Direct quantification of polo-like kinase 1 activity in cells and tissues using a highly sensitive and specific ELISA assay

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Polo-like kinase 1 (Plk1) plays a pivotal role in the regulation of cellular proliferation. Plk1 is overexpressed in ~80% of human tumors of diverse origins, and overexpression of Plk1 promotes neoplastic transformation of human cells. A growing body of evidence suggests that deregulation of Plk1 closely correlates with prognosis of various cancers in humans. Thus, accurate assessment of Plk1 deregulation would provide clear clinical advantages. However, because of the limited amount of cancer tissues available, quantification of the Plk1 activity has not been feasible. Here, we report the development of a rapid, highly sensitive, and specific ELISA-based Plk1 assay that can quantify the level of Plk1 activity with a small amount (2–20 μg) of total cellular proteins. Unlike the conventional immunocomplex kinase assay, this assay directly utilizes total cellular lysates and does not require a Plk1 enrichment step such as immunoprecipitation or affinity purification. Using this assay, we demonstrated that Plk1 activity is elevated in tumors but not in the surrounding normal tissues and that the level of Plk1 activity significantly diminishes after an antiproliferative chemotherapeutic agent, with a small amount (2–20 μg) of total cellular proteins. Unlike the conventional immunocomplex kinase assay, this assay directly utilizes total cellular lysates and does not require a Plk1 enrichment step such as immunoprecipitation or affinity purification. Using this assay, we demonstrated that Plk1 activity is elevated in tumors but not in the surrounding normal tissues and that the level of Plk1 activity significantly diminishes after an antiproliferative chemotherapeutic agent.

Results

Plk1 Kinase Assay Using GST-PBIPtide as a Plk1 Affinity Ligand and in Vitro Substrate. Previous results showed that Plk1 efficiently phosphorylates a centromeric protein PBP1 at T78, and this phosphorylation event generates a docking site for a high-affinity interaction between the PBD of Plk1 and p-T78 PBP1 (11). By taking advantage of the specific Plk1-dependent PBP1 phosphorylation and subsequent interaction, we examined whether a GST-fused PBP1 peptide bearing the T78 motif (hereon referred to as GST-PBP1tide) could precipitate Plk1 through the Plk1-generated p-T78 epitope and whether the precipitated Plk1 could further phosphorylate as yet unphosphorylated GST-PBP1tide. To test this possibility, we first generated GST-PBP1tide (GST-PBP1tide containing 4 repeats of the T78 motif to enhance the phosphorylation level and binding affinity) and expressed it in Escherichia coli. The resulting bead-associated GST-PBP1tide was incubated with mitotic HeLa lysates in a conventional immunoprecipitation buffer, precipitated, and then reacted in a kinase reaction mixture (KC buffer; see Materials and Methods) in the presence of [γ-32P]ATP. The GST-PBP1tide coprecipitated Plk1, which, in turn, phosphorylated and


The authors declare no conflict of interest.

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generated the p-T78 epitope on neighboring GST-PBIPtide molecules (Fig. L4). Under the same conditions, the control GST failed to precipitate Plk1 and generate the p-T78 epitope (Fig. L4). As expected if the Plk1 activity in the total lysates was responsible for generating the p-T78 epitope, Plk1 immunoprecipitated from cultured lysates efficiently phosphorylated GST-PBIPtide and generated the p-T78 epitope in a kinase activity-dependent manner [supporting information (SI) Fig. S1].

We then examined whether the Plk1 activity present in total cellular lysates could be directly measured by incubating the lysates with GST-PBIPtide. To this end, total cellular lysates were prepared from either control luciferase RNAi (shLuc) or Plk1 RNAi (shPlk1)-treated HeLa cells (Fig. S2) in a KC-plus buffer (kinase reaction mixture supplemented with 100 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors), mixed with GST-PBIPtide, and then reacted in the presence of 15 μCi of [γ-32P]-ATP. Consistent with the levels of Plk1 expression (Fig. 1A), the control shLuc cells arrested with nocodazole (M phase) exhibited a high level of T78 phosphorylation onto PBIPtideA4 (Fig. 1B; α-p-T78 in the center column). By contrast, asynchronously growing shLuc cells (Asyn) displayed a significantly diminished level of T78 phosphorylation. However, the same sample exhibited a much higher level of 32P incorporation onto PBIPtideA4 (Fig. 1B; compare the autorad with the α-p-T78 blot in the center column), suggesting that the Thr and Tyr residues other than the T78 residue remain phosphorylated in the nocodazole (Noc)-treated, control luciferase RNAi (shLuc) cells.

**Fig. 1.** Development of a Plk1 kinase assay using GST-PBIPtide as a Plk1 affinity ligand and in vitro substrate. (A) GST-PBIPtide not only precipitates Plk1 but also serves as Plk1 substrate in vitro. (Left) Mitotic HeLa lysates were incubated with either bead-immobilized control GST or GST-PBIPtide in TBSN buffer containing phosphatase inhibitors. Beads were precipitated and washed and then subjected to in vitro kinase assays in the presence of [γ-32P]-ATP. Samples were separated by SDS/PAGE, transferred to PVDF, and then exposed (Autorad). The membrane was immunoblotted with the indicated antibodies and then stained with Coomassie (CBB). Note that the anti-p-T78 signal (α-p-T78 panel) is relatively weak because the incubation was carried out in TBSN at 4 °C. (Right) Schematic diagram illustrating the experimental procedures described in Left. The blue ellipsoids depict bead-associated GST-PBIPtide containing the T78 motif. (B) A direct Plk1 kinase assay with total cellular lysates using GST-PBIPtides as substrate. Asynchronously growing HeLa cells (Asyn) or cells expressing shRNA directed against Plk1 (shPlk1) were harvested. Where indicated, cells were treated with nocodazole (Noc) for 16 h to arrest the cells in prometaphase. Total HeLa lysates (100 μg) were prepared in KC-plus buffer and incubated with the control GST or the indicated GST-PBIPtide in the presence of [γ-32P]-ATP at 30 °C for 30 min. The bead-associated GST-PBIPtides were precipitated and washed with KC-plus buffer before boiling with SDS/PAGE sample buffer. The samples were separated as above, transferred, and then exposed (Autorad). After immunoblotting, the membrane was stained with Coomassie (CBB). The GST-PBIPtide bands were excised, and incorporated 32P was quantified. The signals in the anti-Plk1 and anti-p-T210 immunoblots indicate the amount of Plk1 coprecipitated (Plk1 co-ppt’ed) with the GST-PBIPtide and the level of the p-T210 epitope among the Plk1 precipitates, respectively. Numbers indicate the levels of the p-T78 epitope (α-p-T78 panel) or 32P incorporation (Autorad) relative to those in the nocodazole (Noc)-treated, control luciferase RNAi (shLuc) cells.
but reproducibly, lower level of \( ^{32} \)P incorporation after Plk1 depletion than the original PBIPtide\(_4\) (Fig. 1B; autorad in the right column), suggesting an improved sensitivity to Plk1 activity. In a separate experiment, either mitotic HeLa lysates or Xenopus cytotonic factor (CSF)-arrested extracts efficiently generated the T78 epitope in both PBIPtide\(_4\) and PBIPtide-A\(_6\), and the resulting p-T78-containing PBIPtides interacted with its respective endogenous Plk1 or Plk1 (Fig. S3), demonstrating that the FAF mutations in PBIPtide-A\(_6\) do not alter the efficiency of T78 phosphorylation and subsequent binding to the PBD.

**Fig. 2.** Plk1, but not Plk2 or Plk3, phosphorylates and binds to the T78 motif of GST-PBIPtides. (A) 293T lysates expressing control vector, Flag-Plk1, Flag-Plk2, or Flag-Plk3 were prepared in KC-plus buffer and incubated with the indicated GST-PBIPtides immobilized to the GSH-agarose beads. The GST-PBIPtide precipitates were separated by SDS/PAGE, transferred, and then immunoblotted with anti-Plk1 and anti-p-T78 antibodies. Afterward, the membrane was stained with Coomassie (CBB). The level of Plk3 expression is low because of its cytotoxicity. The low levels of the p-T78 signal detected in the control vector, Plk2, and Plk3-expressing cells are likely due to the endogenous Plk1 activity. The multiple tiers of the p-T78 signals are due to GST-PBIPtide degradation. Arrowheads indicate Plk1 coprecipitated with GST-PBIPtides. (B and C) Plk1-dependent phosphorylation onto the T78 motif of GST-PBIPtide-A\(_6\) is sufficient for the PBD binding. (B) (Top and Middle) Flag-Plk1, Flag-Plk2, and Flag-Plk3 immunoprecipitates prepared from transfected 293T cells were subjected to in vitro kinase assays using both GST-PBIPtide-A\(_6\) and casein as substrates in the same reaction. Samples were separated by SDS/PAGE for autoradiography (Autorad). Arrowheads indicate autophosphorylated signals corresponding to Plk1, 2, or 3 in the gels stained with Coomassie (CBB), whereas asterisks denote nonspecific signals. Incorporation \(^{32} \)P into GST-PBIPtide-A\(_6\) and casein were quantified (Bottom). (C) The Plk1-phosphorylated, bead-bound, GST-PBIPtide-A\(_6\) was incubated with either bead-bound GST-PBD or GST-PBD(H538A K540M) in TBSN buffer. Precipitates were washed and then analyzed as in B.

Plk1, but Not Plk2 or Plk3, Phosphorylates and Binds to the T78 Motif of GST-PBIPtides. Next, we examined whether Plk2 or Plk3 contribute to the generation of the p-T78 epitope (because of a dissimilar PBD-binding module, the distantly related Plk4 has not been tested). To this end, total lysates prepared from cells transfected with Flag-Plk1, Flag-Plk2, or Flag-Plk3, were incubated with the indicated GST-PBIPtides in the KC-plus buffer and then precipitated. Remarkably, lysates expressing Plk1 efficiently generated the p-T78 epitope on GST-PBIPtides, yielding a Coomassie-stainable level of Plk1 binding to the p-T78 GST-PBIPtides (Fig. 2A). In stark contrast, the lysates expressing Plk2 or Plk3 did not significantly generate the p-T78 epitope or bind to the GST-PBIPtides (Fig. 2A). The low levels of the p-T78 epitope in the vector-, Plk2-, and Plk3-expressing lysates are likely due to the endogenous Plk1 activity.

We next tested whether Plk1 directly phosphorylates and generates the p-T78 epitope on GST-PBIPtide-A\(_6\) and whether this event is sufficient for PBD binding. Similar to the previous observation with GST-PBIPtide\(_4\) (12), Plk1 preferentially phosphorylated GST-PBIPtide-A\(_6\) in a kinase reaction that contained both PBIPtide-A\(_6\) and a generic substrate, casein. In contrast, both Plk2 and Plk3 exhibited a strong preference for casein over PBIPtide-A\(_6\) (Fig. 2B). Furthermore, GST-PBD, but not the respective PBD(H538A K540M) phosphate-pincer mutant (13), efficiently coprecipitated the phospho-PBIPtide-A\(_6\) (Fig. 2C).

**Measurement of the Kinase Activity of Plk1 by a T78-Based ELISA.** By exploiting the high specificity in Plk1-dependent PBIPtide phosphorylation and the ensuing PBD-p-T78 interaction, we then generated a PBIPtide-based ELISA designed to measure the Plk1 activity by either of 2 approaches: (i) quantifying the level of the p-T78 epitope (\( \alpha \)-p-T78 antibody) or (ii) measuring the level of Plk1 binding (\( \alpha \)-Plk1 antibody) (Fig. 3A). To perform these assays, a 96-well plate was coated with various amounts of GST-PBIPtide containing the T78 motif and then applied with a range of total cellular lysates prepared in the KC-plus buffer to allow efficient phosphorylation onto PBIPtide during incubation (~2–10 \( \mu \)g of total HeLa lysates were sufficient for reactions with \( \approx 0.5–1 \mu \)g of GST-PBIPtide\(_4\) or GST-PBIPtide-A\(_6\) per well; see Fig. S4). Attesting to the specificity of Plk1-dependent PBIPtide phosphorylation, depletion of Plk1 greatly diminished the levels of both the p-T78 epitope and bound Plk1 on GST-PBIPtides (Fig. 3B). Consistent with the results shown in Fig. 1B, PBIPtide-A\(_6\) exhibited a mildly, but reproducibly, increased sensitivity to Plk1 activity compared with PBIPtide\(_4\). Notably, the level of the Plk1 binding (\( \alpha \)-Plk1 signal) was significantly lower than the level of the p-T78 generation (\( \alpha \)-p-T78 signal) (Fig. 3B), in part because Plk1 binds to a fraction of the total p-T78 epitope generated. Because of the apparently elevated sensitivity, we chose GST-PBIPtide-A\(_6\) for further analysis.
subsequent PBD binding, we then carried out the above assay using purified recombinant proteins. Results showed that the level of the p-T78 epitope generated was proportional to the amount of Plk1 provided. Although the Plk1 amounts that can be quantified in a linear range could be relatively narrow because of the nature of an ELISA-based assay, the increase in the level of the p-T78 epitope also closely paralleled the increase in the amount of Plk1 bound to PBIPtide (Fig. 3C).

In a second experiment, we also found that treatment of cells with a Plk1 inhibitor, BI 2536 (14), for 30 min was sufficient to acutely inhibit the Plk1-dependent p-T78 generation and Plk1 binding (Fig. 3D), further confirming that Plk1 activity is responsible for these events. Notably, Plk1 exhibits only 49–51% sequence identities in the kinase domain with Plk2 and Plk3, suggesting that the substrate recognition modes of these kinases are distinct. Highlighting the functional disparities of these kinases, it has been shown that Plk2 is transiently expressed during the G0/G1 transition (15). However, Plk3 is reported to be active in S and G2 phases of the cell cycle (16, 17), during which Plk1 is also progressively accumulated and activated before mitotic entry. Furthermore, overexpression of Plk3 rescues the growth defect associated with the budding yeast polo kinase cdc5–1 mutation (16). Thus, we examined whether Plk3 contributes to the generation of the p-T78 epitope. Consistent with Plk1-dependent PBIPtide phosphorylation, the level of p-T78 was high in nocodazole-treated (M phase) cells but low in thymidine-arrested (S phase) cells (Fig. 3E). Depletion of Plk1 drastically diminished the level of the p-T78 epitope, whereas depletion of Plk3 did not significantly alter the level of the p-T78 epitope under various conditions examined (Fig. 3E). These results suggest that Plk3 does not contribute to the generation of the p-T78 epitope.

Fig. 3. Development of a Plk1-specific ELISA. (A) Scheme illustrating the Plk1 ELISA. The ELISA wells were coated with soluble GST-PBIPtide containing the T78 motif (dark blue) and then reacted with total cellular lysates. The Plk1 activity in the total lysates generates the p-T78 epitope to which Plk1 itself binds. After reaction, Plk1 activity is quantified by incubating the ELISA wells with either anti-p-T78 antibody (red) to detect the p-T78 epitope generated or anti-Plk1 antibody (blue) to detect Plk1 bound to the p-T78 epitope, followed by HRP-conjugated secondary antibody (the green antibody with a black dot). The yellow and light blue asterisks indicate 3,3’,5,5’-tetramethylbenzidine (TMB) substrate and its reaction product, respectively, generated by HRP. (B) (Left) HeLa cells were silenced for control luciferase (shLuc) or Plk1 (shPlk1) and then treated with nocodazole for 16 h to arrest the cells in prometaphase (a condition that maximizes Plk1 activity). The lysates were then applied onto the ELISA wells coated with GST-PBIPtide, GST-PBIPtide-A6, and ELISAs were carried out as described in Materials and Methods. Buffer indicates no-lysate control. The same lysates were subjected to immunoblotting analysis to determine the level of Plk1 in the lysates (Right). (C) Plk1 generates and binds to the p-T78 epitope in a concentration-dependent manner. GST-PBIPtide-A6-coated ELISA wells were incubated with the indicated amount of recombinant Plk1 purified from Sf9 cells. The level of the p-T78 epitope generated and the amount of Plk1 bound to the p-T78 GST-PBIPtide-A6 were quantified by using anti-p-T78 and anti-Plk1 antibodies, respectively. (D) HeLa cells arrested with nocodazole for 16 h were additionally treated with either control DMSO or a Plk1 inhibitor, BI 2536, for 30 min before harvest. Total lysates were prepared from these cells and applied to the GST-PBIPtide-A6-coated wells. Buffer indicates no-lysate control. (E) HeLa cells silenced for control luciferase (shLuc), Plk1 (shPlk1), or Plk3 (shPlk3) were treated with either thymidine (Thy) or nocodazole (Noc) or left untreated for 16 h before harvest. Total cellular lysates prepared from these cells were subjected to ELISAs as in D. Buffer indicates no-lysate control. Because detection of endogenous Plk3 with currently available antibodies was not reliable, efficiency of Plk3 depletion by shPlk3 was determined by using cells transfected with Flag-Plk3. The target sequence for shPlk3 was described previously (17).
Direct Measurement of Plk1 Activity in Mouse Tissues. To determine whether the above PBIP tide-based ELISA could be used to measure the level of Plk1 activity in mammalian tissues, we compared both the ELISA and the conventional immunocomplex kinase assay using the same lysates prepared from various mouse tissues. We found that the Plk1 activities from the ELISA showed a tight correlation with those from the immunocomplex kinase assays (Fig. 4A; see also immunocomplex kinase assays using GST-PBIP tide-A6 as substrates in Fig. S5). Remarkably, only 4–20 μg of the same lysates (because of low mitotic indices for tissues, we needed more lysates from tissues than from cultured cells) were used for the ELISA compared with 2 mg of total lysates for the immunoprecipitation kinase assay. Thus, the Plk1 ELISA is not only rapid but also sensitive, allowing accurate quantification of Plk1 activity with 100–500-fold smaller amounts of total lysates from cells and tissues.

Taking advantage of this highly sensitive assay, we then investigated whether Plk1 activity alters during tumorigenesis using B16-derived xenografted tumors in nude mice (Fig. 4B). Plk1 activity was low immediately after grafting but soon reached a maximum level during early stages of tumorigenesis (Fig. 4C). However, the level of Plk1 activity began to diminish as the tumor reached a significant volume (~10 mm in diameter). The levels of Plk1 activity closely mirrored those of Plk1 expression (Fig. 4C) and cell proliferation activity (Fig. S6). Measurement of Plk1 activity of tissues taken from different parts of a single tumor revealed that the levels of Plk1 expression and activity tightly correlated with the level of mitotic Cyclin B1 (Fig. S7). These findings suggest that elevated Plk1 activity is critical during early stages of tumorigenesis and strongly support the view that the level of Plk1 functions as an indicator of cell proliferation.

Discussion

Elevated levels of Plk1 expression correlate with poor prognosis for a wide range of human cancers such as non-small-cell lung cancer, oropharyngeal carcinoma, esophageal carcinoma, melanoma, colorectal cancer, hepatoblastoma, and non-Hodgkin lymphoma (5). These observations have marked Plk1 as a potential therapeutic target for cancer, and a number of small-molecule inhibitors of the kinase are being developed for that purpose. Thus, an accurate measurement of Plk1 activity would be important not only as a potential guide to address prognostic issues in individual patients but also as a biomarker to indicate the effectiveness of Plk1-targeted therapy. However, limited supplies of tissue samples have made it difficult to determine Plk1 activity by using conventional Plk1 kinase assays in both experimental models and human material. By exploiting a unique Plk1-dependent PBIP phosphorylation and subsequent binding and by using an ELISA-based technology, we have now developed a rapid, sensitive, and specific assay that measures the Plk1 activity in biological material by using the intracellular ATP present in the lysates. This assay is far more sensitive than conventional immunocomplex kinase assays and thereby enables the detection of an elevated Plk1 activity in small amounts of total lysates obtained from cultured cells or xenografted mouse tumors. In addition, the assay allows for quantification of the Plk1 activity in a given lysate by easily relating to recombinant Plk1 standard or normalizing to a control lysate.

As an extension of this assay and as a further proof of principle, we measured Plk1 activity in normal and tumor tissues obtained from head and neck cancer patients. Although the level of Plk1 activity varied greatly among tumor samples (likely because of the differences in age, diet, genetic background, tumor site, etc.), elevated Plk1 activity in tumor tissue was manifest in most cases where a difference in matched value was detected (Fig. S8A). Furthermore, most tumor samples obtained from patients after antiproliferative chemotherapy exhibited greatly diminished Plk1 activity compared with the tumor samples before therapy (Fig. S8B). The number of patients was too small to draw clinical conclusions, but these preliminary results do suggest that the assay could be used to systematically track patients’ prognosis and therapy response. Most impressive for these human samples was a good correlation of the ELISA results with the cumbersome immuno-
precipitation kinase assay. Furthermore, compared with the amount of total lysates required for the immunocomplex kinase assay, the ELISA required a 100- to 500-fold smaller amount of the sample lysates, which could be further reduced by using europium-labeled antibodies. This level of specificity and sensitivity provides a particular advantage for analyzing small human tumor samples such as needle biopsies as demonstrated in Fig. S8B. Because several Plk1 inhibitors are currently under clinical trials, the ELISA described herein could be of great value in monitoring the early therapy response after the treatment of patients with Plk1 inhibitors.

In light of the importance of Plk1 activity during oncogenesis and its potential as a therapeutic target, this assay could also be applied in several stages in the overall diagnostic and treatment protocols of an individual patient with cancer. Because levels of Plk1 have prognostic value in a number of cancers (5), determining the level of Plk1 activity at the time of initial tissue diagnosis may provide an important clue for proper therapeutic decisions. Because Plk1 is a marker for cells undergoing mitosis, and the level of Plk1 expression is very low in most normal tissues, this assay could also be used to monitor disease remission, progression, and recurrence. Because of its marked sensitivity, the assay may allow for the detection of circulating tumor cells with high mitotic rate from blood samples or shedding mitotic cells from solid tumors and thus foretell cancer recurrence after a broad spectrum of antiproliferative therapy. In this regard, this assay might be useful to predict the choice of certain chemotherapeutic agents that specifically target mitotic cells and to also assess response to therapy. Given that Plk1 overexpression is tightly associated with tumorigenesis and metastasis, the Plk1 ELISA demonstrated here promises to become a useful method in the diagnosis and treatment of Plk1-associated cancers as well as other pathological conditions associated with a high mitotic rate.

Materials and Methods

Construction of GST-PBIPtides and Cell Culture. Construction of GST-PBIPtides and conditions of cell culture are described in SI Text.

GST-PBIPtide Pulldown, Immunoprecipitation, and GST-PBD Pulldown Assays. GST-PBIPtides were expressed and purified from E. coli BL21 by using glutathione (GSH)-agarose (Sigma). For GST-PBIPtide pulldown kinase assays, HeLa cells were lysed in TBSN buffer (20 mM Tris·Cl [pH 8.0], 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EGTA, 1.5 mM EDTA, 20 mM β-nitrophenyl phosphate, and protease inhibitor mixture [Roche]). The resulting lysates were clarified by centrifugation at 16,000 g for 10 min at 4 °C. The resulting pellets were resuspended in 100 μL of total lysates in KC-plus buffer or the indicated amount of recombinant Flag-Plk1 from Sf9 cells for 30 min at 30 °C on an ELISA plate incubator (Boekel Scientific). To terminate the reaction, ELISA plates were washed 4 times with PBST. For detection of the generated p-T78 epitope or bound Plk1, plates were incubated for 2 h with 100 μL per well of anti-p-T78 or anti-Plk1 antibody at a concentration of 0.5 μg/mL. After washing the plates 5 times, 100 μL per well of HRP-conjugated secondary antibody (diluted 1:1,000 in blocking buffer) was added and the plates incubated for 1 h. Plates were then washed 5 times with PBST and then incubated with 200 μL per well of 3,3′,5,5′-tetramethylbenzidine solution (TMB) (Sigma) as substrate until a desired absorbance was reached. The reactions were stopped by the addition of 0.5 M H2SO4. The optical density of the samples was measured at 450 nm by using an ELISA plate reader (Molecular Devices).

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Supporting Information

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SI Text

Construction of GST-PBIPtides. To generate GST-PBIPtide-expressing constructs, we first generated a pUC19 derivative, pUC19N, whose multiple cloning sites were restructured to contain both BamHI and BglII. A small DNA fragment encoding GGPGG-fused-YETFDPLHSTAIYADEE (PBIPtide) was digested with BamHI (5' end) and BglII (3' end) and then inserted into pUC19N digested with the corresponding enzymes. The GGPGG sequence was added at the N terminus of the PBIPtide as a linker between PBIPtide repeats. The resulting pUC19N-GGPGG-PBIPtide was digested with BglII and then ligated with another copy of GGPGG-PBIPtide digested with BamHI (5' end) and BglII (3' end), yielding pUC19N-GGPGG-PBIPtide2. These cloning steps were repeated 2 more times to generate pUC19N-GGPGG-PBIPtide4 (this cloning strategy disables the N-terminal BamHI site of the subsequent GGPGG-PBIPtide fragments inserted). The last GGPGG-PBIPtide fragment bears a stop codon to terminate translation. To generate GST-GGPGG-PBIPtide construct (for simplicity, we refer to this construct as GST-PBIPtide4), pUC19N-GGPGG-PBIPtide4 was digested with BamHI and BglII and then inserted into pGEX-4T-2 (Amersham Biosciences) digested with BamHI. To eliminate potential phosphorylation sites other than the T78 residue, PBIPtide-A form (FEAFDPPPLHSTAIYADEE) that bears 3 mutations (Y68F, T70A, and Y81F) was generated. Construction of GST-PBIPtide-A6, which contains 6 copies of the GGPGG-PBIPtide-A fragment, was carried out in a similar manner as described above.

Cell Culture. Cell cultures were maintained as recommended by American Type Culture Collection. Plasmid transfection was carried out by using Lipofectamine 2000 (Invitrogen). Lentiviruses for chromosome congression and spindle checkpoint signaling. Mol Cell 15:7143–7151.

Immunoprecipitation Kinase Assays. Immunoprecipitation and in vitro kinase assays were carried out essentially as described previously (4) using anti-Plk1 antibody (N-19) (Santa Cruz Biotechnology). To determine the substrate preference for the Flag-Plk1, Flag-Plk2, or Flag-Plk3 immunoprecipitates, reactions were carried out in a kinase reaction mixture [KC; 50 mM Tris-Cl (pH 7.5), 10 mM MgCl2, 2 mM DTT, 2 mM EGTA, 0.5 mM Na2VO4, and 20 mM p-nitrophenyl phosphate] in the presence of 10 μM ATP (10 μCi of [γ-32P]ATP; 1 Ci = 37Gbq). To determine the substrate preference, both GST-PBIPtide-A6 and casein were provided as substrates in the same reaction. Reactions were terminated by the addition of sample buffer, separated by SDS/PAGE, and then exposed. Where appropriate, phosphorylation onto GST-PBIPtide was evaluated by autoradiogram and/or anti-p-T78 immunoblotting analysis.

GST-PBD Pulldown Assays. To examine the interaction between PBD and the p-T78 of PBIPtide-A6, bead-bound GST-PBIPtide was first reacted with immunoprecipitated Plk1, Plk2 or Plk3 with constant agitation in the presence of [γ-32P]ATP. Subsequently, the reacted GST-PBIPtide was digested with thrombin (Invitrogen), and then the resulting soluble 32P-labeled PBIPtide-A6 was incubated with the bead-bound, bacterial, GST-PBD or GST-PBD(H538A K540M) (5) in TBSN for 1 h at 4°C. Precipitates were washed in the binding buffer, separated by SDS/PAGE, stained with Coomassie (CBB), and then exposed (Aurorad).

Immunoblotting Analysis. Primary antibodies used for this study were anti-PBI1 p-T78 antibody (Rockland Immunologicals, Inc.), anti-Plk1 antibody (F-8) (Santa Cruz Biotechnology), anti-Plk1 p-T210 antibody (BD Biosciences), anti-Flag antibody (Sigma), anti-GFP antibody (MBL International), anti-Plk1 antibody (Jim Maller, University of Colorado, Aurora, CO), and anti-Cyclin B1 antibody (Abcam, Inc.). Proteins that interact with the primary antibodies were decorated with appropriate HRP-conjugated secondary antibodies and detected by using the enhanced chemiluminescence (ECL) western detection system (Pierce).

Nude Mouse Xenografting. Female athymic (NCr-nu/nu) mice were injected s.c. with the indicated numbers of B16 mouse tumor cells. At the indicated days after grafting, mice were killed and photographed. The resulting tumors were surgically removed, lyzed in KC-plus buffer, and then subjected to either ELISA or immunoprecipitation kinase assay.

H&E and BrdU Staining Analyses. Nude mice bearing xenografted B16 tumors were injected with BrdU (0.2 ml, 10 mM BrdU/100 g of body weight) 2 hours before termination. Tumors were collected and fixed in Formalin for 24 h and then transferred into 70% ethanol. Fixed tumors were embedded in paraffin and sectioned with 5-μm intervals. Consecutive tumor sections were subjected to either anti-BrdU or H&E staining analyses. Photographs were taken using a Nikon microscope.

Fig. S1. Wild-type Plk1, but not the respective kinase-inactive form, efficiently phosphorylates GST-PBIPtides. (A) Endogenous Plk1 immunoprecipitated from either asynchronously growing (Asyn) or nocodazole-treated (Noc) HeLa cells were subjected to kinase reactions in the presence of [γ-32P]-ATP. Both GST-PBIPtides (GST-PBIPtide4 and a longer form of PBIPtide, GST-PBIPtide-Z4) and an in vitro Plk1 substrate, casein, were used as substrates in a single reaction tube. Samples were separated by SDS/PAGE, exposed (Autorad), and then immunoblotted with anti-Plk1 antibody. (B) Plk1 was immunoprecipitated with anti-GFP antibody from HeLa cells expressing either EGFP-Plk1 or the corresponding kinase-inactive Plk1(K82M). Immunoprecipitates were then subjected to kinase reactions using GST-PBIPtides as substrates. Samples were separated by SDS/PAGE, exposed (Autorad), and then blotted with anti-p-T78 antibody to examine the level of the p-T78 epitope generated. Later, the same membrane was stained with Coomassie (CBB). Dots indicate the positions of each substrate.
Efficiency of Plk1 depletion by shPlk1. HeLa cells were infected with lentivirus expressing either control shLuc or shPlk1, treated with nocodazole for 16 h where indicated, and then harvested for immunoblotting analyses with the indicated antibodies. The same membrane was stained with Coomassie (CBB). The levels of actin and the CBB staining serve as loading controls.

Fig. S2.
Fig. S3. GST-PBIPTide efficiently precipitates Plk1 and its Xenopus homolog, Plx1, from the respective total lysates. (A) Mitotic HeLa lysates were prepared in KC-plus buffer and incubated with bead-bound GST or GST-PBIPTides. Anti-Plk1 immunoprecipitation with a commercially available anti-Plk1 antibody (N-19; Santa Cruz Biotechnology) was carried out as a comparison. Precipitates were separated and then immunoblotted with the indicated antibodies. Afterward, the same membrane was stained with Coomassie (CBB). (B) CSF-arrested egg extracts from Xenopus laevis were diluted in KC-plus buffer and incubated with the indicated ligands immobilized to the beads. Precipitates were washed and then subjected to in vitro kinase reaction in the presence of $[^{32}P]ATP$. The resulting samples were separated by SDS/PAGE, transferred, and then exposed (Autorad). Subsequently, the same membrane was immunoblotted with the indicated antibodies and stained with Coomassie (CBB). Arrows indicate weakly detectable Plx1 precipitated by GST-PBIPTides.
Fig. S4. Examples of Plk1 ELISA using GST-PBIPtide as Plk1 substrate. (A and B) ELISA wells were coated with the indicated amount of either GST-PBIPtide_4 or GST-PBIPtide-A_6. The wells were incubated with the designated amount of total cellular lysates prepared from HeLa cells treated with thymidine (S phase) or nocodazole (M phase) for 16 h. Because of the high sensitivity of the p-T78-based assay as shown in Fig. 3B, only the p-T78 antibody was used for analyses. Note that the reactions were more sensitive with GST-PBIPtide-A_6 than with GST-PBIPtide_4. With a given amount of total cellular lysates, the reactions in B were saturated with 0.3 μg of GST-PBIPtide-A_6. Under the conditions used, all of the reactions were terminated in 10 s because the signals for the 20-μg lysates were already saturated.
Fig. S5. Anti-Plk1 immunocomplex kinase assays with various mouse tissues using GST-PBIPtide-A6 as substrate. Anti-Plk1 immunoprecipitates from various tissues were subjected to in vitro kinase assays under the same conditions as in Fig. 4A except that GST-PBIPtide-A6 was used as substrate. Asterisk indicates that only half the amount of total lysates (1 mg) and anti-Plk1 antibody (3 μg) was used for ovary immunoprecipitation because of the limited amount of the tissue. Note that the relative levels of GST-PBIPtide-A6 phosphorylation by immunoprecipitated Plk1 are in line with those of the casein phosphorylation in Fig. 4A.
Fig. S6. The level of cell proliferation is high during early stages of tumorigenesis. Tumor sections from the nude mice bearing xenografted B16 tumors in Fig. 4B were prepared and subjected to H&E and BrdU stainings as described in Materials and Methods. Largely correlating with the levels of Plk1 activity in Fig. 4C, the BrdU-positive, proliferating, cells are highly concentrated at the growing edge of the tumors during early stages of tumorigenesis (2, 4, and 8 days). In contrast, the 12- and 16-day tumors exhibit sparsely populated proliferating cells, suggesting a diminished level of cell proliferation activity.
Fig. S7. Close correlation between the levels of Plk1 expression and activity and those of mitotic Cyclin B1 in a B16-derived tumor. (A) Athymic mice were s.c. grafted with B16 tumor cells. One of the large tumors was surgically removed and then divided into 9 sections. (Scale bar: 1 cm.) (B Upper) Total proteins prepared from each section were subjected to immunoblotting analyses with the indicated antibodies and then stained with Coomassie (CBB). Asterisk indicates a cross-reacting protein with anti-Cyclin B1 antibody. (B Lower) Plk1 ELISA assays were carried out with 20 μg of the same total lysates. Likely because of differences in the degree of senescence among different parts of the tumors, the levels of Plk1 activities vary significantly in #1 to #9 samples. However, it should be noted that the levels of Plk1 expression and activity tightly correlate with those of cyclin B1, suggesting that Plk1 is a marker for cell proliferation. Bars indicate the standard deviation.
Fig. S8. Quantification of Plk1 activity in tumor and normal tissues from various head and neck cancer patients. (A) To determine whether the Plk1 ELISA can be used to quantify the Plk1 activity with human tissue samples, pairs of frozen tumors and its surrounding normal tissues were initially obtained from 7 white European head and neck cancer patients. Total lysates were prepared in KC-plus buffer and subjected to anti-Plk1 immunocomplex kinase assays with 2 μg of lysates using casein as substrate (top) and Plk1 ELISA assays with 20 μg of lysates using GST-PBIPtide-A6 as substrate (bottom). Note that the order of sample loading for patient #1 and #2 is different from the rest of the samples. The casein phosphorylation activity for the tumor sample from patient #1 is significantly higher than the ELISA-based Plk1 activity, raising the possibility that a contaminating kinase(s) co-precipitated with Plk1 immunoprecipitates could have contributed to the phosphorylation level of the generic substrate casein in vitro. Our attempts to detect endogenous Plk1 by immunoblotting analysis with the total lysates failed due to a low percentage of mitotic cells in tissue samples. T, tumor tissues; N, normal tissues. Bars, standard deviation. (B) Tumor biopsies were collected from white European head and neck cancer patients, who were placed under 3 rounds of a 3-week cycle chemotherapy regimen with Docetaxel (75 mg/m²), Cisplatinum (100 mg/m²), and 5-Fluorouracil (1000 mg/m²). All of the biopsy samples collected before and after the 3 rounds of chemotherapy (a total of 10 biopsy pairs) were examined to determine the effect of the therapy on Plk1 activity. The samples before chemotherapy are needle biopsies, whereas the samples after chemotherapy are biopsies prepared immediately after surgical removal of the primary tumors. All of the samples were inspected by 2 independent pathologists. Plk1 ELISA assays were carried out twice as in A with 20 μg of total lysates prepared from the respective tumors independently. Evaluation of tumor stages was conducted within 7 days before chemotherapy by carrying out magnetic resonance imaging or computed tomography of the head and neck tumors. Staging was based on the size of the primary tumor (T), the degree of regional lymph node involvement (N), and the absence or presence of distant metastases (M). T1–T4, size and/or extent of the primary tumor; N0, no regional lymph node involvement; N1–N3, extent of spread into regional lymph nodes; M0, no distant metastasis; Mx, distant metastasis cannot be evaluated. Although the levels of Plk1 activity varied from one patient to another, this assay did indicate that 7 of the 10 pairs of samples exhibited significantly decreased Plk1 activity after the chemotherapeutic treatment. Interestingly, the 2 patients (marked with asterisks), who were delinquent in their scheduled second round (#8 patient) or third round (#4 patient) of treatment, exhibited high levels of Plk1 activity even after therapy. This could be due to reprovifion of tumor cells after their recovery from an incomplete chemotherapeutic treatment. Subsequent analyses with a large cohort of patient samples will be required to determine whether Plk1 activity measurement can be used as a clinical tool to monitor early therapy response and/or to predict therapeutic success.