Plx1 is the 3F3/2 kinase responsible for targeting spindle checkpoint proteins to kinetochores

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Dynamic attachment of microtubules to kinetochores during mitosis generates pulling force, or tension, required for the high fidelity of chromosome separation. A lack of tension activates the spindle checkpoint and delays the anaphase onset. A key step in the tension–response pathway involves the phosphorylation of the 3F3/2 epitope by an unknown kinase on untensed kinetochores. Using a rephosphorylation assay in *Xenopus laevis* extracts, we identified the kinetochore-associated Polo-like kinase Plx1 as the kinase both necessary and sufficient for this phosphorylation. Indeed, Plx1 is the physiological 3F3/2 kinase involved in checkpoint response, as immunodepletion of Plx1 from checkpoint extracts abolished the 3F3/2 signal and blocked association of xMad2, xBubR1, xNdc80, and xNuf2 with kinetochores. Interestingly, the kinetochore localization of Plx1 is under the control of the checkpoint protein xMps1, as immunodepletion of xMps1 prevents binding of Plx1 to kinetochores. Thus, Plx1 couples the tension signal to cellular responses through phosphorylating the 3F3/2 epitope and targeting structural and checkpoint proteins to kinetochores.

Introduction

Accurate segregation of sister chromatids, which is essential for cell proliferation, is mediated by the attachment of microtubules to kinetochores during mitosis. This dynamic attachment generates pulling forces, or tension, across sister kinetochores to align chromosomes to the metaphase plate. Tension, in turn, stabilizes microtubule binding to kinetochores and is an indicator for functional attachment. A lack of tension on kinetochores activates a surveillance mechanism, the spindle checkpoint, which delays the onset of anaphase until all kinetochores are under tension and all chromosomes are properly aligned at the metaphase plate (Shah and Cleveland, 2000). The checkpoint signal originates from and is amplified and transduced at kinetochores. It then leaves kinetochores and diffuses into the cytosol to inhibit the anaphase-promoting complex/cyclosome (APC/C), which is a ubiquitin ligase whose activation is required for the separation of sister chromatids and exit from mitosis. In addition to arresting the cell cycle progression, components in the tension–response pathway are likely to contribute to the attachment of microtubules to kinetochores and to the establishment of tension.

Several spindle checkpoint proteins have been characterized in vertebrate cells. These include four kinases, Mps1, Bub1, BubR1, and Aurora B, as well as three additional checkpoint proteins, Mad1, Mad2, and Bub3 (for reviews see Millband et al., 2002; Musacchio and Hardwick, 2002; Lens and Medema, 2003; Taylor et al., 2004). Upon activation of the checkpoint, Mad2 and BubR1 synergistically bind to Cdc20, an activator of APC/C, and thereby prevent premature activation of APC/C and anaphase onset (Sudakin et al., 2001; Tang et al., 2001; Fang, 2002). The biochemical pathway upstream from Mad2 and BubR1 remains poorly understood.

Key steps in the response to a lack of tension involve protein phosphorylation on kinetochores. Indeed, unattached or untensed kinetochores are hyperphosphorylated (Gorbsky and Ricketts, 1993; Nicklas et al., 1998) and all four checkpoint kinases are localized to these kinetochores (for reviews see Lens and Medema, 2003; Taylor et al., 2004). A phosphorylation step crucial to responses to a lack of tension across sister kinetochores is revealed by the 3F3/2 antibody, a monoclonal antibody (Cyert et al., 1988) that recognizes a phosphoepitope of a yet unidentified protein. Studies in mammalian culture cells indicate that the presence of the 3F3/2 phosphoepitope at kinetochores tightly correlates with the absence of tension in prophase/prometaphase cells and in cells treated with taxol, which stabilizes microtubules and abolishes the tension across sister kinetochores (Gorbsky and Ricketts, 1993; Nicklas et al., 1995, 1998). Once sister kinetochores achieve tension through bipolar attachment at metaphase, the 3F3/2 epitope is dephosphorylated and the 3F3/2 signal is substantially reduced and

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Abbreviations used in this paper: APC/C, anaphase-promoting complex/cyclosome; CSF, cytostatic factor; NEM, *N*-ethyl maleimide; Plx1-KD, Plx1 kinase-dead mutant; RNAi, RNA interference; siRNA, small interfering RNA; xMps1-KD, xMps1 kinase-dead mutant; XTC, *Xenopus laevis* tissue culture.

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eventually disappears at the onset of anaphase, suggesting that the 3F3/2 epitope responds to tension (Nicklas et al., 1995). Microinjection of the 3F3/2 antibody into mitotic cells preserves the 3F3/2 epitope and prolongs the checkpoint arrest, suggesting a role of the 3F3/2 phosphorylation in checkpoint arrest in mammalian cells (Campbell and Gorbsky, 1995). It has been postulated that the 3F3/2 kinase and its substrate are tension-sensitive components of the spindle checkpoint (Nicklas et al., 1995, 1998). At the molecular level, the 3F3/2 epitope and the kinase remain to be identified, although in vitro rephosphorylation assay suggests that the kinase is associated with the kinetochores (Nicklas et al., 1998; Campbell et al., 2000). Given the important role of tension in controlling the fidelity of chromosome separation, it is critical to determine the molecular identity of the 3F3/2 antigen and the 3F3/2 kinase and to investigate the mechanism of their response to a lack of tension.

Multiple kinases are localized to mitotic kinetochores. In addition to the four checkpoint kinases, Polo-like kinase (Plk1) is also associated with kinetochores in prometaphase and metaphase cells. Plk1 is a key mitotic kinase that controls centrosome maturation, mitotic entry, bipolar spindle assembly, sister chromatid cohesion/separation, and cytokinesis (for review see Barr et al., 2004). The diverse function of Plk1 in mitosis makes its activity at kinetochores difficult to investigate. Plk1 has been reported to be required for spindle assembly, and a reduction of the Plk1 protein level by RNA interference (RNAi) in human cells activates the spindle checkpoint as a result of defects in the mitotic spindle (Sumara et al., 2004; van Vugt et al., 2004), suggesting a lack of requirement of Plk1 in the checkpoint arrest. However, this conclusion is complicated by a possibility that the residual Plk1 protein in knockdown cells, although not adequate for spindle assembly, may be sufficient to carry out its function at kinetochores.

To understand the tension-sensitive response, we set out to investigate the biochemical mechanism for phosphorylation of the 3F3/2 epitope at kinetochores and report here the identification of the 3F3/2 kinase. To avoid pleiotrophic effects of mitotic kinases in living cells, we used a *Xenopus laevis* cell-free system to investigate the tension-sensitive phosphorylation. Among the six kinetochore-associated kinases tested, only the *X. laevis* Plk1 homologue, Plx1, is both necessary and sufficient for phosphorylation of the 3F3/2 epitope at kinetochores. Immunodepletion of Plx1 abolished the 3F3/2 signals at kinetochores and substantially reduced the amounts of xMad2, xBubR1, xNdc80, and xNuf2 localized to kinetochores. Interestingly, localization of Plx1 to kinetochores requires the checkpoint protein xMps1, indicating that Plx1 itself is under the control of the spindle checkpoint. We propose that Plx1 is involved in the tension-sensitive response by phosphorylating the 3F3/2 epitope and targeting structural and checkpoint proteins to kinetochores. The function of Plx1 as the 3F3/2 kinase is conserved during evolution, as knockdown of human Plk1 also abolished the kinetochore-associated 3F3/2 epitope in HeLa cells.

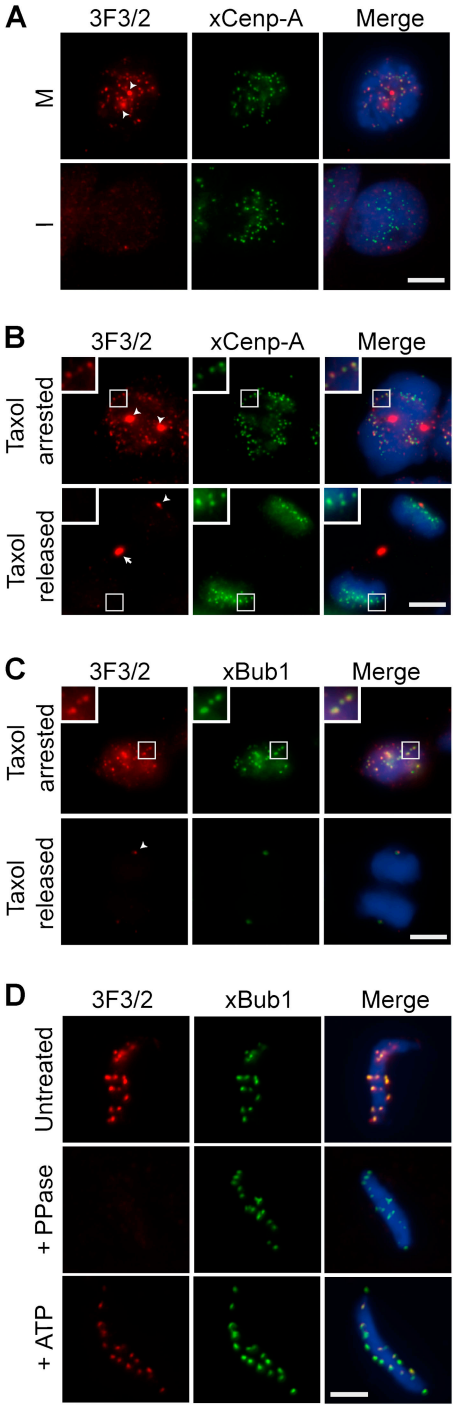

Figure 1. **The 3F3/2 epitope in *X. laevis* is a phosphospecific kinetochore antigen.** (A) Asynchronous XTC cells in mitosis (M) and in interphase (I) were stained with the 3F3/2 antibody and with an anti–xCenp-A antibody. The majority of the xCenp-A dots in M cells contained the 3F3/2 signals, although a few lacked the 3F3/2 staining, likely because of the dephosphorylation that occurred during sample processing. (B and C) XTC cells arrested with taxol or released from taxol arrest were stained for the 3F3/2 antigen and for xCenp-A or xBub1. Insets show a magnified image of the boxed areas. (D) Nuclei purified from checkpoint extracts (top) were dephosphorylated with λ-phosphatase (middle) and then rephosphorylated with ATP (bottom). (A–D) Red, 3F3/2; green, xCenp-A or xBub1. Arrowheads and arrow point to the 3F3/2 signals on centrosomes and at the midbody, respectively. Bars, 5 μm.

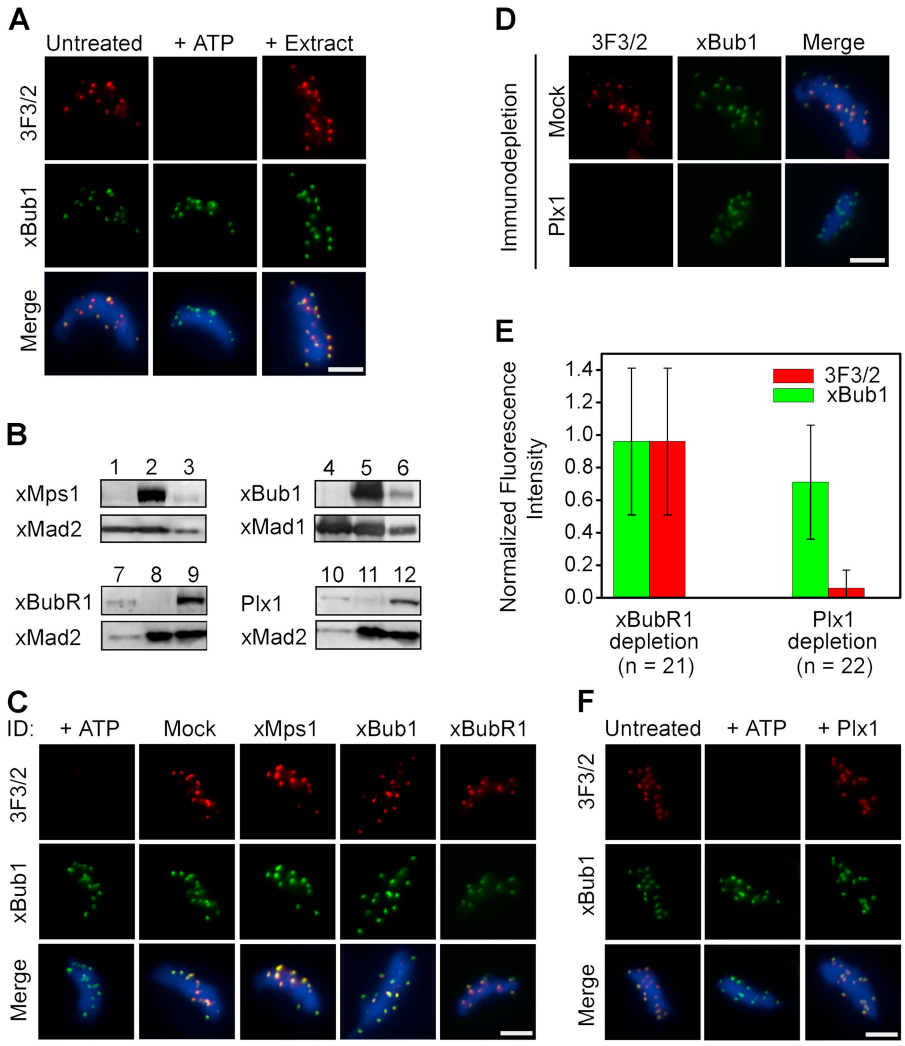

Figure 2. **Plx1 is both necessary and sufficient to phosphorylate the 3F3/2 epitope at kinetochores.** (A) Nuclei purified from checkpoint extracts carrying the 3F3/2 epitope (left) were dephosphorylated, treated with NEM, and rephosphorylated with either ATP (middle) or ATP plus CSF extract (right). Nuclei were then stained for the 3F3/2 epitope and xBub1. (B) Immunodepletion of kinetochore-associated kinases from CSF extracts. CSF extracts were depleted of xMps1 (lane 1), xBub1 (lane 4), xBubR1 (lane 8), and Plx1 (lane 11). 1 μl of depleted extracts and mock-depleted extracts (lanes 2, 5, 9, and 12) and 0.05 μl of input extracts (lanes 3, 6, 7, and 10) were analyzed by Western blotting to determine the depletion efficiency. xMad1 and xMad2 were shown here to demonstrate the specificity of the immunodepletion. (C and D) Rephosphorylation of 3F3/2 by extracts depleted of kinetochore-associated kinases. Nuclei were prepared as in A and rephosphorylated with either ATP (first column in C) or ATP plus depleted extracts prepared in B. ID, immunodepletion. (E) Mean kinetochore fluorescence intensity of xBub1 (green) and 3F3/2 (red) signals from samples rephosphorylated with xBubR1- or Plx1-depleted extracts. The fluorescence intensity was normalized to the corresponding values derived from mock-depleted extracts. Error bars represent SD. (F) Nuclei from checkpoint extracts were dephosphorylated and rephosphorylated with ATP or with ATP plus recombinant His_6-Plx1. (A, C, D, and F) Red, 3F3/2; green, xBub1. Bars, 5 μm.

Results

The *X. laevis* 3F3/2 epitope is a phosphospecific kinetochore antigen

To investigate the biochemistry of the 3F3/2 pathway, we analyzed the 3F3/2 epitope in *X. laevis* cells and extracts. Asynchronous *X. laevis* tissue culture (XTC) cells were costained with a 3F3/2 antibody and an antibody against xCenp-A, which is a structural component of kinetochores. The 3F3/2 epitope was present only in mitotic cells, but not in interphase cells (Fig. 1 A). In addition to centrosomal staining, the 3F3/2 epitope colocalized with xCenp-A in mitotic cells, indicating that the 3F3/2 antibody recognizes a mitotic kinetochore epitope (Fig. 1 A). To determine the sensitivity of the 3F3/2 epitope to tension across sister kinetochores, XTC cells were treated with taxol. In early prometaphase cells treated with taxol, strong 3F3/2 signals colocalized with xCenp-A and xBub1, which is a kinetochore-associated checkpoint protein (Fig. 1, B and C). As cells were released from taxol arrest, both the 3F3/2 epitope and xBub1 were diminished from kinetochores, whereas xCenp-A remained at kinetochores (Fig. 1, B and C). Thus, in *X. laevis* cells, the 3F3/2 antibody recognizes unattached kinetochores that are not under tension, a finding that is consistent with previous reports in mammalian cells (Gorbsky and Ricketts, 1993; Nicklas et al., 1995).

We then determined the localization of the 3F3/2 antigen in *X. laevis* checkpoint extracts. Nuclei were purified from *X. laevis* checkpoint extracts onto the coverslip by centrifugation, fixed, and then stained with the 3F3/2 antibody. The 3F3/2 epitope colocalized with xBub1 (Fig. 1 D). To determine whether the 3F3/2 epitope corresponds to a phosphoepitope, purified nuclei on coverslips were incubated with λ-phosphatase. Phosphatase treatment had no effect on the localization of xBub1, but it removed the 3F3/2 epitope from kinetochores (Fig. 1 D). To determine whether the 3F3/2 kinase is associated with kinetochores, nuclei purified from checkpoint extracts were first treated with λ-phosphatase and then rephosphorylated by the addition of ATP. Interestingly, the 3F3/2 epitope was regenerated in the presence of ATP (Fig. 1 D). Thus, the 3F3/2 kinase in *X. laevis* extracts is localized at kinetochores in proximity to its substrate, which is consistent with a previous paper in a mammalian system (Campbell et al., 2000).

Plx1 is both necessary and sufficient to phosphorylate the 3F3/2 epitope at kinetochores

We used a candidate approach to identify the 3F3/2 kinase. Six kinetochore-associated mitotic kinases—xMps1, xBub1, xBubR1, xAurora B, Plx1, and Cdc2/cyclin B (for reviews see Lens and Medema, 2003; Taylor et al., 2004)—were tested. To assay for the 3F3/2 kinase activity, we modified a rephosphorylation assay developed in mammalian cells (Nicklas et al., 1995). Nuclei purified from *X. laevis* checkpoint extracts were treated first with λ-phosphatase to remove the phosphoepitope and then with *N*-ethyl maleimide (NEM), a sulfhydryl alkylating agent that covalently modifies cysteine residues, to inactivate kinetochore-associated kinases. The resulting nuclei were used as substrates in an in vitro kinase assay. Addition of ATP alone did not recover the 3F3/2 signals, whereas ATP plus *X. laevis* meiotic metaphase extracts (cytostatic factor [CSF] extracts) regenerated the 3F3/2 epitope (Fig. 2 A), indicating that CSF extracts contain the 3F3/2 kinase.

We then examined the three known spindle checkpoint kinases—xMps1, xBub1, and xBubR1—for their ability to generate the 3F3/2 epitope. Checkpoint kinases were individually immunodepleted from CSF extracts with respective antibodies to an extent that was >95% (Fig. 2 B). Surprisingly, the depleted extracts retained the ability to rephosphorylate the 3F3/2 epitope (Fig. 2 C). For example, a quantitative analysis indicated that xBubR1- and mock-depleted extracts had comparable mean levels of 3F3/2 fluorescence intensity at kinetochores (Fig. 2 E). Thus, none of these three checkpoint kinases is the 3F3/2 kinase.

Next, we analyzed the kinetochore kinase Plx1. Plx1-depleted extracts lost the ability to rephosphorylate the 3F3/2 epitope (Fig. 2 D). An analysis of the mean 3F3/2 fluorescence intensity at kinetochores indicated that the intensity from Plx1-depleted extracts was only 6% of that from control-depleted extracts (Fig. 2 E). As a control, we found no significant difference in the xBub1 intensity between nuclei treated with Plx1- versus control-depleted extracts (Fig. 2 E). To rule out the possibility that the lack of phosphorylation is attributable to the coprecipitation of a kinase associated with Plx1, purified recombinant His_6-Plx1 was used in the rephosphorylation assay. Recombinant Plx1 was sufficient to regenerate the 3F3/2 phosphoepitope (Fig. 2 F), whereas the active recombinant GST-xAurora B and the active Cdc2/cyclin B kinase failed to restore any 3F3/2 signal (not depicted). Together, these results show that Plx1 is both necessary and sufficient to phosphorylate the 3F3/2 epitope at kinetochores.

Plx1 is the physiological kinase for the 3F3/2 epitope

If Plx1 is indeed the 3F3/2 kinase, immunodepletion of Plx1 should abolish the 3F3/2 epitope on kinetochores in spindle checkpoint extracts. To test this prediction, we immunodepleted Plx1 from CSF extracts to >99.5%, and then assembled the checkpoint extracts by adding sperm nuclei and nocodazole (Fig. 3 A). Immunodepletion of Plx1 abolished its kinetochore staining (Fig. 3 B), indicating that Plx1 is indeed a kinetochore protein in *X. laevis* extracts. Upon depletion of Plx1, the 3F3/2 epitope was also absent from kinetochores, whereas the localization of xCenp-A was not affected (Fig. 3 B). To rescue the Plx1-depletion phenotype, we translated recombinant Plx1, using in vitro–transcribed Plx1 mRNA, in *X. laevis* CSF extracts that had been depleted of endogenous Plx1 to >99.5%. Addition of translated Plx1 to the depleted extracts at only ∼5% of the endogenous Plx1 level efficiently restored both the Plx1 and 3F3/2 signals at kinetochores. In contrast, addition of an in vitro–translated Plx1 kinase-dead mutant (Plx1-KD) failed to restore the 3F3/2 signals, despite the fact that Plx1-KD was efficiently targeted to kinetochores (Fig. 3 B). A quantitative analysis of 3F3/2 kinetochore fluorescence intensity in these samples further supported the requirement of the Plx1 kinase activity for phosphorylating the 3F3/2 epitope (Fig. 3 C). Together, our data (Figs. 2 and 3) indicate that Plx1 is the physiological kinase for the 3F3/2 epitope in checkpoint extracts. We found that Plx1 has an extremely high affinity for kinetochores, as immunodepletion of Plx1 by 99% affected neither its localization at kinetochores nor the phosphorylation of the 3F3/2 epitope in checkpoint extracts (not depicted). This high affinity of Plx1 to kinetochores also explains why the addition of only 5% of wild-type Plx1 to the depleted extracts is sufficient to rescue the 3F3/2 signal. Therefore, all the depletion experiments in this paper were performed with a depletion efficiency of >99.5% for Plx1.

Plx1 targets xBubR1 and xMad2 to kinetochores

We next investigated the function of Plx1 in checkpoint response. The fact that Plx1 is required for a calcium-triggered exit from the CSF arrest (Descombes and Nigg, 1998) precludes the use of conventional checkpoint assays, such as DNA morphology, H1 kinase activity, and cyclin B stability, to assess the status of the spindle checkpoint in Plx1-depleted extracts. Taking an alternative approach, we examined whether the localization of the checkpoint proteins is dependent on Plx1. We reasoned that if Plx1 is involved in checkpoint control, it may act to control the localization of checkpoint proteins at kinetochores. To test this prediction, we focused on xMad2 and xBubR1, two essential kinetochore-associated checkpoint proteins required for the inhibition of APC/C (Sudakin et al., 2001; Tang et al., 2001; Fang, 2002).

In nuclei prepared from Plx1-depleted extracts ($n = 117$), the fluorescence intensity of xMad2 at kinetochores fell into three groups. The first group of nuclei, which corresponded to 53% of nuclei, had no detectable xMad2 staining on kinetochores (Fig. 3 D, second column). The second group of nuclei, which corresponded to 39% of nuclei, contained only weak signals on kinetochores (∼25% of the intensity found in mock-depleted samples; Fig. 3 D, third column). The third group of nuclei, which corresponded to 8% of the nuclei, had strong xMad2 staining with intensity resembling that of mock-depleted samples (Fig. 3 D, compare first and fourth columns). In contrast, the majority (92%, $n = 107$) of nuclei from mock-depleted extracts had strong xMad2 staining (Fig. 3 D, first column), whereas only 6 and 2% of nuclei had weak and no xMad2 staining, respectively (not depicted).

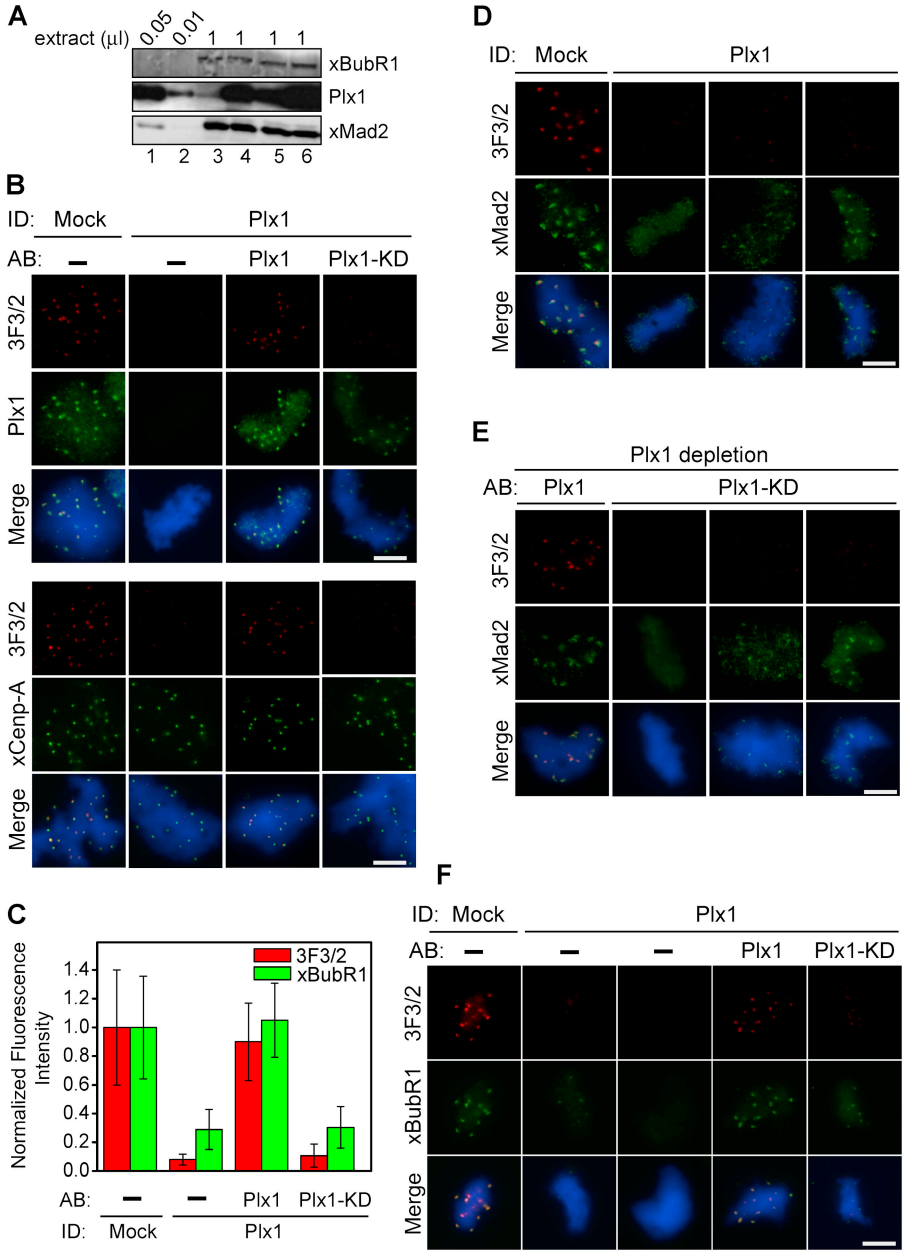

Figure 3. **Plx1 is the physiological 3F3/2 kinase.** (A) Extracts were mock depleted (lane 6) or depleted of Plx1 (lanes 3–5). Plx1 (lane 4) or Plx1-KD (lane 5) was translated in CSF extracts that had been depleted of endogenous Plx1 and then added to the Plx1-depleted extracts. Lanes 1 and 2 show input extracts. The volumes of extracts loaded were as indicated. Different amounts of input extracts were loaded to quantify the degree of depletion and add-back. (B and D–F) 3F3/2, Plx1, xCenp-A, xMad2, and xBubR1 staining of nuclei purified from checkpoint extracts that had undergone immunodepletion (ID) and add-back (AB) of the indicated proteins. Red, 3F3/2; green, Plx1, xCenp-A, xMad2, and xBubR1. (C) Mean kinetochore fluorescence intensity (from 15 randomly selected kinetochores) of xBubR1 (green) and 3F3/2 (red) signals from samples that were depleted of Plx1 and then added back with the indicated proteins. The fluorescence intensity was normalized to the corresponding values derived from mock-depleted extracts. Error bars represent SD. Bars, 5 μm.

Upon addition of translated wild-type Plx1 to the Plx1-depleted extracts, the phenotype reverted back to that of the mock-depleted samples, with 94 and 6% of nuclei (n = 116) showing strong and weak xMad2 signals on kinetochores, respectively (Fig. 3 E). The ability of the exogenously added Plx1 to rescue the defect in xMad2 localization requires the kinase activity, as the addition of Plx1-KD did not rescue the defect in xMad2 localization, although Plx1-KD itself was efficiently targeted to kinetochores. In the Plx1-KD add-back extracts, 35, 51, and 14% of nuclei (n = 120) had no, weak, and strong xMad2 kinetochore signals, respectively, numbers comparable to those observed in Plx1-depleted extracts (Fig. 3, D and E).

Similarly, depletion of Plx1 reduced the amount of xBubR1 recruited to kinetochores (Fig. 3 F, compare second and third columns with first column). A quantitative analysis of xBubR1 fluorescence intensity indicated that depletion of Plx1 reduced, on average, ~70% of xBubR1 signals on randomly selected kinetochores (Fig. 3 C). In addition, no BubR1 signal was detectable in a small number (5%) of nuclei from depleted extracts (Fig. 3 E, third column). The addition of translated wild-type Plx1, but not Plx1-KD, rescued the defect in kinetochore localization of xBubR1 (Fig. 3, C and F). The lack of kinetochore localization for xBubR1 and xMad2 was not attributable to codepletion of these two proteins with Plx1, as the levels of both proteins were not affected by immunodepletion (Fig. 3 A). We conclude that the Plx1 kinase activity is required to target xMad2 and xBubR1 to kinetochores. Our observation is consistent with the previous finding that phosphorylation of kinetochore components is required for the binding of Mad2 to kinetochores in mammalian cells (Waters et al., 1999). Similarly, it has been reported that localization of

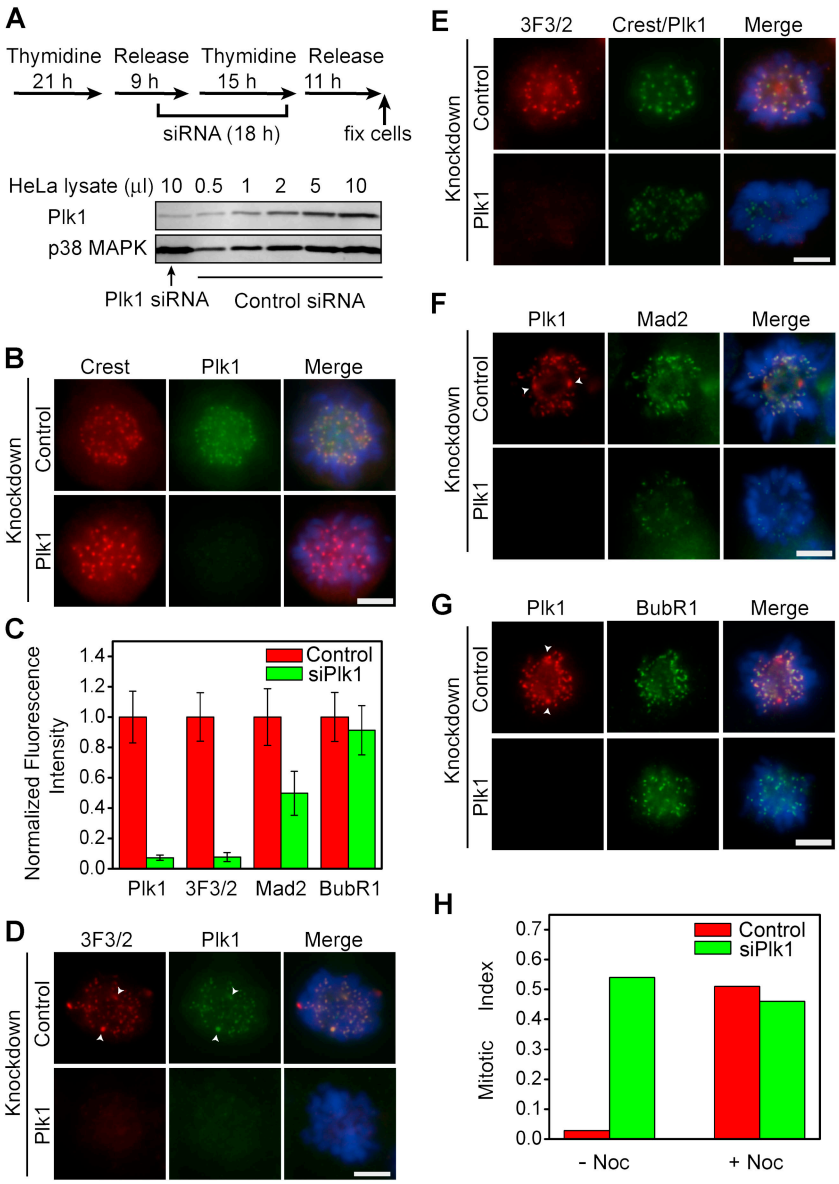

Figure 4. **Plk1 is required for the generation of the 3F3/2 epitope in HeLa cells.** (A, top) Schematic of double-thymidine synchronization and siRNA transfection of HeLa cells. (bottom) Western blot analysis of Plk1 and control knockdown cell lysates to determine the knockdown efficiency. p38 MAPK was used as a loading control. The volumes of HeLa cell lysates loaded were as indicated. Different amounts of cell lysates were loaded from the control knockdown sample to quantify the degree of Plk1 knockdown. (B and D–G) Plk1 or control knockdown cells were fixed at 11 h after release from the second thymidine arrest. Prometaphase cells were stained with the following antibodies: (B) Crest serum (red) and anti-Plk1 (green); (D) 3F3/2 antibody (red) and anti-Plk1 (green); (E) 3F3/2 antibody (red), Crest serum (green), and anti-Plk1 (green); (F) Plk1 (red) and Mad2 (green); (G) Plk1 (red) and BubR1 (green). In D, F, and G, arrowheads point to 3F3/2 and Plk1 signals at spindle poles. Bars, 5 μm. (C) Mean kinetochore fluorescence intensity (from randomly selected kinetochores of multiple prometaphase cells) of Plk1, 3F3/2, Mad2, and BubR1 signals from Plk1 (green) or control knockdown (red) cells. The fluorescence intensity was normalized to the corresponding values derived from control knockdown cells. Error bars represent SD. (H) HeLa cells were synchronized by double-thymidine arrest/release and transfected with siRNAs as described in A, except that transfected cells were released from the second thymidine arrest in either the presence (+ Noc) or absence (− Noc) of 100 ng/ml nocodazole. At 14 h after release from the second thymidine arrest, cells were fixed, and the mitotic index was counted ($n > 150$ cells for each sample).

BubR1 is dependent on phosphorylation of kinetochore components in *Drosophila melanogaster* S2 cells (Logarinho et al., 2004).

Generation of the 3F3/2 epitope depends on Plk1 in HeLa cells

To address whether the relationship between Plx1 and the 3F3/2 epitope is conserved during evolution, we tested the role of Plk1 in phosphorylation of the 3F3/2 epitope in HeLa cells. We transfected small interfering RNAs (siRNAs) into synchronized HeLa cells to achieve the maximal knockdown and analyzed the immediate phenotype during the first mitosis after transfection (Fig. 4 A). Western blot analysis indicated that the level of Plk1 was efficiently reduced through RNAi by 95% (Fig. 4 A). The reduction of Plk1 is also drastic on kinetochores at the cellular level. Whereas 100% of prometaphase cells ($n = 56$) in the control-transfected sample had strong Plk1 signals on kinetochores, 89.9% of prometaphase cells ($n = 148$) in the Plk1 knockdown sample had no detectable Plk1 signal on kinetochores (Fig. 4 B). The remaining 10.1% of cells had weak but visible Plk1 signals on kinetochores. To quantify the level of Plk1 knockdown on individual kinetochores, cells were co-stained with Crest serum and Plk1. Crest serum recognizes structural components at inner centromeres not affected by Plk1 knockdown (Fig. 4 B) and was used here as an internal reference to normalize the Plk1 fluorescence intensity. Upon knockdown of Plk1, the normalized fluorescence intensity of Plk1 signals at prometaphase kinetochores was reduced to ~7.3% of that of control cells (Fig. 4, B and C). We next examined the level of the kinetochore 3F3/2 epitope in prometaphase cells in which Plk1 was not detectable on kinetochores. Prometaphase cells in control knockdown cells contained strong 3F3/2 signals at kinetochores (Fig. 4, D and E). In agreement with our results from extract experiments, the 3F3/2 signals were absent in Plk1 knockdown cells (Fig. 4, C and D). To exclude the possibility that Plk1 is generally required for

the assembly of mitotic kinetochores, but not specifically involved in the phosphorylation of the 3F3/2 epitope, we triple stained the Plk1 knockdown cells with 3F3/2, Plk1, and Crest serum. Cells with efficient knockdown of Plk1 were identified by the absence of Plk1 signals at the spindle poles. In these cells, the 3F3/2 epitope was absent, but the Crest signals persisted, indicating that knockdown of Plk1 does not affect the integrity of the inner centromere structure (Fig. 4 E). Thus, Plk1 is also required for the formation of the 3F3/2 phosphoepitope in mammalian cells, suggesting a common mechanism for tension sensing/response from *X. laevis* to human.

Plk1 contributes to the loading of Mad2 onto kinetochores in HeLa cells

We next investigated whether Plk1 controls the kinetochore localization of the spindle checkpoint proteins Mad2 and BubR1 in HeLa cells. In prometaphase cells with undetectable Plk1 signals, the level of Mad2 at the kinetochores was reduced to ~50% of that of control cells (Fig. 4, C and F), suggesting that, under our experimental conditions, Plk1 contributes to but is not required for the loading of Mad2 to kinetochores in HeLa cells. In contrast, the kinetochore BubR1 level was not affected in Plk1 knockdown cells (Fig. 4, C and G), indicating a lack of requirement for Plk1. These data raise the possibility that differences may exist in the kinetochore assembly pathway and in the spindle checkpoint signaling pathway between *X. laevis* extracts and mammalian cells.

We next examined the role of Plk1 in the checkpoint arrest. Upon release from the second thymidine arrest, control-transfected cells peaked in mitosis between 10 and 12 h after release (unpublished data). At 14 h after release, nearly all of the control-transfected cells had exited mitosis, whereas >50% of the Plk1 knockdown cells remained at prometaphase (Fig. 4 H and not depicted), indicating a mitotic arrest in Plk1 knockdown cells. In the presence of nocodazole, the mitotic index of control-transfected cells became comparable to that of Plk1 knockdown cells (0.51 and 0.46, respectively; Fig. 4 H), indicating the activation of the spindle checkpoint in both samples. These observations are consistent with those of previous studies showing that Plk1 is not required for checkpoint arrest in mammalian cells (Sumara et al., 2004; van Vugt et al., 2004). Instead, knockdown of Plk1 activates spindle checkpoint because of defects in spindle structure.

Localization of Plx1 to kinetochores is controlled by the checkpoint protein xMps1

We next asked whether Plx1 itself is also under the control of upstream checkpoint components, such as xMps1. xMps1 was depleted from CSF extracts before the assembly of the checkpoint extracts, and the kinetochore localization of Plx1 was examined in the depleted checkpoint extracts. Interestingly, depletion of xMps1 completely abolished the Plx1 signal at kinetochores without affecting the level of the Plx1 protein in extracts (Fig. 5, A and B). Addition of in vitro–translated wild-type xMps1, but not the xMps1 kinase-dead mutant (xMps1-KD), rescued the kinetochore localization of Plx1

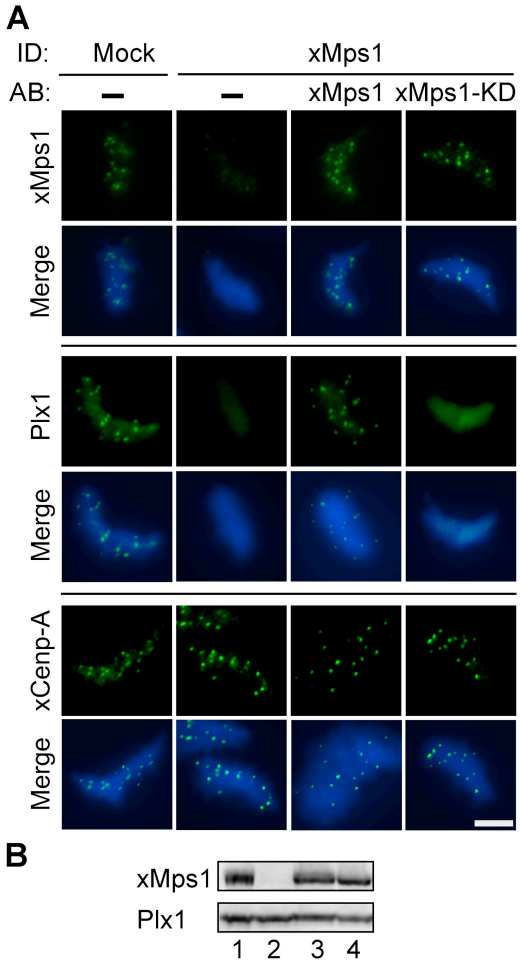

Figure 5. **xMps1 controls the kinetochore localization of Plx1.** (A) CSF extracts were either mock depleted or depleted of xMps1 and then added back with the indicated proteins. Nuclei were purified from depleted checkpoint extracts and stained for xMps1, Plx1, and xCenp-A. ID, immunodepletion; AB, add-back. Bar, 5 μm. (B) Depletion efficiency was determined by Western blot analysis of equal volumes of mock- (lane 1) or xMps1-depleted (lane 2) extracts as well as xMps1-depleted extracts with the add-back of xMps1 (lane 3) or xMps1-KD (lane 4).

(Fig. 5 A). In all cases, xCenp-A localization was not affected, indicating that depletion of xMps1 did not affect the structural integrity of inner centromeres. We conclude that the checkpoint kinase xMps1 is required to recruit Plx1 to kinetochores.

xMps1 and xPlx1 control the outer kinetochore structure

To determine whether xMps1 and Plx1 control the outer kinetochore structure, nuclei from xMps1- or Plx1-depleted extracts were stained with xNdc80 and xNuf2. xNdc80 and xNuf2 form a complex required for microtubule attachment to kinetochores (DeLuca et al., 2002). Depletion of xMps1 completely removed xNdc80 and xNuf2 from the kinetochores, and their kinetochore localization can only be rescued by the addition of wild-type xMps1 but not xMps1-KD (Fig. 6 A). Similarly, Plx1 is also required for the kinetochore localization of xNdc80 and xNuf2 (Fig. 6 B). Interestingly, addition of either wild type or Plx1-KD was sufficient to rescue the kinetochore localization

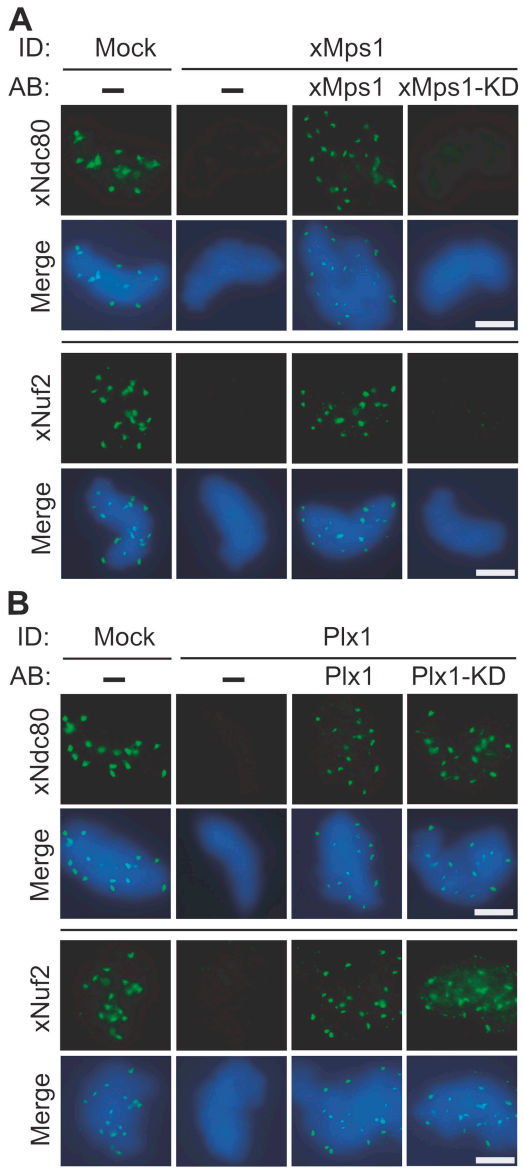

Figure 6. **xMps1 and Plx1 are required for the assembly of the outer kinetochore structure.** (A) Nuclei were purified from mock-, xMps1-, or xMps1-depleted extracts with the add-back of the indicated proteins, as described in Fig. 5. Purified nuclei were stained for xNdc80 or xNuf2. (B) Nuclei were purified from mock-, Plx1-, or Plx1-depleted extracts with the add-back of the indicated proteins, as described in Fig. 3. Purified nuclei were stained for xNdc80 and xNuf2. ID, immunodepletion; AB, add-back. Bars, 5 μm.

of xNdc80 and xNuf2, indicating a kinase-independent function of Plx1 at kinetochores, although we cannot exclude the possibility that the residual endogenous Plx1, even with >99.5% depletion, acted together with Plx1-KD to mediate the rescue. Together, our data suggest that both xMps1 and Plx1 control the assembly of the outer kinetochore structure.

Discussion

A lack of tension across sister kinetochores activates downstream cellular responses, including the spindle checkpoint, which delays the onset of anaphase and thereby ensures the fidelity of chromosome separation. A key cellular component that couples the tension sensor to downstream responses is the 3F3/2 epitope, whose phosphorylation is triggered by the lack of tension. We have shown here that the Polo-like kinase Plx1 is both necessary and sufficient for phosphorylation of the 3F3/2 epitope in vitro and is the physiological 3F3/2 kinase in vivo. Thus, Plx1 has a direct role in the cellular responses to the lack of tension, and possibly in the spindle checkpoint control. Consistent with this, our results show that Plx1 is localized to kinetochores and that this localization is under the control of xMps1, a checkpoint kinase required for tension-sensitive cell cycle arrest (Dorer et al., 2005). Furthermore, Plx1 targets the checkpoint proteins xMad2 and xBubR1 as well as structural proteins xNdc80 and xNuf2 to kinetochores, further linking Plx1 to checkpoint responses in *X. laevis* extracts.

What is the physiological role of Plx1 in spindle checkpoint control? Cellular responses to checkpoint activation usually consist of two branches, one targeting the cell cycle machinery to halt the cell cycle progression and the other targeting the repair mechanism to fix cellular defects, leading to inactivation of the checkpoint. Indeed, emerging evidence suggests that components in the spindle checkpoint pathway are bifunctional and act in both branches. For example, the checkpoint kinases Mps1, Bub1, BubR1, and Aurora B are not only involved in kinetochore-mediated signaling to inhibit APC/C but also control spindle assembly, attachment of microtubules to kinetochores, and biorientation of sister chromatids (Tanaka et al., 2002; Lampson et al., 2004; Jones et al., 2005; Lampson and Kapoor, 2005; Meraldi and Sorger, 2005). Similarly, the checkpoint protein Mad2 not only functions as an inhibitor of APC/C to prevent premature anaphase onset but also contributes to the biorientation of homologous chromosomes during meiosis (Shonn et al., 2003). Consistent with a role of Plk1 in response to the lack of tension, it has been reported that Plk1 promotes the formation of the bipolar spindle and is required for the generation of tension across sister kinetochores in mammalian cells (Sumara et al., 2004; van Vugt et al., 2004). We report here that Plk1/Plx1 is the kinase that phosphorylates the 3F3/2 epitope in the absence of tension and propose that one function of the tension-sensitive pathway is to promote tension across sister kinetochores through the action of the Plk1/Plx1 kinase. In agreement with this model, our data reveal that Plx1 is required for the assembly of the xNdc80–xNuf2 complex onto kinetochores in *X. laevis* extracts, indicating that Plx1 controls certain aspects of the outer kinetochore structure. Because this complex mediates the attachment of microtubules to kinetochores (DeLuca et al., 2002), Plx1, through the loading of the xNdc80–xNuf2 complex, may act to promote the binding of microtubules to kinetochores and the establishment of tension across sister kinetochores. The presence of tension then stabilizes microtubule attachment and changes the dynamics of checkpoint proteins on kinetochores, leading to the inactivation of the checkpoint pathway (Howell et al., 2000, 2004; Shah et al., 2004). This model predicts that Plk1 is a key mediator in the tension–response pathway and that the tension sensor controls either the recruitment/activation of Plk1 or the accessibility of the 3F3/2 antigen on untensed kinetochores.

Another cellular response to the lack of tension is to arrest the cell cycle progression by inhibiting APC/C (Shah and Cleveland, 2000). Is Plx1 involved in cell cycle arrest in *X. laevis* checkpoint extracts? To release CSF extracts from the meiotic metaphase II, Plx1 is required to activate APC/C through degradation of xErp1, which is a checkpoint-independent inhibitor of APC/C (Descombes and Nigg, 1998; Schmidt et al., 2005). Thus, we were not able to directly assay the status of the checkpoint arrest in Plx1-depleted extracts in the current study (unpublished data). However, the fact that Plx1 is required to target xMad2 and xBubR1 to kinetochores supports a direct role of Plx1 in cell cycle arrest in the extract system. Mad2 and BubR1 are two checkpoint effectors that directly bind to Cdc20 and prevent premature activation of APC/C at prometaphase (Sudakin et al., 2001; Tang et al., 2001; Fang, 2002). Both Mad2 and BubR1 exist in two populations, kinetochore-bound forms and cytosolic diffusive forms, and these two populations exchange with ultrafast kinetics (Howell et al., 2000, 2004; Shah et al., 2004). It has been proposed that the kinetochore-bound forms receive checkpoint signals from unattached or untensed kinetochores, which convert them into diffusive inhibitors to inactivate Cdc20 throughout the cytosol (Shah and Cleveland, 2000; Musacchio and Hardwick, 2002). Indeed, cell biological analyses indicate that the kinetochore-bound checkpoint proteins are essential for checkpoint-mediated cell cycle delay (Meraldi et al., 2004). In addition, biochemical and cell biological studies suggest that Mad2 undergoes a conformational change mediated through self-oligomerization at kinetochores and that this kinetochore-dependent conformational change is required for inhibition of Cdc20 (Fang et al., 1998; De Antoni et al., 2005). The lack of xMad2 and xBubR1 on kinetochores in the absence of Plx1 indicates that Plx1 is likely required for the checkpoint arrest in *X. laevis* extracts. This conclusion is further strengthened by our observation that the localization of Plx1 at kinetochores is under the control of the spindle checkpoint protein xMps1 and that Plx1 is required for kinetochore localization of xNdc80 and xNuf2, which form a complex required for checkpoint arrest both in *X. laevis* extracts and in mammalian cells (McCleland et al., 2003; Meraldi et al., 2004). These observations suggest that the xMps1-dependent association of xMad2 (Abrieu et al., 2001) and xNdc80–xNuf2 (our study) with kinetochores is likely mediated through the action of Plx1, and are consistent with the previous finding that the binding of Mad2 to kinetochores depends on the phosphorylation of kinetochore components in mammalian cells (Waters et al., 1999).

We found that Plk1 is also required for phosphorylation of the 3F3/2 epitope in human cells. A recent study, published when the final version of the current paper was under review, also showed that Plk1 is the 3F3/2 kinase in mammalian cells (Ahonen et al., 2005). Thus, there may exist a common tension–response mechanism upstream from the 3F3/2 epitope between human and *X. laevis* systems. On the other hand, we noted that our conclusions about Plx1's role in checkpoint arrest in *X. laevis* extracts are in sharp contrast to the mitotic function of Plk1 reported in mammalian cells (for review see Barr et al., 2004). For example, a reduction of the Plk1 level by RNAi activates, rather than abolishes, the spindle checkpoint because of defects in spindle assembly (Sumara et al., 2004; van Vugt et al., 2004). In the current study, we also found that the knockdown of Plk1 in HeLa cells activates the spindle checkpoint, even though the levels of kinetochore-associated Mad2, but not BubR1, are reduced, which is consistent with the findings of a recent study (Ahonen et al., 2005). In Plk1 knockdown cells, the 3F3/2 signals are also substantially reduced, and yet the checkpoint arrest persists. Although a tight correlation between the 3F3/2 epitope and the activation of the tension-sensitive checkpoint has been observed (Gorbsky and Ricketts, 1993; Nicklas et al., 1995, 1998), our data suggest that the 3F3/2 phosphoepitope is not required for checkpoint-mediated mitotic arrest, per se, in mammalian cells. In addition, the differential requirement of the Polo-like kinase in the loading of BubR1 onto kinetochores between *X. laevis* extracts and human cells suggests that differences may exist in the kinetochore assembly pathway and in the checkpoint signaling pathway between these two systems.

Alternatively, this discrepancy between the two systems could be the result of an incomplete knockdown of Plk1 in mammalian cells, as has been reported for other checkpoint proteins. For example, both xBub1 and xNdc80 are required for checkpoint arrest in *X. laevis* extracts (Sharp-Baker and Chen, 2001; McCleland et al., 2003). In mammalian cells, partial knockdown of either Bub1 or Ndc80 activates the spindle assembly checkpoint, whereas complete knockdown of either protein abolishes the checkpoint (DeLuca et al., 2002; Martin-Lluesma et al., 2002; Meraldi et al., 2004; Tang et al., 2004; Meraldi and Sorger, 2005). Plx1 has an exceedingly high affinity to kinetochores, as 1% of residual Plx1 in immunodepleted *X. laevis* extracts was sufficient to mask the requirement of Plx1 for the loading of xMad2, xBubR1, and xNdc80–xNuf2 onto kinetochores (unpublished data). The maximal degree of knockdown we achieved in mammalian cells was 95%; therefore, it is possible that the residual Plk1 activity in mammalian knockdown cells, although not adequate for spindle assembly, is sufficient for checkpoint arrest. Consistent with a role of Plk1 in checkpoint control in mammalian cells, it has been reported that preservation of the 3F3/2 phosphoepitope by injected 3F3/2 antibody prolongs the checkpoint arrest and delays the onset of anaphase during mitosis, suggesting that the 3F3/2 epitope contributes, either directly or indirectly, to the checkpoint arrest in mammalian cells (Campbell and Gorbsky, 1995). A clear causal link between Plk1 and checkpoint arrest in mammalian cells awaits the isolation of a potent, small molecule inhibitor of Plk1 or the determination of the molecular identity of the 3F3/2 epitope.

What is the nature of the 3F3/2 antigen? Several candidates, such as DNA topoisomerase II and subunits of APC/C (Daum and Gorbsky, 1998; Daum et al., 2000), have been found to contain the 3F3/2 epitope, although none has been directly linked to the tension–response pathway at kinetochores. The demonstration that the Polo-like kinase phosphorylates the 3F3/2 epitope, a mechanism conserved both in *X. laevis* extracts and in human cells, provides an entry point for examining the potential function of the 3F3/2 epitope in checkpoint control and an opportunity to investigate the molecular mechanism of the tension-sensitive signaling pathway in mitosis.

Materials and methods

Recombinant proteins and antibodies

The following recombinant proteins were expressed and purified: His_6-xMad1 (aa 321–562), His_6-xMad2, GST-xBub1 (aa 1–670), GST-xBubR1 (aa 1–197), GST-xMps1 (kinase-dead mutant), and His_6-Plx1 (aa 225–598). All recombinant proteins were expressed in *Escherichia coli*, except xMps1, which was expressed in Sf9 cells. Rabbit antibodies were raised against the aforementioned recombinant proteins and affinity purified. Antisera against Plx1 were provided by R. Heald (University of California, Berkeley, Berkeley, CA). Anti-Ndc80 and -Nuf2 antibodies were provided by T. Stukenberg (University of Virginia, Charlottesville, VA), and anti-Cenp-A antibody was provided by A. Straight (Stanford University, Stanford, CA). Rabbit antibodies against human Mad2 and BubR1 were described previously (Fang, 2002). Commercial antibodies were obtained as follows: 3F3/2 ascite from Boston Biologicals, mAb and rabbit antibody against Plk1 (for immunofluorescence in HeLa cells) from Santa Cruz Biotechnology, Inc., monoclonal Plk1 antibody (for Western blotting) from Zymed Laboratories, and Crest serum from Antibodies, Inc.

GST-xAurora B was expressed in *E. coli* and purified with glutathione-Sepharose beads. The recombinant xAurora B was tested for its ability to phosphorylate Histone H3 and was found to be highly active, although it is possible that the kinase by itself is not able to recognize all the physiological substrates in the absence of INCENP and Survivin. Active Cdc2/cyclin B kinase was purchased from New England Biolabs, Inc. Recombinant Plx1 protein was provided by P. Jackson (Stanford University, Stanford, CA).

Preparation of *X. laevis* egg extracts, immunodepletion, and translation in extracts

Meiotic metaphase extracts (CSF extracts) from *X. laevis* eggs and demembranated sperm nuclei were prepared as described previously (Minshull et al., 1994).

For immunodepletion, 75 μg of affinity-purified antibodies were coupled to 8 μl of Affi-prep protein A beads (Bio-Rad Laboratories) by dimethyl pimelimidate (Pierce Chemical Co.). The antibody beads were then incubated with 50 μl of CSF extracts for 1 h at 4°C and pelleted at 4°C. The depletion conditions were as follow: one round of immunodepletion for xMps1, two successive rounds for xBub1 and xBubR1, and three successive rounds for Plx1. To achieve reproducible high-depletion efficiency of Plx1 (>99.5%), anti-Plx1 antibodies were not cross-linked to the protein A beads, as cross-linking reduced the depletion efficiency.

For in vitro translation in extracts, mRNAs were transcribed from linearized plasmids encoding xMps1 and Plx1 using mMessage mMachine transcription kit (Ambion). Translation reactions were performed in CSF extracts that had been depleted of the protein of interest. Proteins translated in depleted extracts were used in rescue experiments. xMps1 construct was provided by D. Cleveland (University of California, San Diego, San Diego, CA), Plx1 by J. Maller (University of Colorado, Denver, CO), and xBub1 by R.H. Chen (Cornell University, Ithaca, NY). The kinase-dead mutants of xMps1 and Plx1 used in this paper were xMps1 D685A and Plx1 N172A (Qian et al., 1998; Abrieu et al., 2001).

Spindle checkpoint extracts were prepared as described previously (Minshull et al., 1994) with the following modifications: CSF extracts (fresh or depleted of the protein of interest) were incubated with demembranated sperm nuclei and nocodazole. For rescue experiments, in vitro–translated proteins were added to depleted extracts and incubated for 10 min at RT before the addition of sperm nuclei and nocodazole.

Immunofluorescence microscopy

Spindle checkpoint extracts were fixed in fixation buffer (80 mM Pipes, pH 6.8, 2 mM $MgCl_2$, 1 mM EGTA, 30% glycerol, 0.5% Triton X-100, 0.5 μM microcystin-LR, and 2% formaldehyde). Each sample was then layered onto a centrifuge tube filled with CSF-XB (10 mM Hepes, pH 7.8, 50 mM sucrose, 100 mM KCl, 2 mM $MgCl_2$, and 1 mM EGTA) plus 0.5% Triton X-100 and 40% glycerol. A poly-L-lysine–coated coverslip was placed at the bottom of each tube, and nuclei were spun onto the coverslip. Coverslips were recovered, fixed with 2% formaldehyde in CSF-XBT (CSF-XB with 0.1% Triton X-100), blocked by CSF-XBT plus 3% BSA, and incubated with primary and secondary antibodies (Molecular Probes). Images were captured on a microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) equipped with a 100× 1.4 NA lens, a digital charge-coupled device camera (Hamamatsu Photonics), and Openlab 4.0.2 (Improvision). For quantitative comparison of fluorescence intensities (Figs. 2 E, 3 C, and 4 C), antibody concentrations were titrated to ensure a linear response of immunofluorescence signals to the antigen concentrations, and images were taken with identical exposure time and processed identically. The mean fluorescence intensity values and their standard deviations were calculated from ≥15 kinetochores taken from multiple nuclei.

XTC cells were grown at RT on poly-L-lysine–coated coverslips in L-15 medium supplemented with 10% FBS and 1% penicillin-streptomycin. HeLa cells were grown in DME supplemented with 10% FBS and 1% penicillin-streptomycin. Samples were fixed with 0.1% PFA in CSF-XB plus 0.5 μM of microcystin LR, permeabilized in CSF-XB plus 0.5% Triton X-100 and 0.5 μM of microcystin LR, and incubated in CSF-XBT and 0.5 μM of microcystin LR. Subsequently, the samples were fixed again with 2% PFA in CSF-XBT plus 0.5 μM of microcystin LR, and then blocked in CSF-XBT plus 3% egg albumin before being processed for immunofluorescence staining. For taxol-treated samples, cells were grown in the presence of 100 nM taxol for 15 h and then fixed immediately or released into fresh medium for 15 min before fixing.

Dephosphorylation and rephosphorylation reactions

Nuclei were purified onto coverslips from spindle checkpoint extracts. To remove the 3F3/2 phosphoepitope, coverslips were incubated with λ-phosphatase (New England Biolabs, Inc.) at 2 U/μl for 15 min.

For rephosphorylation in Fig. 1 D, coverslips were treated with the phosphatase and rinsed twice with RPB (50 mM Tris-HCl, pH 7.7, 5 mM $MgCl_2$, and 1 μM of microcystin LR), and phosphorylation reaction was done in RPB plus 2 mM ATP. Coverslips were then processed for immunofluorescence staining.

For all other rephosphorylation reactions, coverslips were incubated with phosphatase and then with 5 mM NEM (Sigma-Aldrich) in 50 mM Tris-HCl, pH 7.7, and 5 mM $MgCl_2$ to inactivate endogenous kinases. The reaction was quenched by 1 mM DTT in CSF-XBT and blocked in CSF-XBT plus 3% BSA, 0.5 μM of microcystin LR, and 1 mM DTT. For rephosphorylation with extracts, coverslips were incubated, in the presence of 2 mM ATP, 1 mM DTT, and 1 μM of microcystin LR, with 35 μl of CSF extracts either mock depleted or depleted of an endogenous kinase (xMps1, xBub1, xBubR1, or Plx1). For rephosphorylation with recombinant kinase, coverslips were incubated with 0.5 μg His_6-Plx1 in 40 μl RPB plus 2 mM ATP and 1 mM DTT. Coverslips were then processed for immunofluorescence staining.

Knockdown of Plk1 in HeLa cells

HeLa cells were grown on coverslips and synchronized by double-thymidine arrest and release (Fig. 4 A). SmartPool siRNAs against Plk1 (Dharmacon, Inc.) were transfected 3 h before the second thymidine arrest by a DF1 reagent (Dharmacon, Inc.), and the transfection mix was present throughout the second thymidine arrest. Cells were fixed and assayed by immunofluorescence staining 11 to 14 h after release from the second thymidine arrest.

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