoresis and immunoblotting

After resolved on 10% SDS-PAGE, the protein samples were transferred onto nitrocellulose filters in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 1 hour at 100 V (while 350 mA as long as 3 hours for PCM1), the filter was blocked in TTBS (20 mM Tris-HCl [pH 7.4], 500 mM NaCl and 0.3% Tween 20) containing 3% non-fat milk at room temperature for 1 hour, and then probed with antibody (diluted 1:1,000 in TTBS with 3% nonfat milk) overnight at 4℃. The membranes were then blotted with horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson, diluted 1:5,000 in TTBS with 3% nonfat milk) at room temperature for 1 hour. After final wash in TTBS, the filter was developed for visualization by enhanced chemiluminescence and X-ray films.

RNA interference

The sequence of the oligonucleotide against PCM1 was 5′-UCAGCUUCGUGAUUCUCAG-3′; and the sequence against Plk1 was 5′-CGGCACCGUGCAGAUUAACUU-3′. The oligonucleotides were synthesized by Sunbio Company, then annealed and ligated into p-Super retro vector. The positive clones were sequenced by Invitrogen Company. The p-Super-PCM1 and p-Super-Plk1 were transfected into HeLa or NIH3T3 cells with Lipofectamine 2000 (Invitrogen).

Live cell imaging

NIH3T3 cells transfected with GFP–Plk1 and RFP–H2B were used for live cell imaging. Cells were transfected with GFP–Plk1 and RFP–H2B for 24 hours and the culture dish was placed onto a heated sample stage within a heated chamber (37℃). Live-imaging was performed using a DeltaVision live cell imaging system (Applied Precision) equipped with an Olympus IX-71 inverted microscope and a 100×/1.40 oil objective. Images were captured with 200 ms exposure times in 10-minute intervals by a CoolSnap HQ2 CCD camera, and different Z sections then were projected by SoftWorx suite.

Acknowledgements

We thank Dr Andreas Merdes for *PCM1* cDNA and antibody. We also thank Dr. Kunsoo Rhee for providing us with *PCNTB*, *Plk^{CA}-PCNTB* and *Plk1^{KD}-PCNTB* cDNA. We acknowledge other members of this laboratory for helpful comments and critical discussion of the paper prior to submission.

Author contributions

C.Z., G.W. and Q.C. conceived the project and designed experiments. G.W. and Q.C. performed experiments. G.W., Q.C., X. Zhang, B.Z., X. Zhuo, J.L., Q.J. and C.Z analyzed the data. C.Z., Q.J. and G.W. wrote the manuscript.

Funding

This work was supported by the State Key Basic Research and Development Plan of Ministry of Science and Technology of China [grant number 2010CB833705 to C.Z.]; and the National Natural Science Foundation of China [grant numbers 30900726, 31030044 and 90913021 to C.Z.].

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.114918/-/DC1

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Journal of Cell Science

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