Current Biology 21, 1145–1151, July 12, 2011 ©2011 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2011.05.047

Report

Plk1 Controls the Nek2A-PP1γ Antagonism in Centrosome Disjunction

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Summary

In human cells, separation of the centrosomes and formation of a bipolar spindle are essential for correct chromosome segregation [1]. During interphase, centrosomes are joined together by the linker proteins C-Nap1 and rootletin [2–4]. At the onset of mitosis, these linker proteins are phosphorylated and displaced from centrosomes by the Nek2A kinase, which is regulated by two Hippo pathway components, Mst2 kinase and the scaffold protein hSav1. The kinesin-5 motor protein Eg5 promotes centrosome separation in a parallel pathway to Nek2A [5]. Here, we report that Polo-like kinase 1 (Plk1) functions upstream of the Mst2-Nek2A kinase module in centrosome disjunction as well as being important for Eg5 localization at centrosomes. Plk1 regulates Mst2-Nek2A-induced centrosome disjunction by phosphorylating Mst2. The absence of Plk1 phosphorylation of Mst2 promotes assembly of Nek2A-PP1γ-Mst2 complexes, in which PP1γ counteracts Nek2A kinase activity. In contrast, Plk1 phosphorylation of Mst2 prevents PP1γ binding to Mst2-Nek2A, allowing Nek2A activity to promote centrosome disjunction. We propose that centrosome disjunction is regulated by Plk1, providing a well-balanced control between the counteracting Nek2A and PP1γ activities on the centrosome linker.

Results and Discussion

Plk1 and Aurora A but Not Cdk1 Kinase Activity Are Essential for Centrosome Disjunction

Two distinct pathways regulate the separation of mammalian centrosomes in mitosis. The NIMA-related kinase Nek2A, regulated by the Hippo pathway components Mst2 and hSav1, triggers the disassembly of the linker proteins C-Nap1 and rootletin by phosphorylation, which is known as disjunction [2, 3, 6, 7]. Kinesin-5 (human Eg5) motor activity functions in parallel to Nek2A in centrosome separation [8, 9]. Eg5 is even able to sever the linker in the absence of the Mst2-hSav1-Nek2A pathway as long as motor activity is sufficiently high [5].

Regulation of centrosome disjunction and separation by the cell-cycle machinery is largely unclear. Cdk1 (cyclin-dependent kinase 1), Plk1, and Aurora A are mitotic kinases that have been implicated in centrosome disjunction in different organisms [10–15]. We evaluated the contribution of these kinases to centrosome disjunction using small-molecule inhibitors (Plk1 [BI2536], Aurora A [VX-680], or Cdk1 [RO3306] [11, 16, 17]). Human retinal pigment epithelial (RPE-1) cells expressing centrin-GFP were treated as outlined in Figure 1A. Cdk1-inhibited cells remained in mitosis upon MG132 addition, in contrast to a previous study applying complete inhibition of all Cdks [18]. Inhibition of Cdk1 activity in prometaphase did not affect centrosome disjunction compared to untreated control cells (Figure 1B; Cdk1i versus UT). In contrast, inhibition of Plk1 or Aurora A (Figure 1B; Plk1i and AurAi) reduced the distance between the two centrosomes (Figure 1C) and maintained C-Nap1 and rootletin at centrosomes (Figures 1D and 1E). Notably, Plk1 inhibition was more efficient than Aurora A inhibition in maintaining centrosomal C-Nap1 and rootletin, despite the nearly equal centrosome distance (Figures 1C–1E). The residual amount of linker proteins in Aurora A-inhibited cells was probably sufficient to join the two centrosomes together. We could confirm the role of Plk1 in centrosome disjunction using RPE-1-eGFP-Plk1^{as} cells [19]. In these cells, the endogenous *PLK1* gene has been replaced by a genetically modified allele (eGFP-Plk1^{as}), whose ATP-binding pocket can accommodate bulky purine analogs, which do not inhibit wild-type kinases (see Figures S1A and S1B available online).

The failure to disassemble the C-Nap1/rootletin linker suggests that the Mst2-Nek2A centrosome disjunction pathway is not functional in cells without Plk1 and Aurora A kinase activity. Therefore, we monitored C-Nap1 phosphorylation by Nek2A using phosphospecific C-Nap1-Ser2417/2421 antibodies [5]. Phosphorylation of C-Nap1-Ser2417/2421 was drastically reduced in cells that were treated with the Plk1 inhibitor (Figure 1F). Aurora A inhibition was less efficient than Plk1 inhibition in reducing C-Nap1 phosphorylation (Figure 1F). This may reflect the fact that Aurora A kinase merely stimulates Plk1 kinase activity by phosphorylating Thr210 in the T loop of Plk1 [20, 21]. Accordingly, inhibition of both kinases did not significantly increase the localization of C-Nap1 and rootletin at centrosomes compared to inhibition of only Plk1 (Figures S1C–S1E). These data are consistent with the notion that Aurora A and Plk1 function in a linear pathway where Aurora A phosphorylates and activates Plk1 [20, 21].

We next asked whether centrosomes remain joined together in cells without Plk1 activity because of a persistent C-Nap1 linker. We treated RPE-1-centrin-GFP cells with the Plk1 inhibitor BI2536 and depleted the linker protein C-Nap1 by small interfering RNA (siRNA). In control siRNA-treated cells (Figure 1G, UT) that were treated with Plk1 inhibitor, centrosome disjunction failed. In contrast, C-Nap1-depleted cells disjoined the centrosomes despite Plk1 inhibition (Figures 1G and 1H). These data suggest a function of Plk1 in dissolving the centrosomal linker.

Plk1 Is Essential for Directing the Kinesin-5 Motor Eg5 to Centrosomes

Centrosome separation is completely blocked only when both the Mst2-Nek2A kinase and Eg5-dependent motor pathways [5] are inactive. Because cells without Plk1 activity arrested in prometaphase with adjacent centrosomes that were still connected by the C-Nap1/rootletin linker (Figures S1F and S1G), we tested whether Plk1 inhibition also affects Eg5 localization. Eg5 is dispersed throughout the cytoplasm in interphase cells but begins to accumulate at centrosomes in

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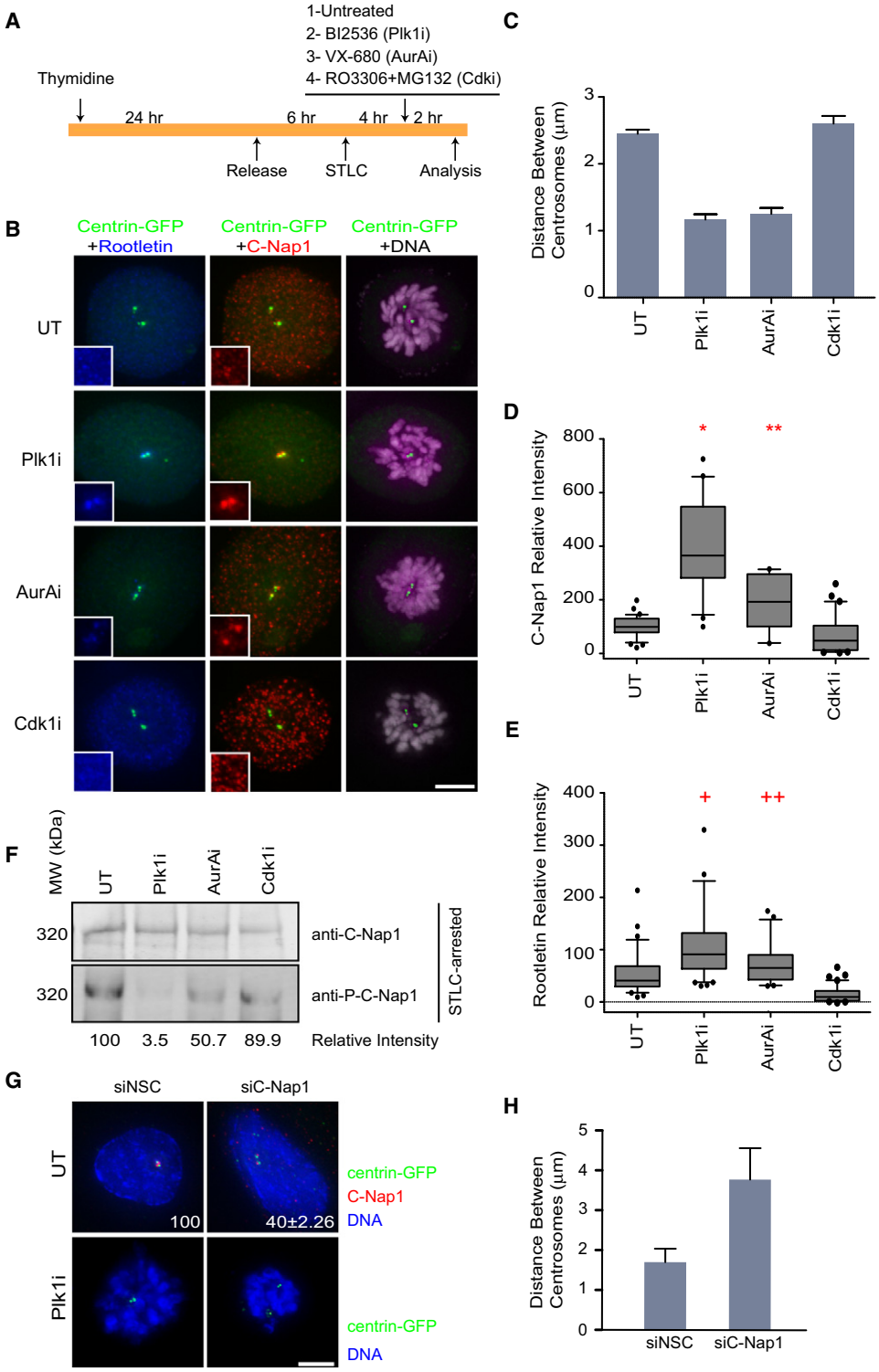

Figure 1. Inhibition of Plk1 Prevents Centrosome Disjunction by Affecting Phosphorylation of the Centrosomal Linker Protein C-Nap1 in Early Mitosis

(A) Schematic outline of experiment in (B)–(F). RPE-1-centrin-GFP cells were enriched in G2 by a single thymidine block/release and treated with the indicated inhibitors together with STLC to trap cells in prometaphase. To prevent Cdk1-inhibited cells from exiting mitosis, we added the proteasome inhibitor MG132.

(B) Cells were fixed and stained with C-Nap1 and rootletin antibodies. Scale bar represents 5 μm.

(C) Distances between the two centrosomes of cells in (B). Results are from three independent experiments; 20 cells were analyzed for each condition. Data are mean ± standard error of the mean (SEM).

(D and E) Intensity of C-Nap1 (D) and rootletin (E) signals at centrosomes from cells in (B) was quantified and corrected to centrin-GFP. Results are from two independent experiments. n > 20 cells were analyzed for each condition (*p < 0.0001, UT/Plk1i; **p < 0.0001, UT/AurAi; +p = 0.0009, UT/Plk1i; ++p = 0.01, UT/AurAi).

prophase [22]. Eg5 accumulation at centrosomes was dramatically decreased by inhibition of Plk1 (Figures 2A and 2B; Figure S2A) without significantly affecting the total amount of Eg5 (Figure 2C). Similar results were obtained with RPE-1-eGFP- Plk1^{as} cells treated with 3MB-PP1 (Figure S2B). Thus, we concluded that Plk1 is required for the targeting of Eg5 to centrosomes.

In human cells, it is unclear how Eg5 is targeted to spindle poles. We found no evidence for involvement of Cdk1 in this process (Figure S2C). On the other hand, in cells treated with low concentrations of nocodazole, Eg5 localized to remnants of microtubules, whereas in cells with completely depolymerized microtubules, Eg5 was dispersed throughout the cytoplasm (Figure S2D). This demonstrates the requirement of intact microtubules for centrosomal Eg5 localization. Plk1 inhibition might cause Eg5 to be trapped on microtubules that then prevents localization to the poles. Alternatively, because Plk1 inhibition causes a decrease in microtubule nucleation capacity of the spindle poles [11], Eg5 localization might be indirectly affected. Although the molecular mechanism of centrosomal targeting of Eg5 by Plk1 remains elusive, it confirms the pivotal role of Plk1 for centrosome separation.

Plk1 Functions Upstream of the Mst2-Nek2A Module

The results presented above suggest that Plk1 functions upstream of C-Nap1 in the Mst2-Nek2 centrosome disjunction pathway. We next tested on which level Plk1 regulates the Mst2-Nek2 kinase module. Mst2 phosphorylates Nek2A on four residues. When overexpressed, the phosphomimicking Nek2A-4D (serine-to-aspartate mutant) is able to bypass the requirement of Mst2 in centrosome disjunction [5]. If Plk1 is acting upstream of Mst2, then Nek2A-4D should bypass the need for Plk1 in centrosome disjunction. To test this, we overexpressed Nek2A-WT, Nek2A-4A (serine-to-alanine mutant), and Nek2A-4D in cells that were incubated with or without the Plk1 inhibitor. In the absence of Plk1 inhibition, Nek2A-WT and Nek2A-4D, but not Nek2A-4A, were able to initiate premature centrosome separation [5] (Figures 2D and 2E). In striking contrast, we observed that in cells without Plk1 activity, Nek2A-WT overexpression did not initiate centrosome separation, whereas the Nek2A-4D mutant was efficient in this process (Figures 2D and 2E). Consistently, overexpression of Nek2A-4D allowed hyperphosphorylation of C-Nap1 in Plk1-inhibited cells (Figure 2F). These data suggest that Plk1 acts upstream of Mst2-Nek2A module in centrosome disjunction.

Plk1 Inhibits PP1γ

Next, we tested whether Plk1 directly regulates Nek2A kinase. However, Plk1 inhibition did not change localization or activity of Nek2A (Figures S3A–S3C). An alternative possibility is that Plk1 inhibition induces upregulation of PP1γ phosphatase. PP1γ was previously found in complex with Nek2A and shown to counteract the ability of Nek2A to phosphorylate C-Nap1 in vitro [23]. Supporting this hypothesis, depletion of PP1γ by siRNA rescued the centrosome disjunction defect and allowed efficient displacement of C-Nap1 in cells upon Plk1 inhibition (Figures 2G–2I). Similar results were obtained with protein phosphatase inhibitors (Figures S3D and S3E). Hence, we suggest that the ability of PP1γ to counteract Nek2A is inhibited by Plk1. Different isoforms of PP1 (PP1α and PP1γ) have been proposed to antagonize Nek2A [23, 24]. Although we cannot rule out a possible additive regulation of PP1α on Nek2A, we did not observe changes in PP1α levels when we depleted PP1γ (data not shown), suggesting that PP1γ is the major PP1 isoform that counteracts Nek2A.

Plk1 Selectively Binds and Phosphorylates Mst2

To understand how Plk1 regulates Mst2-hSav1-Nek2A via PP1γ, we performed in vitro kinase assays using recombinant proteins (Figure S3F). We found that among all of the proteins tested, Plk1 only phosphorylated Mst2 (Figure 3A). In addition, in vitro binding assays using recombinant proteins revealed that Plk1 bound directly to Mst2, but not to hSav1 or Nek2A (Figure 3B). This specific binding was also observed in immunoprecipitation experiments (Figure 3C; Figures S3G and S3H). Thus, Plk1 interacts with and phosphorylates Mst2.

Using mass spectrometry analysis of Mst2-KD incubated with recombinant Plk1 in the presence of ATP, we determined three sites that corresponded to the consensus sequence of Plk1, all of which were recently identified by in vivo phosphoproteome analyses [25–27] (Figure 3D).

Plk1 Regulates Binding of PP1γ to Nek2A via Mst2 Phosphorylation

To analyze the functional significance of Mst2 phosphorylation by Plk1, we generated nonphosphorylatable Mst2^{S15A,S18A,S316A} (Mst2-3A) and the corresponding phosphomimicking Mst2^{S15D,S18D,S316D} (Mst2-3D) mutants. We then investigated the formation of complexes between Mst2, Nek2A, and PP1γ. We cotransfected cells with Mst2, Mst2-3A, or Mst2-3D together with Nek2A-WT-GFP; immunoprecipitated Nek2A-GFP; and analyzed bound endogenous PP1γ. Additionally, in one set of HA-Mst2-WT-transfected cells, we inhibited Plk1 with BI2536. Interestingly, when cells either were treated with Plk1 inhibitor or expressed Mst2-3A, we observed a consistent increase in PP1γ and Mst2 binding to Nek2A compared to cells expressing Mst2 or Mst2-3D (Figure 4A). This finding suggests that lack of Plk1 phosphorylation on Mst2 increases complex formation between Mst2, Nek2A, and PP1γ.

We confirmed the regulation of complex formation between Nek2A and PP1γ by Plk1 with an in vitro binding experiment. We failed to identify interaction between Mst2 and PP1γ, whereas a strong interaction was observed between Nek2A and PP1γ (Figure S4A) [23]. Next, we asked whether Mst2 regulates the Nek2A-PP1γ interaction. Recombinant His-tagged Mst2-KD kinase that was in part phosphorylated by endogenous Plk1 was purified from insect cells. To obtain defined phosphorylated and nonphosphorylated conditions, we dephosphorylated Mst2-KD with λ-phosphatase (Figure 4B, lane 4) or preincubated Mst2-KD with WT Plk1 (Figure 4B, lane 6) to hyperphosphorylate the protein. We incubated GST-PP1γ-coupled beads together with His-tagged

(F) Extracts of untreated (UT) and RO3306 (Cdk1i)+MG132-, BI2536 (Plk1i)-, and VX-680 (AurAi)-treated RPE-1-centrin-GFP cells were analyzed by immunoblotting using C-Nap1 and C-Nap1-Ser2417/2421 antibodies.

(G) RPE-1-centrin-GFP cells were treated with unspecific control (siNSC) or C-Nap1 siRNA oligos (siC-Nap1). Top: fixed cells were stained with C-Nap1 antibodies to show C-Nap1 depletion. Numbers indicate the level of C-Nap1 depletion in interphase cells. Data are the average of 20 cells ± SEM. Bottom: cells treated with siC-Nap1 or siNSC siRNA oligos were incubated with the Plk1 inhibitor BI2536 (Plk1i). Scale bar represents 5 μm.

(H) Distances between the two centrosomes of cells in (G). Results are from two independent experiments; 20 cells were analyzed for each condition. Data are mean ± SEM.

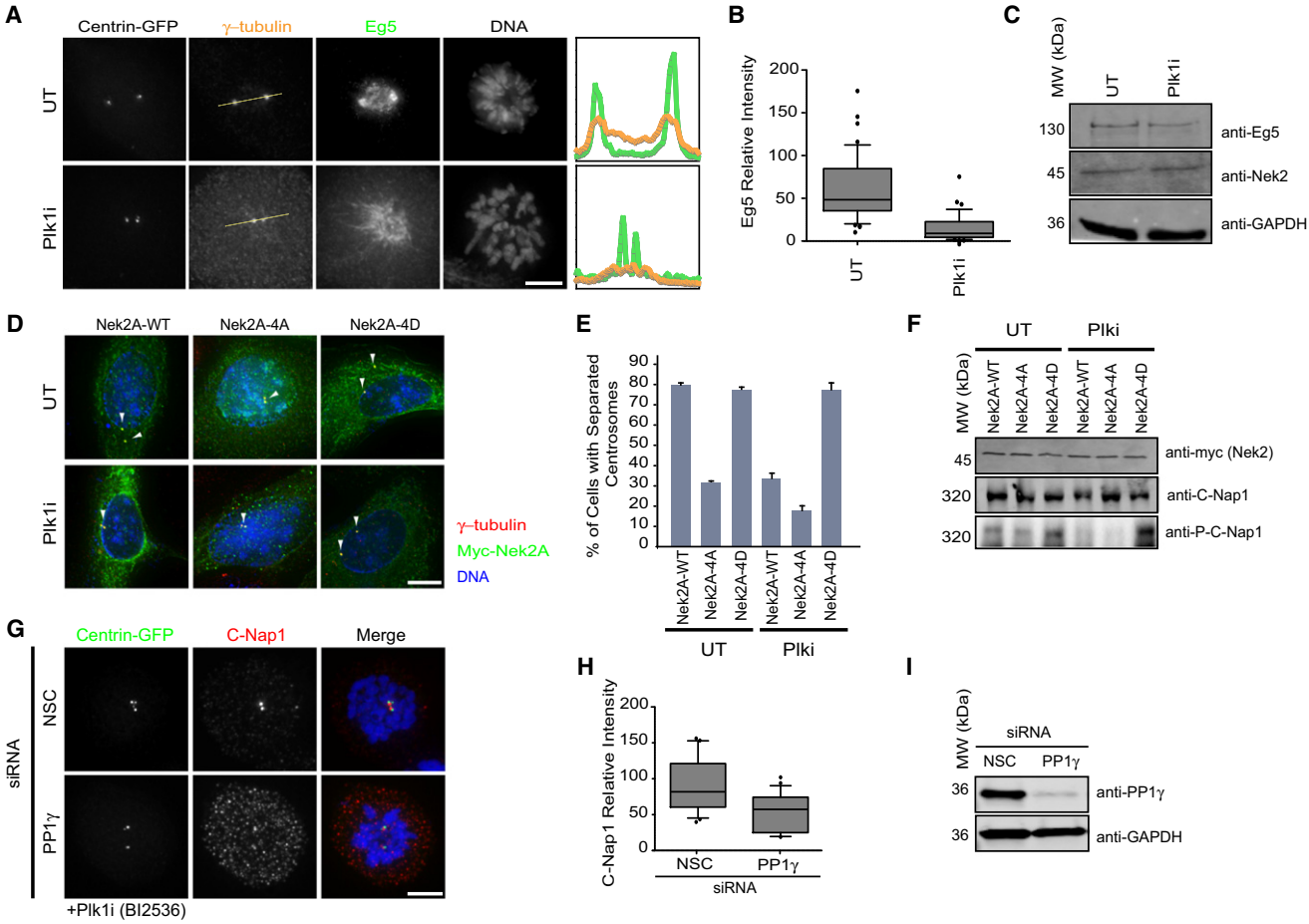

Figure 2. Plk1 Functions Upstream of the Eg5 and the Mst2-Nek2A Kinase Pathways

(A) RPE-1-centrin-GFP cells were arrested in prometaphase with BI2536 (Plk1i) or kept untreated (UT). Cells were fixed and stained with Eg5 and γ-tubulin antibodies. Right: line scans of γ-tubulin (green) and Eg5 signals (orange) along the line shown in the γ-tubulin images. Scale bar represents 5 μm.

(B) Centrosomal association of Eg5 was quantified and corrected for corresponding centrin-GFP signals from cells in (A). Results are from two independent experiments; n > 20 cells were analyzed for each condition ($p < 0.0001$, UT/Plk1i).

(C) Extracts of cells treated as in (A) were analyzed by immunoblotting.

(D) Myc-tagged Nek2A (-WT, -4A, or -4D) constructs were transfected into U2OS cells, and cells were treated with (Plk1i) or without (UT) Plk1 inhibitor. Cells were stained with myc and γ-tubulin antibodies. Arrows highlight centrosomes. Scale bar represents 10 μm.

(E) Distances between the two centrosomes of cells in (D). Centrosome distance > 2 μm was counted as separated. Results are from three independent experiments; n > 20 cells were analyzed for each condition. Data are mean ± SEM.

(F) Extracts of cells treated as in (D) were analyzed by immunoblotting.

(G) RPE-1-centrin-GFP cells were treated with siRNA oligos against PP1γ, synchronized in prometaphase, and treated with BI2536. Fixed cells were stained with C-Nap1 antibodies. Scale bar represents 5 μm.

(H) Centrosomal association of C-Nap1 of cells in (G) was quantified and corrected for centrin-GFP. Results are from two independent experiments; n > 20 cells were analyzed for each condition ($p = 0.0002$, NSC/PP1γ).

(I) Extracts of cells treated as in (G) were analyzed by immunoblotting.

Nek2A-KD in the presence (Figure 4B, lanes 3–7) and absence (Figure 4B, lane 2) of His-Mst2-KD and determined the amount of bound versus unbound His-tagged proteins on the GST-PP1γ-coupled beads. In agreement with the immunoprecipitation experiment (Figure 4A), we observed a strong increase in Nek2A-KD binding to PP1γ when Mst2-KD was dephosphorylated (Figure 4B, compare lanes 4 and 6). Taking these data together, we propose that phosphorylation of Mst2 by Plk1 regulates Mst2-PP1γ-Nek2A complex formation.

Counteracting Activities of Nek2 and PP1γ Regulate C-Nap1 Phosphorylation

From the experiments described above, three scenarios were plausible. PP1γ in the Mst2-PP1γ-Nek2A complex could counteract phosphorylation of either Mst2 by Plk1 or Nek2A by Mst2. Alternatively, PP1γ might reduce phosphorylation of the linker protein C-Nap1 by competing with Nek2A. In order to test the first two possibilities, we established an in vitro system where we could study Mst2 phosphorylation and activity. We preincubated Mst2-WT with either Plk1-KD or Plk1-WT in the presence of ATP and then used Nek2A-KD or Nek2A-KD-PP1γ as substrates together with [γ-^{32}P]ATP. As reported [28], Mst2 showed autophosphorylation, and the presence of Plk1 further increased the incorporation of [γ-^{32}P]ATP into Mst2 (Figure 4C, lanes 3, 4, 7, and 8). The level of Mst2 phosphorylation was unaffected by the presence of PP1γ, suggesting that Mst2 is not a substrate of the phosphatase. Because Plk1 only phosphorylated Mst2 in these

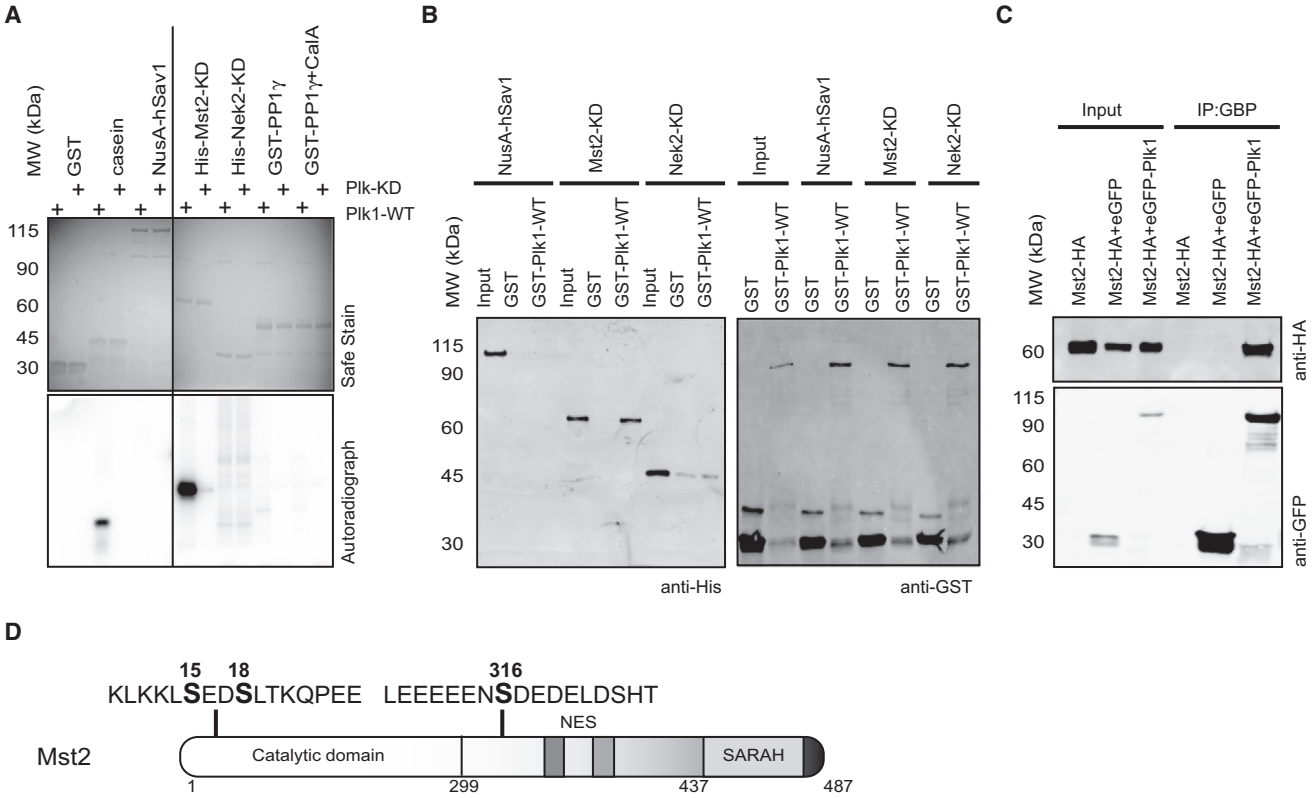

Figure 3. Plk1 Selectively Phosphorylates Mst2 Kinase

(A) Recombinant proteins were incubated with wild-type (WT) or kinase-dead (KD) versions of Plk1 in the presence of [γ-^{32}P]ATP. Proteins were separated by SDS-PAGE and analyzed by Safe Stain and autoradiography.

(B) Purified recombinant GST or GST-Plk1 was incubated with recombinant His-tagged Mst2-KD, hSav1, or Nek2A-KD. GST-tagged proteins were precipitated with glutathione Sepharose beads, and bound proteins were analyzed by immunoblotting.

(C) HA-Mst2 together with GFP or GFP-Plk1 was coexpressed in HEK293 cells. GFP-tagged proteins were immunoprecipitated with GFP-binder protein (GBP)-coupled NHS-activated Sepharose beads. Coimmunoprecipitation of HA-tagged Mst2 was determined by immunoblotting.

(D) Plk1 phosphorylation sites on Mst2 identified by liquid chromatography-tandem mass spectrometry analysis.

reactions (Figure S4B), Nek2A-KD phosphorylation is a reflection of Mst2 activity. Importantly, Nek2A phosphorylation was not reduced by the addition of PP1γ (Figure 4C, lanes 2, 4, 6, and 8), suggesting that PP1γ does not act on Nek2A. In summary, PP1γ in the Mst1-PP1γ-Nek2A complex regulates neither Mst2 nor Nek2A but instead may constantly dephosphorylate substrates of Nek2A.

To test this possibility, we followed C-Nap1 phosphorylation. For this experiment, we cotransfected cells with WT-Nek2A-GFP and Mst2, Mst2-3A, or Mst2-3D. Nek2A-GFP was immunoprecipitated, supplemented with recombinant active or inactive PP1γ to a concentration that reflected the situation in cells, and incubated with recombinant C-Nap1-CTD (C-terminal domain) in the presence of ATP. Finally, phosphorylation of C-Nap1-CTD was monitored with phosphospecific C-Nap1 antibodies [5]. Prior inhibition of PP1γ with CalA (after immunoprecipitation) effectively prevented dephosphorylation of C-Nap1 (Figure 4D, lanes 3 and 4). When active but not inactive PP1γ was added to Mst2-WT/Nek2A, C-Nap1 was efficiently dephosphorylated (Figure 4D, lanes 1 and 2). Similar results were obtained with Mst2-3A/Nek2A (Figure 4D, lanes 5 and 6). In contrast, PP1γ did not efficiently dephosphorylate C-Nap1 in presence of the phosphomimicking Mst2-3D (Figure 4D, lane 8). This indicates that the phosphorylation state of Mst2 controls the balance between Nek2A and PP1γ to phosphorylate C-Nap1. Together, our results support a model in which Plk1 selectively phosphorylates Mst2. This decreases the formation of Mst2-PP1γ-Nek2A complexes and thereby promotes C-Nap1 phosphorylation by Nek2A.

Duplicated centrosomes need to be separated at the G2/M transition in order to form a bipolar spindle in human cells. It has been reported that upon Plk1 inhibition, cells arrest in prometaphase with two adjacent centrosomes [11]. Data presented here now reveal that Plk1 is crucial for Eg5 targeting to centrosomes at the time of nuclear envelope breakdown. Additionally, Plk1 selectively phosphorylates Mst2 and provides control of C-Nap1 phosphorylation through regulating the counteracting activities of Nek2A and PP1γ. Thus, centrosome disjunction is regulated by Plk1 by providing a balanced control between the counteracting Nek2A and PP1γ activities on C-Nap1. It will be interesting to see whether Plk1 also regulates the function of Mst2 in the Hippo growth control pathway.

Experimental Procedures

Cell Lines and Treatments

For thymidine block and release experiments, cells were incubated in 2.5 mM thymidine-containing medium for 24 hr and released in fresh medium without thymidine for 6 hr. For inhibition of Eg5 motor activity, S-trityl-L-cysteine (STLC) was used at a concentration of 5 μM. To inhibit mitotic kinases, we used BI2536 at 100 nM (unless indicated otherwise), 3MB-PP1 at 10 μM, RO3306 at 5 μM, and VX-680 at 1 μM. For inhibition of phosphatases, calyculin A was used at 50 ng/ml and okadaic acid at 1 nM.

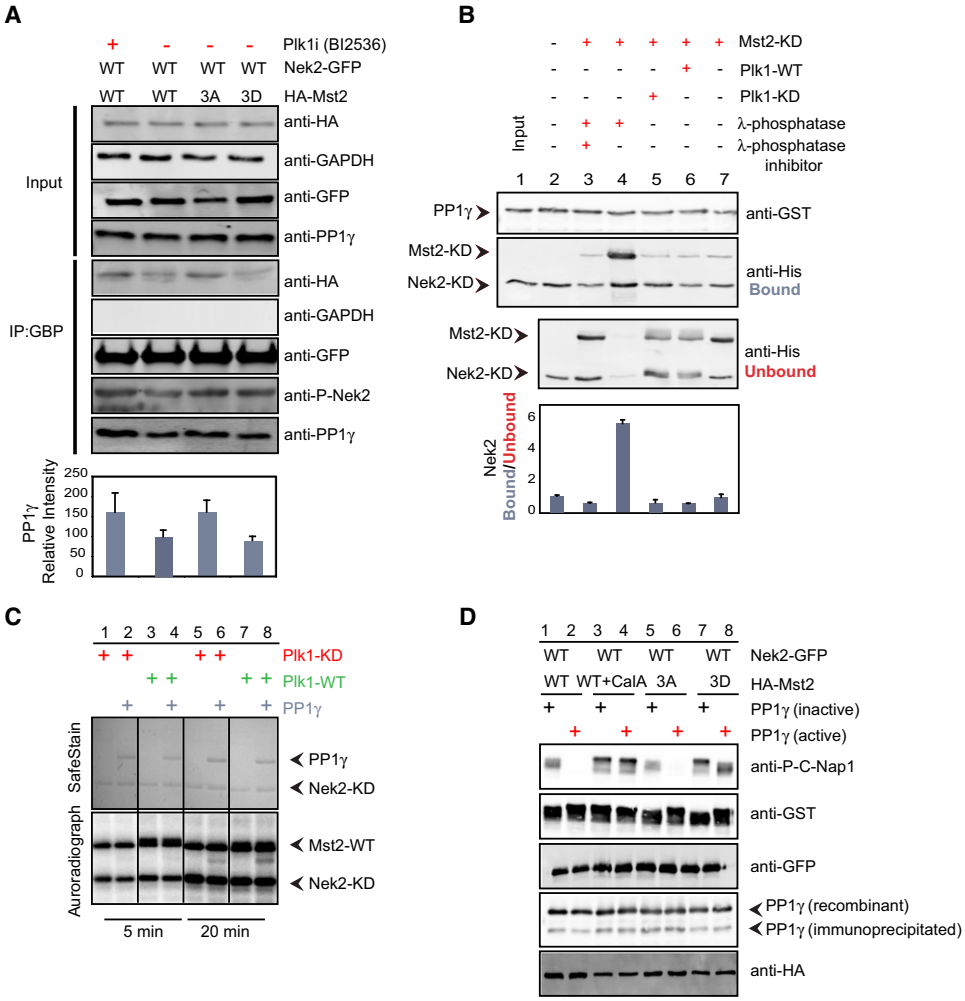

Figure 4. Plk1 Phosphorylation of Mst2 Disrupts Formation of the Nek2A-PP1γ Complex

(A) HA-Mst2 variants together with GFP-Nek2A were coexpressed in HEK293 cells. Cells were kept with or without BI2536 for 2 hr. GFP-tagged proteins were immunoprecipitated with GBP beads. Coimmunoprecipitation of HA-Mst2 and endogenous PP1γ was determined by immunoblotting. GAPDH was used as control for IP specificity. Bottom panel: bound PP1γ was quantified, corrected for corresponding GFP-IP signals, and normalized to WT. Data are mean ± SEM.

(B) Equimolar amounts of recombinant Mst2-KD from insect cells were dephosphorylated by λ-phosphatase (lane 4), incubated with λ-phosphatase in the presence of λ-phosphatase inhibitors (lane 3), incubated with Plk1-WT (lane 6) or Plk1-KD (lane 5) in the presence of ATP, or kept untreated (lane 7). Mst2 proteins were then mixed with recombinant Nek2A-KD and PP1γ-coupled GST Sepharose. After washing, bound and unbound fractions of Mst2-KD and Nek2A-KD were analyzed by immunoblotting. Bottom: ratios of bound to unbound fractions of Nek2A were determined. Averages from three independent experiments are shown. Data are mean ± SEM.

(C) Recombinant Mst2-WT kinase was preincubated with Plk1-WT or Plk1-KD in the presence of ATP. [γ-^{32}P]ATP was added, and Mst2 was used as kinase for in vitro kinase assays with Nek2A-KD or Nek2A-KD+PP1γ as substrates. The reactions were stopped after 5 and 20 min. Samples were analyzed by SDS-PAGE and autoradiography.

(D) HA-Mst2 constructs and GFP-Nek2A were coexpressed in HEK293 cells. GFP-tagged proteins were immunoprecipitated with GBP Sepharose beads. Immunoprecipitated samples were incubated with either active or inhibited PP1γ (+CalA) together with recombinant C-Nap1-CTD as a substrate. Phosphorylation of C-Nap1 was analyzed using phosphospecific C-Nap1-Ser2417/2421 antibodies.

Immunofluorescence Microscopy

Imaging was performed at 25°C on a DeltaVision RT system (Applied Precision) with an Olympus IX71 microscope equipped with FITC, TRITC, and Cy5 filters (Chroma Technology); Plan Apo 100× NA 1.4 and 60× NA 1.4 oil-immersion objectives (Olympus); a CoolSNAP HQ camera (Photometrics); a temperature controller (Precision Control); and Softworx software (Applied Precision).

Protein Methods

Protein purification, in vitro binding experiments, and immunoprecipitation experiments were carried out as described elsewhere [5]. For details, see Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.05.047.

Acknowledgments

We thank A.M. Fry, I. Hoffmann, P. Jallepalli, A. Khodjakov, T. Mayer, and G. Pereira for contributing reagents. S. Heinze is acknowledged for excellent technical support. We are grateful to T. Glatter, A. Schmidt, and A. Spang (Biozentrum, University of Basel) for mass spectrometry analysis; A.M. Fry

for helpful comments and discussions; and A. Khmelinskii for critical reading of the manuscript.

Received: March 10, 2011
Revised: April 28, 2011
Accepted: May 24, 2011
Published online: June 30, 2011

References

1. Lim, H.H., Zhang, T., and Surana, U. (2009). Regulation of centrosome separation in yeast and vertebrates: Common threads. Trends Cell Biol. *19*, 325–333.
2. Yang, J., Adamian, M., and Li, T. (2006). Rootletin interacts with C-Nap1 and may function as a physical linker between the pair of centrioles/basal bodies in cells. Mol. Biol. Cell *17*, 1033–1040.
3. Mayor, T., Stierhof, Y.D., Tanaka, K., Fry, A.M., and Nigg, E.A. (2000). The centrosomal protein C-Nap1 is required for cell cycle-regulated centrosome cohesion. J. Cell Biol. *151*, 837–846.
4. Mayor, T., Hacker, U., Stierhof, Y.D., and Nigg, E.A. (2002). The mechanism regulating the dissociation of the centrosomal protein C-Nap1 from mitotic spindle poles. J. Cell Sci. *115*, 3275–3284.
5. Mardin, B.R., Lange, C., Baxter, J.E., Hardy, T., Scholz, S.R., Fry, A.M., and Schiebel, E. (2010). Components of the Hippo pathway cooperate with Nek2 kinase to regulate centrosome disjunction. Nat. Cell Biol. *12*, 1166–1176.
6. Fry, A.M., Mayor, T., Meraldi, P., Stierhof, Y.D., Tanaka, K., and Nigg, E.A. (1998). C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. J. Cell Biol. *141*, 1563–1574.
7. Bahe, S., Stierhof, Y.D., Wilkinson, C.J., Leiss, F., and Nigg, E.A. (2005). Rootletin forms centriole-associated filaments and functions in centrosome cohesion. J. Cell Biol. *171*, 27–33.
8. Sawin, K.E., LeGuellec, K., Philippe, M., and Mitchison, T.J. (1992). Mitotic spindle organization by a plus-end-directed microtubule motor. Nature *359*, 540–543.
9. Roof, D.M., Meluh, P.B., and Rose, M.D. (1992). Kinesin-related proteins required for assembly of the mitotic spindle. J. Cell Biol. *118*, 95–108.
10. Lane, H.A., and Nigg, E.A. (1996). Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. J. Cell Biol. *135*, 1701–1713.
11. Lenart, P., Petronczki, M., Steegmaier, M., Di Fiore, B., Lipp, J.J., Hoffmann, M., Rettig, W.J., Kraut, N., and Peters, J.M. (2007). The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. Curr. Biol. *17*, 304–315.
12. Hannak, E., Kirkham, M., Hyman, A.A., and Oegema, K. (2001). Aurora-A kinase is required for centrosome maturation in *Caenorhabditis elegans*. J. Cell Biol. *155*, 1109–1116.
13. Crasta, K., Huang, P., Morgan, G., Winey, M., and Surana, U. (2006). Cdk1 regulates centrosome separation by restraining proteolysis of microtubule-associated proteins. EMBO J. *25*, 2551–2563.
14. Berdnik, D., and Knoblich, J.A. (2002). *Drosophila* Aurora-A is required for centrosome maturation and actin-dependent asymmetric protein localization during mitosis. Curr. Biol. *12*, 640–647.
15. Mori, D., Yano, Y., Toyo-oka, K., Yoshida, N., Yamada, M., Muramatsu, M., Zhang, D., Saya, H., Toyoshima, Y.Y., Kinoshita, K., et al. (2007). NDEL1 phosphorylation by Aurora-A kinase is essential for centrosomal maturation, separation, and TACC3 recruitment. Mol. Cell. Biol. *27*, 352–367.
16. Harrington, E.A., Bebbington, D., Moore, J., Rasmussen, R.K., Ajose-Adeogun, A.O., Nakayama, T., Graham, J.A., Demur, C., Hercend, T., Diu-Hercend, A., et al. (2004). VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo. Nat. Med. *10*, 262–267.
17. Collins, I., and Garrett, M.D. (2005). Targeting the cell division cycle in cancer: CDK and cell cycle checkpoint kinase inhibitors. Curr. Opin. Pharmacol. *5*, 366–373.
18. Potapova, T.A., Daum, J.R., Pittman, B.D., Hudson, J.R., Jones, T.N., Satinover, D.L., Stukenberg, P.T., and Gorbsky, G.J. (2006). The reversibility of mitotic exit in vertebrate cells. Nature *440*, 954–958.
19. Burkard, M.E., Randall, C.L., Larochelle, S., Zhang, C., Shokat, K.M., Fisher, R.P., and Jallepalli, P.V. (2007). Chemical genetics reveals the requirement for Polo-like kinase 1 activity in positioning RhoA and triggering cytokinesis in human cells. Proc. Natl. Acad. Sci. USA *104*, 4383–4388.
20. Seki, A., Coppinger, J.A., Jang, C.Y., Yates, J.R., and Fang, G. (2008). Bora and the kinase Aurora A cooperatively activate the kinase Plk1 and control mitotic entry. Science *320*, 1655–1658.
21. Macurek, L., Lindqvist, A., Lim, D., Lampson, M.A., Klompmaker, R., Freire, R., Clouin, C., Taylor, S.S., Yaffe, M.B., and Medema, R.H. (2008). Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery. Nature *455*, 119–123.
22. Sawin, K.E., and Mitchison, T.J. (1995). Mutations in the kinesin-like protein Eg5 disrupting localization to the mitotic spindle. Proc. Natl. Acad. Sci. USA *92*, 4289–4293.
23. Helps, N.R., Luo, X., Barker, H.M., and Cohen, P.T. (2000). NIMA-related kinase 2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1. Biochem. J. *349*, 509–518.
24. Mi, J., Guo, C., Brautigan, D.L., and Larner, J.M. (2007). Protein phosphatase-1alpha regulates centrosome splitting through Nek2. Cancer Res. *67*, 1082–1089.
25. Daub, H., Olsen, J.V., Bairlein, M., Gnad, F., Oppermann, F.S., Korner, R., Greff, Z., Keri, G., Stemmann, O., and Mann, M. (2008). Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. Mol. Cell *31*, 438–448.
26. Dephoure, N., Zhou, C., Villen, J., Beausoleil, S.A., Bakalarski, C.E., Elledge, S.J., and Gygi, S.P. (2008). A quantitative atlas of mitotic phosphorylation. Proc. Natl. Acad. Sci. USA *105*, 10762–10767.
27. Oppermann, F.S., Gnad, F., Olsen, J.V., Hornberger, R., Greff, Z., Keri, G., Mann, M., and Daub, H. (2009). Large-scale proteomics analysis of the human kinome. Mol. Cell. Proteomics *8*, 1751–1764.
28. Deng, Y., Pang, A., and Wang, J.H. (2003). Regulation of mammalian STE20-like kinase 2 (MST2) by protein phosphorylation/dephosphorylation and proteolysis. J. Biol. Chem. *278*, 11760–11767.